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ENTEROENDOCRINAL BIOACTIVITY OF A PROTEIN EXTRACT

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INDEX

Abstract

By secreting hormones that regulate hunger and energy homeostasis, the intestine plays a crucial endocrine role. This study aimed to evaluate the enteroendocrinal bioactivity of a protein extract through *ex vivo* and *in vivo* approaches. Rat ascending colon and proximal jejunum explants were stimulated with various digested and non-digested versions of the extract in order to evaluate the release of ghrelin, cholecystokinin (CCK), gastric inhibitory peptide (GIP), glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY). Also, *in vivo* effects on body weight and food intake were evaluated in a cafeteria-diet induced obesity model in Wistar rats.

On upper jejunum, while GIP secretion responded to various kinds of digestions the effect of the protein extract (digested or not) on CCK stimulation was subtle. Intestinal and non-digested conditions increased ghrelin secretion. On colonic samples only intestinal and complete secretions boosted GLP-1 and PYY secretions. Intestinal and non-digested conditions paradoxically increased ghrelin secretion. When compared to chow diet controls, the cafeteria diet resulted in a considerable increase in weight, as expected. While 20X had no discernible effect, 1X displayed a trend towards reduced body weight gain and a slight reduction in food intake after chronic supplementation. Regardless of supplementation, food intake was significantly higher in all cafeteria-fed groups.

These findings imply that the protein extract has the ability to modulate the release of enterohormones, specifically stimulating the secretion of anorexigenic hormones in the colon. However, *in vivo* results suggest that its effects in food intake and body weight are mild and might not be dose-dependant. Although other mechanisms aside appetite modulation may be implicated, these results warrant further investigation of protein-derived bioactive compounds as possible metabolic modulators.

1. Introduction

Even though the most well-known functions of the intestine are nutrient absorption and working as a selective physical and biochemical barrier, it is also an endocrine organ containing enteroendocrine cells that secrete enterohormones. Although enteroendocrine cells only represent 1% of the whole population of intestinal cells[1] they are the largest endocrine organ in the body. Generally, enteroendocrine cells are open[2], being connected to the intestinal lumen so they can receive signals from metabolites and nutrients and, in response, secrete hormones with autocrine and paracrine effects[3]. Mainly, enterohormones modulate in different ways the digestive process but also can modulate metabolic parameters or processes that have an effect on energy homeostasis[4]. The release of these hormones and its influence on food intake is mostly mediated by the presence or absence of nutrients in the gastrointestinal tract, acting via vagus nerve or via systemic circulation to exert their effect on the brain[5]. Generally, hormones show a low secretion rate while fasting and rise after feeding, but others like ghrelin appear to show an inverse profile[6].

Ghrelin is a peptide hormone synthesized and secreted from the stomach and small intestine X cells[5], being fasting (or absence of nutrients) the principal trigger[4,7]. It is the main ligand for growth-hormone secretagogue receptor (GHS-R)[8]. High levels of ghrelin in plasma are linked to hyperphagia[4], being the only enterohormone that levels lower postprandially. To be active, preproghrelin is cleaved to obtain the 28 amino acid active form[7]. Orexigenic action is mediated via NPY and AgRP neurons, decreasing food intake and secondarily decreasing metabolic rate[8] and gastric emptying rate[8], being a long-term body weight regulator.

Cholecystokinin (CCK) was first characterized as a hormone with a role in controlling gut motility, pancreatic secretions and gall bladder contractions. CCK has multiple roles in our physiology and acts generally as a neurotransmitter or

as a hormone, being involved in regulation of food intake, learning and memory, behavioral expression and some functions related to dopaminergic, serotonoic and opioid systems[9]. There are multiple CCK forms, being CCK-8 (duodenum) and CCK-33 the most important forms, and two receptors have been characterized: CCK-1 and CCK-2, triggering biological actions of the peptide. One of the major functions of CCK is the inhibition of feeding, mainly mediated by CCK-1 receptor interaction with CCK-8 giving a signal of satiety[10]. CCK is released after nutrient stimulation in duodenum and jejunum, being fat and protein the macronutrients that stimulate the most, being a short-term meal-reducing signal[4].

Gastric inhibitory peptide (GIP) is secreted from intestinal K cells, mostly placed in upper jejunum, and its principal role is regulating glucose homeostasis, playing a paper in energy balance and weight homeostasis[2,4,11]. GIP is an incretin, enhancing insulin secretion in response to oral glucose intake[12,13] and is synthesized from the proglucagon gene as a pro-peptide and cleaved to the active form. The secretion of GIP is mostly stimulated by dietary fats but also by carbohydrates[1], being rapidly turned over by DPP-IV[4,14] and acts as an anabolic hormone increasing insulin levels, glycogen, fatty acid synthesis and inhibiting fat breakdown via g-coupled protein receptor (GPCR) by increasing AMPc and releasing insulin[14].

Glucagon-like peptide-1 (GLP-1) is a peptide derived from preproglucagon gene[1,15], also secreted by L cells, located in colon and ileum in the human gastrointestinal tract. GLP-1 secretion is biphasic, consisting in an acute peak in the first 15-20 min after eating and a second one, with less intensity, between 1h and 2h after food intake[16]. Although all three macronutrients stimulate GLP-1 secretion, glucose and fat are the strongest stimulators[17] after ingestion via SGLT-1 and GCRP[16], respectively. Together with GIP, is responsible for incretin effect, binding to β pancreatic cells leading to a rise of Ca^{2+} concentration and insulin secretion[13,17]. In the same way that happens with GIP, DPP-IV is the main

inhibitor, giving a short half-life to the hormone[16]. GLP-1 is also involved in decrease of gastric emptying, inhibition of food intake and others[18].

Peptide tyrosine tyrosine (PYY) is a proglucagon derived peptide member of the pancreatic polypeptide family that also includes pancreatic polypeptide and neuropeptide-Y[4] secreted by L cells in colonic cells[1]. PYY is a ligand for NPY receptor family, and when it is processed, selectively binds to receptor Y2 in the arquate nucleus to inhibit food intake[4]. Secretion is proportional to nutrients abundance and its caloric density[19], being fats and carbohydrates the principal stimulators[20].

Metabolic syndrome refers to the union of several well-known risk factors including abdominal fat, insulin resistance, dyslipidemia and hypertension, among others, which are related between them and identifies individuals with high risk of suffering a cardiometabolic pathology[21]. In concrete, obesity is a health epidemic affecting at least 20% of western population and its incidence shows a rising trend in recent years[22]. Metabolic syndrome and obesity can influence enterohormone secretion, altering it in some cases. Some kinds of obesity have reduced sensitivity to CCK or even an absent response to it[23]. GIP levels and sensitivity in obesity and type 2 diabetes mellitus (T2DM) vary between studies, but generally levels are higher due to hyperplasia of K cells, playing a role in fat accumulation in adipose tissue[14]. Ghrelin levels can vary due to body weight changes, trying to compensate for the variation of the body weight[8]. Subjects with obesity in early stages have lower levels of ghrelin but a loss of weight increases basal ghrelin levels[8], being partly responsible for voluntary hyperphagia when trying to lower weight. PYY release is impaired in patients with obesity or metabolic syndrome. Obese patients show lower levels in plasma both in basal and postprandial situations[5]. GLP-1, opposite to GIP, shows an impaired secretion profile secreting lower levels in T2DM but if administered exogenous GLP-1 is still able to normalize both fasting and postprandial glucose concentrations[13].

Obesity and metabolic syndrome are multifactorial conditions, and although there are some available drugs to treat or alleviate their comorbidities, most of them are usually accompanied by undesirable side effects. Protein extracts and non-drug natural products are arising as promising candidates to help in these situations playing a relevant role in reinforcing treatment against it or help being an adjuvant to change the lifestyle. Bioactive peptides derived from protein hydrolysis have been shown to possess different bioactivities to the original protein. Different enzymes hydrolyze peptide bonds in different ways and generate peptides of diverse sizes and properties that could positively modulate physiological functions and reduce disease risk. It has been shown the potential of some bioactive peptides with antidiabetic, antioxidant, antimicrobial or immunomodulatory properties derived from different sources: dairy, egg, fish, plants and legumes among others[24]. Also, many natural compounds can act as modulators of food intake and body weight, especially for early stages of metabolic conditions[25,26]. Hydrolyzed whey proteins have shown to directly stimulate the enteroendocrine system, promoting the release of GIP, CCK and GLP-1[27,28], also shown by hydrolyzed gelatin protein[29] and hydrolyzed egg protein[30]. Thus, studying and characterizing the beneficial effects of novel bioactive peptides is highly relevant, as they may play a key role in the development of new supplements and functional foods aimed at improving certain pathological conditions.

2. Hypothesis and objectives

The main hypothesis of this project is that the protein extract modulates the secretion of enterohormones involved in appetite regulation. These hormonal alterations are expected to cause beneficial effects in a context of metabolic syndrome.

The main objective of this study is **to characterize the effects of a given protein extract with a clear focus on food intake modulation**. Secondary objectives derived from this main objective are (i) **to characterize the enterohormone secretory profile ex vivo** and (ii) **to study variations in body weight and food intake in a cafeteria-induced obesity animal model**.

3. Materials and methods

Source and composition of the extract is confidential.

3.1 *Ex vivo* studies

3.1.1 Tissue obtention

Segments of the upper jejunum and ascending colon were obtained from euthanized rats for other studies (female Wistar rats (Rj:WI), receiving only standard chow diet -Teklad 2014 Envigo- for 13 weeks. Euthanasia was performed at 21 weeks of age.). After rinsing the tissues with Krebs-Ringer (KRB) buffer with D-mannitol 10mM, they were mounted to facilitate scraping of fatty layers. Then, they were sliced longitudinally, and circles of tissue were obtained using a biopsy punch. Samples were kept at low temperature with ice-cold buffer during the entire procedure.

3.1.2 Secretion study

Each piece of upper jejunum or ascendant colon was placed on a 24-well plate containing 350 µL of KRB buffer with D-mannitol 10 mM, pre-warmed to 37 °C for 15 min. After this first incubation period, the buffer was replaced by the same volume of pre-warmed treatments diluted in KRB buffer with D-glucose 10mM. In this case, as we were studying enterohormone profile secretion, complete, gastric and intestinal digestions and non-digested samples[31,32] were used as treatments, trying to simulate the main stages of *in vivo* digestion. As this model tries to match what happens physiologically, digestion consists in 3 steps: oral, consisting in samples treated with amylase for 2 minutes; gastric, consisting in samples being treated with pepsin for 2h and intestinal, with samples being treated with biliar salts and pancreatine for 2h, all three steps performed at 37ºC[31]. In our study, complete digestion consists in all three steps, gastric digestion consists in oral and gastric digestions and intestinal digestion consists in oral and intestinal digestions. Although this does not occur physiologically, intestinal digestion was

also used as a condition to better know if there are interesting bioactive peptides, as they can be used by the industry with different applications. Peptone was used as positive control (50mg/mL, 16% protein)[33], KRB D-glucose as negative control of secretion. The incubation period was 30 min for the total GLP-1, GIP, PYY and CCK secretion studies and 90 min for the total ghrelin secretion study. After the incubation period, the medium was collected in aliquots and stored at -80ºC for further analysis. Secreted enterohormones were analyzed using commercial ELISA kits. Total GLP-1 (Catalogue no. EGLP1T-36K), GIP (Catalogue no. EZRMGIP-55K) and total ghrelin (Catalogue no. EZGRT-91K) kits were purchased from Millipore (Billerica, MA, USA). Total CCK (Catalogue no. EKE-069-04) and PYY (catalogue no. FEK-059-03) kits were purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA).

3.2 *In vivo* studies

Thirty-two, eight-week-old, Wistar female rats (Janvier, Le Genest-Saint-Isle, France) were chosen as the model of the *in vivo* study. The animals spent 10 days under standard conditions through the adaptation process. The first 3 days, rats were caged in pairs at 22ºC with a standard 12h light-dark cycle, ventilation, *ad-libitum* access to water and a standard Teklad diet (Cat no: Teklad 2014, Envigo+++, Barcelona, Spain) consisting of 67% carbohydrates, 13% fats and 20% protein. After this short period, animals were caged individually in the same conditions stated before.

After the adaptation period, the animals were weighed before distribution in groups. 8 rats were placed on the standard group (STD), defined as the healthy control and were not exposed to cafeteria diet. 24 rats were placed on a cafeteria diet for 4 weeks to trigger the development of metabolic syndrome[34]. After these four weeks, rats exposed to cafeteria diet were divided into 3 groups, with the same average initial body weight (BW) for each group. The groups were as it follows: cafeteria diet (CAF), defined as the obesity control; 1X, who were given the treatment with the protein extract at a low concentration and 20X, who were given the treatment with the protein extract at a high concentration. Treatments

were administered daily by voluntary feeding until the end of the study 30 minutes before lights went off. Food was removed at 13:00 and provided again when lights went off (17:00) every day. The cafeteria diet given to the animals consisted of bacon, sausages, carrots, biscuits with paté, muffins and milk with sugar, providing 20.73kJ/g. This diet was given alongside chow diet, inducing voluntary hyperphagia[35].

During the study, various approaches were employed to assess the effects of the supplementation with the protein extract, including weekly BW monitoring, food intake measurements, enterohormone secretion, gastric emptying, indirect calorimetry, intestinal motility and faecal sample collections for microbiota analysis. At the end of week 10, the rats were euthanized, and relevant tissues and organs were collected. Due to time constraints, only a subset of the results has been fully analyzed and included in this report. Further analysis is currently ongoing.

3.2.1 Food intake

Food intake is measured once a week with the aim to monitor energy intake. To measure it, food is weighed at the start and end of the period and the difference between both values is stated as the food intake (20 hours record of food intake from 17:00 to 13:00 next day). The cafeteria groups were given a high-sucrose emulsion (HS)[36]containing in weight: 10% powdered skimmed milk, 40% sucrose, 4% lard and 0.35% xanthan gum (Sigma-Aldritch, St Louis, MO) given together with chow diet and in the STD group, food intake was measured using standard chow diet alone.

To better characterize the effects on food intake of the protein extract, a deeper analysis was performed, named food intake timing, at weeks 4 and 8. For this, rats were fasted at 13:00, the vehicle or pretein extract was supplied to the animals at 16:30 and at 17:00, as lights go out and rats start their active period, food was provided to the animals and it was monitored 1h, 5h, 20h after the lights were off.

3.2 Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). Shapiro-Wilk test was applied to test normality of the data. Data was analysed using Friedman test with Dunn's Multiple Comparison *posthoc* test to compare multiple groups when samples are paired, Kruskal-Wallis test when they were not or Mann Whitney U test to examine differences between two groups. P-values under 0.05 were considered statistically significant. All calculations were performed by IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp and GraphPad Prism version 8.0.1 for Windows, GraphPad Software, Boston, Massachusetts USA.

4. Results

4.1 *Ex vivo* enteroendocrine secretions

To assess and characterize enteroendocrine secretions of ghrelin, CCK, GIP in upper jejunum and GLP-1 and PYY in colonic samples, we exposed rat explants for 30 minutes (90 minutes for ghrelin) to different conditions to see differential stimulation of the secretion of enterohormones.

Only positive control induced a significant increase in CCK secretion compared to the negative control and gastric digestion (Figure 1A). None of the treatments tested—complete, gastric digestion, intestinal digestion, or non-digested—were able to induce CCK levels statistically higher than the negative control. GIP secretion was significantly elevated in response to complete digestion, intestinal digestion, and non-digested treatments compared to the negative control (Figure 1B). No significant differences were observed among these three treatments, suggesting a robust and nonspecific response to different types of stimuli derived from the protein extract studied. Surprisingly, the orexigenic hormone ghrelin was most highly secreted in response to the non-digested condition, intestinal digestion and positive control (Figure 1C). These groups differed significantly from the negative control, which had the lowest levels. These results suggest that the presence of undigested or partially digested material may stimulate ghrelin secretion.

Total GLP-1 levels showed significant differences in response to the different treatments (Figure 2A). Treatment with the intestinal digestion induced the highest GLP-1 secretion, followed by gastric digestion which also increased GLP-1 levels compared to the negative control, suggesting that the intestinal and gastric digestion are the most effective stimulus for the release of GLP-1 in ascending colon. On the other hand, Figure 2B shows that complete and intestinal digested treatments promoted higher levels of PYY secretion compared

to the negative control. This indicates that PYY, an anorexigenic hormone secreted in the ileum and colon, can be stimulated by peptides present in complete or partially digested products in the intestine.

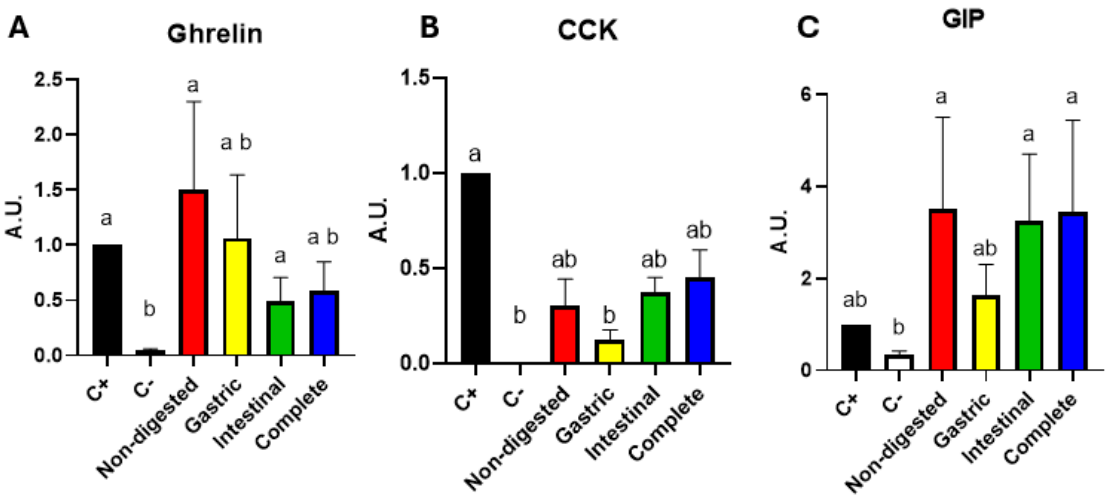

Figure 1. Levels of total ghrelin (A) , CCK (B) and GIP (C) after stimulation (3mg protein/mL). Treatments are peptone 5mg/mL (C+), KRB-glucose (C-) and complete, gastric, intestinal digested samples and non-digested condition. Results were normalized respect to the C+ and presented as mean ± SEM, n=8. A.U, arbitrary units. Friedman test with Dunn's Multiple Comparison posthoc test for multiple comparisons. Different letters (a, b, c) indicate significant differences (*p-value* < *0.05*).

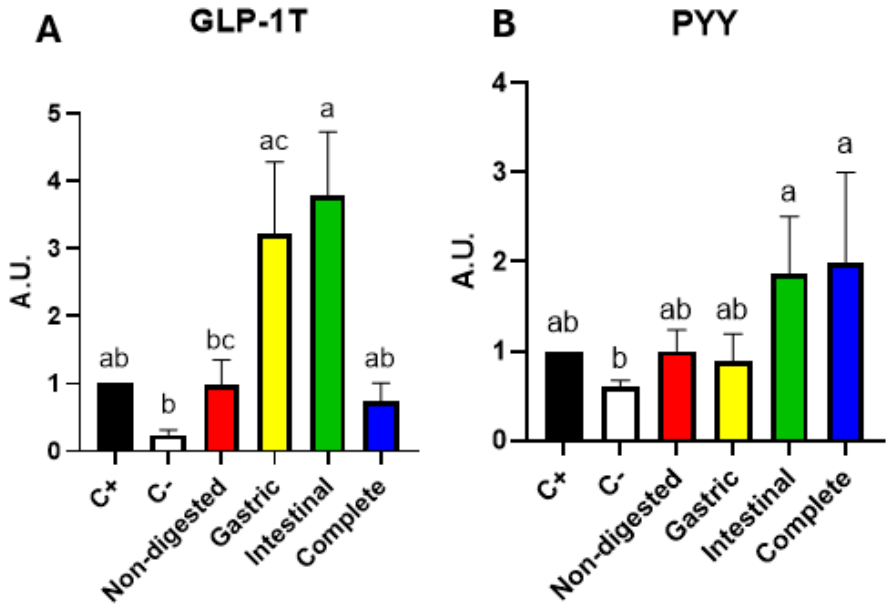

Figure 2. Levels of total GLP-1 (A) and PYY (B) after stimulation (1mg protein/mL). Treatments are peptone 5mg/mL (C+), KRB-glucose (C-) and complete, gastric, intestinal digested samples and non-digested condition. Results were normalized respect to the C+ and presented as mean ± SEM, n=8. A.U, arbitrary units. Friedman test with Dunn's Multiple Comparison posthoc test for multiple comparisons. Different letters (a, b, c) indicate significant differences (*p-value* < *0.05*).

4.2 *In vivo* outcomes

4.2.1 Body weight

Body weight was monitored weekly and analyzed only until week 8, since after that rats suffered different stressful procedures (not included in this project) that could produce altered results.

In Figure 3A, we can see that STD group shows a gradual increase in weight, expected in a normal growth process. The CAF group showed a more pronounced weight increase, being significantly higher than STD group since week 4 (Figure 3B). The 1X and 20X groups showed a weight gain trend similar to the STD group during first week of treatment (week 5) and later continued to increase weight to a lesser extent than the CAF group, although no statistical differences were observed. At the end of the study (week 8), only CAF and 20X group were statistically different from the STD group.

The CAF group body weight was still higher than the STD group after 8 weeks of cafeteria diet, compared to the STD group receiving the standard diet, as expected (Figure 3C). The three cafeteria-fed (CAF, 1X, and 20X groups) showed a higher than the STD group although the groups receiving the protein extract for 4 weeks showed a subtle reduction in body weight compared with the STD group, not statistically different. Regarding the body weight gain during the last 4 weeks (weeks 5 to 8), the lower dose of the protein extract (1X) seemed to be more effective in limiting body weight increase caused by the cafeteria diet, since the 20X group showed no differences compared with CAF group while a trend was observed between 1X and STD groups (Figure 3D).

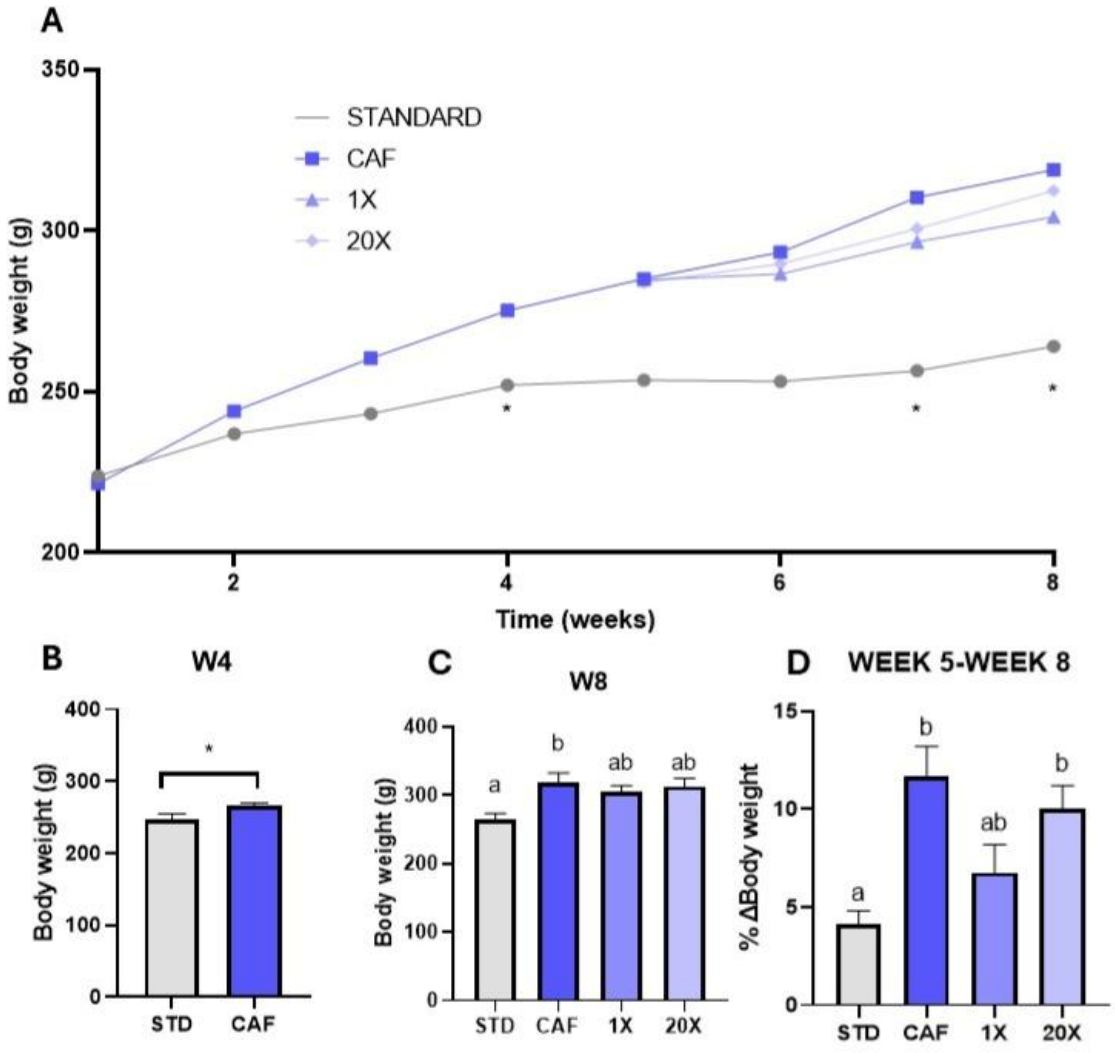

Figure 3. Body weight evolution over time in different experimental groups (A), body weight at week 4 (B) and week 8 (C), and percentage change in body weight between week 5 and week 8 (D). The groups were: STD (standard diet), CAF (cafeteria diet), 1X (low dose of the protein extract), and 20X (high dose of the protein extract). The asterisk (*) indicate significant differences ($p<0.05$) between the indicated group and CAF group. Bars with different letters (a, b) indicates significant differences ($p<0.05$) between groups according to Friedman's test followed by Dunn's posthoc test for multiple comparisons.

4.2.2 Food intake

Food intake was monitored weekly until week 8, since after that rats suffered different stressful procedures that could alter the results.

Figure 4A shows the average weekly food intake (in kcal) for the different experimental groups from week 4 (W4) to week 8 (W8). The STD group

consistently showed significantly lower weekly food intake compared to the other three groups throughout the weeks analyzed. The CAF, 1X, and 20X groups showed similar weekly food intakes throughout the study, with no significant differences between them in each week analyzed. Slight variability in weekly food intake was observed within each group over time, even though it was not significant, except for the STD group that was quite constant as shown in the graph.

At week 4 the acute effects on food intake of a unique dose of the protein extract was evaluated in more detail. Figure 4B shows the food intake measured at different timepoints (1, 5, 15, and 20 hours) after lights-out, the active phase of the animals. The four experimental groups are compared at each timepoint. At 1 hour after lights-out, only group 1X had a significantly higher intake compared to STD group. At 5 hours after lights-out, a significant increase in food intake was observed also in 20X group and the 1X group still had a significantly higher intake compared to STD group, while CAF group only showed a trend to a higher intake. At 15 and 20 hours after lights out, food intake continued to be increased in the CAF groups as well as in the supplemented groups (1X, 20X), showing no significant effect of the protein extract food intake modulation. No significant differences were observed between the supplemented groups at any of the analyzed timepoints.

Figure 4C presents the average cumulative food intake for each group at week 8, after a chronic supplementation for 4 weeks and cafeteria diet. 1 hour after lights-out, food intake was similar between cafeteria fed groups, with the CAF group showing an intermedium profile between STD and supplemented groups. From 5 to 20 hours after lights-out, CAF, 1X and 20X groups show significantly higher cumulative intake, like the observed at week 4.

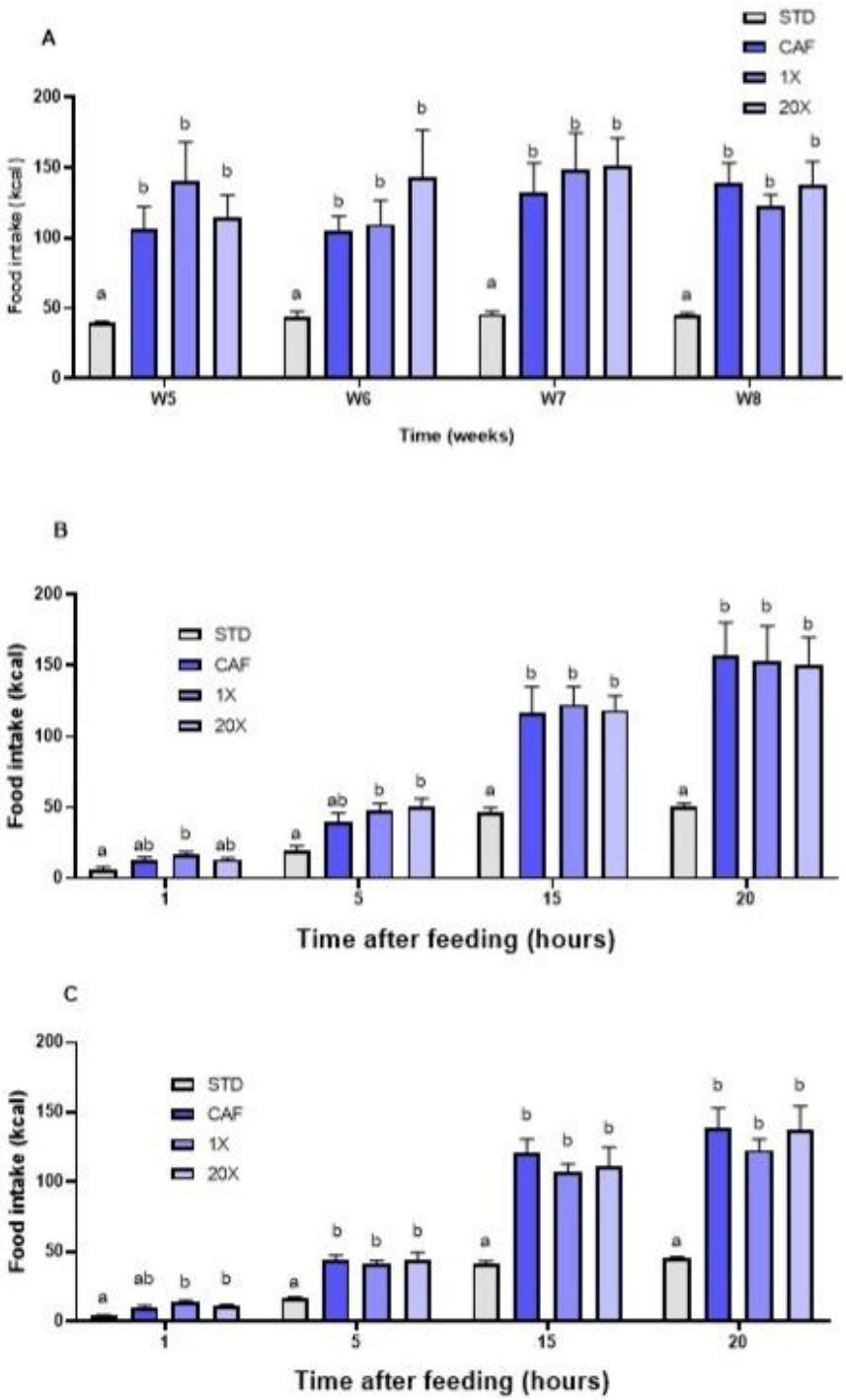

Figure 4. Weekly food intake (20h) from week 5 to week 8 (A), cumulative food intake at different times after lights-out in week 4 (B), and cumulative food intake at different times after lights-out in week 8 (C) in the different experimental groups: STD (standard diet), CAF (cafeteria diet), 1X (low dose of the protein extract) and 20X (high dose of the protein extract). Bars with different letters (a, b) indicate significant differences ($p<0.05$) between groups according to Friedmann's test followed by Dunn's posthoc test for multiple comparisons. Analysis is performed comparing between groups in the same week (A) and comparing groups in the same timepoint (B, C).

5. Discussion

In the present study, we first compared the enterohormone secretion profile in rat ex vivo samples of jejunum with different digestions[31], as explained before.

We assayed the enterohormonal response to different conditions in upper jejunum, showing that no digestion of the protein extract could increase the CCK secretion, while GIP secretion was significantly modified by intestinal, non-digested and complete digestions and ghrelin secretion by intestinal and non-digested conditions. Regarding CCK, physiologically is more expressed in duodenum and jejunum[9]. In our study, the 3 types of digestions and the non-digested peptide extract were able to increase the secretion in comparison to C- even though not statistically significant. Other studies found that gastrically and completely digested proteins stimulate the CCK secretion in pig duodenum explants[37]. Also, different amino acids stimulate CCK secretion in rat duodenum[38], being an area with higher expression of CCK than jejunum, because most of the I cells (CCK secretory cells) are present in higher spots of the small intestine[39,40]. CCK is generally secreted after stimulus like protein hydrolysates, amino acids or LCFA's, but in our model it was only stimulated significantly by C+[41], that is a protein extract but from a different source, which can tell us that the protein type, rather than the hydrolysis degree, might be relevant for CCK secretion in jejunum.

In regard to GIP, non-digested, intestinal and complete digestions are the conditions that stimulated the secretion of the hormone. GIP secreting-cells are mostly placed in upper jejunum[11], meaning that mainly gastric digestions and non-digested should be the conditions that stimulate GIP secretion at higher levels, mimicking physiological conditions. But in our study, the gastric digestion of the protein extract was not able to stimulate GIP secretion. On the other hand, the peptides generated by the intestinal and complete digestions promoted

significantly higher secretion of GIP, together with the non-digested extract. Some of our results contradict other results already published that showed that non-digested protein was unable to increase GIP levels[42]. However, other findings support our results, even though some of them are not in animal models, stating that secretion levels may rise if protein is more degraded[41–47], meaning that intestinal and complete digestions would be the best to increase GIP secretion.

Concerning ghrelin, it is secreted mostly in the stomach but also in lower levels by X cells in the duodenum and upper jejunum[4,7]in fasting conditions. Our results show that non-digested and intestinal digested samples surprisingly stimulate ghrelin secretion, when physiologically levels should be lower after food ingestion, due to the nature of its effects[8]. Other studies from our group have shown that a GSPE extract also increases ghrelin levels after an acute or sub-chronic treatment administered to rats[48] but not when used *ex vivo* or *in vitro* and that monomeric molecules also stimulate ghrelin secretion *in vitro*, while oligomeric molecules decrease them[49]. On the other hand, other studies show that proteins and amino acids successfully reduce ghrelin levels in different animal models[37,50–53]. Hence, the nature of the molecules and/or the degree of hydrolysis can severely affect the results depending on the model of study. These discrepancies may happen because in *ex vivo* models we don't have any regulation from the central nervous system. The stimulation via the non-digested protein extract in jejunum may as well suggest that the components of the raw extract can trigger ghrelin secretion and induce more satiety through the ghrelin system than the peptides released after gastric digestion.

These results suggest that there is not a unique digestion product that provides a stable secretory profile in upper jejunum and it varies between hormones.

In ascendant colon, we also measured secretion of GLP-1 and PYY. Both gastric and intestinal digested conditions increase GLP-1 secretion compared to negative control, as expected for the intestinal digestion, due to the nature of this hormone, secreted mostly in colon[1]. Other studies from our group have found that different protein stimulus enhance GLP-1 secretion both in cells[16] and rats[16,37,54]

and similar results were found by other studies[55–58]. Surprisingly, complete digestion does not stimulate GLP-1 secretion while the gastric one does. Therefore, there might be bioactive peptides generated from the gastric enzymes that can stimulate GLP-1 directly in colon explants. PYY secretion is increased by complete and intestinal digestions as expected since most of the secretion of this hormone is happening in colonic cells[1] physiologically. Some authors have noticed that PYY secretion is increased by proteins[55,59–61], but further studies are needed to increase specificity.

These results suggest that intestinal digestion is the best enhancer of the secretion of colonic secreted hormones, meaning that peptides derived from intestinal digestion provide the best secretion profile in ascendant colon.

In reference to *in vivo* outcomes and results, rats showed significant differences in body weight (STD and CAF groups), meaning that cafeteria diet made its effect and rats were a proper model to evaluate protein extract effects under obesogenic conditions. As protein extract supplementation starts (week 5), there's no difference in body weight between both supplemented groups (1X and 20X) and CAF. However, 1X group showed a promising trend of reducing the increase of body weight the further the study went, being even more noticeable in the last stances of the study (week 8), even though it was not statistically significant. The 20X group did not show a slowdown in body weight increase compared with the CAF group, which means that the protein extract effects may not have a lineal dose-response effect on body weight. Previous studies have shown that different protein sources can increase body weight, by changing the main protein source[62] or by injection of a peptide[63] but also contradictory results were found that protein-based diets can lower body weight gain[64]. Therefore, the protein source can be the cause of those different results.

Regarding food intake, STD and CAF diets showed clear differences as expected, meaning that cafeteria diet model was correctly established. However, supplementation with the protein extract does not appear to have significant effects on food intake modulation, nor on acute or chronic time points. On the

other hand, these results imply that the hyperphagic effect of the palatable and energetically dense cafeteria diet are stronger than the effects that the protein extract could exert, neither in low nor high concentrations. Other studies have shown multiple peptides reduce that food intake via interactions with enterohormones[37,53,63,65,66].

Comparing body weight gain and food consumption, the results are similar except for 1X group. The lower dose of the protein extract significantly reduced body weight gain during the 4 weeks of supplementation although with no food intake modulation. This may suggest that the protein extract may not follow a linear dose-response effect, being the lower one the more effective in the modulation of body weight gain.

6. Conclusions

In the present study, the hypothesis that a protein extract modulates the secretion of enterohormones involved in appetite regulation, thereby reducing energy intake and attenuating weight gain, was evaluated in rats fed a cafeteria-type diet.

In the present *ex vivo* study, anorexigenic hormones (CCK, GIP, GLP-1 and PYY) were mostly stimulated by intestinal digestion in a general way, even though the secretion of some of them was also stimulated significantly by other digestions. However, these finding were not completely translated to an obesity *in vivo* rat model. Nevertheless, formulation strategies aimed at promoting intestinal release of the active peptides could enhance the satiating effect of the extract in the future. On the other hand, only relevant changes in body weight after supplementation with a low dose of the protein extract were observed, which suggests that the protein extract may not follow a dose-response effect and that the mechanisms behind the reduction in body weight gain may be more on the metabolic side, changing metabolic rate rather than being anorexigenic.

To better understand these results, further analysis on plasma levels of GLP-1, PYY, GIP, CCK and ghrelin after acute and chronic administration of the extract might be helpful.

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