Grape seed proanthocyanidins target the enteroendocrine system in cafeteria diet-fed rats.

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List of abreviations: CCK, Cholecystokinin; GLP-1, Glucagon-like peptide-1; PYY, Peptide YY; T2DM, type 2 diabetes mellitus; GI, gastrointestinal; RQ respiratoy quotient; STD, control group; CAF, cafeteria treatment; SIT-CAF, Simultaneous-Intermittent-Treatment-CAF; CORR500-CAF, corrective treatment with 500 mg GSPE/kg; CORR100-CAF, corrective treatment with 100 mg GSPE/kg; KRB buffer, Krebs-Ringer Bicarbonate buffer;

SCFA, short chain fatty acid; FID, ionization detector; SEM, error of the mean; GOAT, ghrelin-activating enzyme; SEM-standard error of the mean.

Abstract

Scope: we analysed the effects on the enteroendocrine system of three different grape seed proanthocyanidin extract (GSPE) treatments in rats on a cafeteria diet for 17 weeks.

Methods and results: GSPE was administered in a corrective manner (15 last days of the cafeteria diet) at two doses, 100 and 500 mg GSPE/kg bw. A third longer treatment where GSPE (500 mg/kg bw) was administered daily every other week during the 17 weeks of the cafeteria diet was also tested. Most of GSPE treatments led to ghrelin accumulation in the stomach, limited CCK secretion in the duodenum and increased GLP-1 and PYY mRNA in colon. GSPE also increased caecal hypertrophy and reduced butyrate content. When the treatment was administered daily every other week during 17 weeks, there was too an increase in colon size. These effects were accompanied by a reduced food intake at the end of the experiment when GSPE was administered at 500mg GSPE/kg during the last 15 days, but not on the other treatments, despite a reduction in body weight had been observed in the longer treatment.

Conclusion: GSPE modulates the enteroendocrine system in models in which it also reduces food intake or body weight.

1. Introduction

Obesity is a primary risk factor for the most prevalent diseases affecting the worldwide population, including cardiovascular disease, type 2 diabetes mellitus (T2DM) and inflammation [1]. It is regulated by a complex biochemical process and its pathological mechanisms have been widely studied in adipose tissue, the liver and muscle [2,3]. The gastrointestinal (GI) tract plays a role in controlling the metabolism through peptide hormones secreted by enteroendocrine cells. These hormones from the gut play a central role in nutrient intake signalling, and regulating appetite and energy expenditure. There is evidence that specific enterohormones administered at physiological concentrations can influence the appetite of rodents and humans (reviewed in [4]). 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 PYY secretion and is used to treat obesity. Therefore the modulation of enterhormone signalling may represent an important target for preventing obesity and related/associated pathologies.

Natural compounds could be used to prevent the development of overweight and obesity-related problems from early preclinical stages [5]. Of these, grape-derived proanthocyanidins have been described as potential bioactive compounds that exhibit a wide array of beneficial effects on health. They have been reported to improve lipid [6] and glucose [7] metabolism, and although there are discrepancies in the literature, several studies show reduction in body weight gain and increase in energy expenditure (reviewed in [8]). Over the years *in vitro*, *ex vivo* and *in vivo* studies as well as clinical trials have provided ever-increasing evidence of the role polyphenols play as potential health compounds [9, 10]. However, the scientific evidence shows that the beneficial effects of polyphenols on health are directly linked to their absorption, distribution, metabolism and excretion. Some mechanisms used by flavonoids are exerted in the intestine, such as the inhibition of the enzymes that participate in carbohydrate digestion and the reduction of glucose absorption through the inhibition of the transporters involved in glucose uptake (reviewed in [7]). Moreover,

flavonoids have also been reported as having effects on the incretin system, which could be related to an improvement in glucose homeostasis (reviewed in [11]). Grape seed proanthocyanidins have been shown to modulate the enteroendocrine system. Acute grape seed proanthocyanidin extract (GSPE) treatment promotes the secretion of GLP-1 *in vivo* [12], while *ex vivo* studies also show modulation of PYY secretion [13]. GSPE and the specific pure compounds it contains modulate ghrelin production and secretion in cell lines, and *in vivo* studies also show acute and subchronic effects of GSPE on this hormone [14]. However, these effects have been shown in animals fed a standard diet or as acute effects on a palatable diet; whether these effects will be maintained under obesogenic diet, where the metabolic flexibility has been disrupted, remains unclear.

We have recently shown that 500 mg GSPE/kg bw administered daily every other week for a period of 17 weeks in animals fed a cafeteria diet reduces body weight gain and respiratoy quotient (RQ) [15]. In this study, we analyse the effects on the enteroendocrine system of this GSPE treatment in the context of an obesogenic diet intervention, and compare it with the effects of GSPE administered in a corrective manner (for only 15 days).

2. Material and methods

2.1 Proanthocyanidin extract

The grape seed extract enriched in proanthocyanidins (GSPE) was kindly provided by Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE composition used in this study (Batch number: 124029) contains monomers of flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5-13 units; 31.7%) of proanthocyanidins. A detailed analysis of the monomeric to trimeric structures can be found in Margalef et al. [16].

2.2 <u>Animal experiments</u>

Female rats weighing 240-270g were purchased from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, the rats were individually caged in animal quarters at 22°C with a 12-hour light/12-hour dark cycle and were fed *ad libitum* with a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. After a period of acclimation, the animals were randomly distributed into 5 experimental groups (n=7-10/group) and fed *ad libitum* a standard chow diet for the whole duration of the experiment. The control group (**STD**) received only the standard chow diet. All the other groups, received a cafeteria diet as a model of a high fat/high sucrose diet. The cafeteria diet consisted of bacon, sausages, biscuits with pâté, carrots, muffins and sugared milk, which induces voluntary hyperphagia, in addition to the standard chow [17]. This diet was provided fresh *ad libitum* every day to the animals for 17 weeks. One of these groups was used as a control for cafeteria (**CAF**), while the three remaining groups received the cafeteria diet and also received an oral GSPE supplementation (see 2.2 Dosage information).

Body weight was monitored weekly. 20-hour food intake was measured right before the corrective treatments (week 15) and in the last week of the experiment (week 17). Food intake was calculated for each individual animal by weighting the food, and the caloric intake was calculated according to the caloric content of each food provided by the manufacturer. On the days of the measurement a simplified cafeteria diet was used, which consisted on the usual above mentioned cafeteria diet with the exception of muffins and biscuits with paté, that were not included.

All the procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (code: 0152S/4655/2015).

2.3 Dosage information.

Study intervention is summaryzed in Figure 1. The treatments performed to the animals fed a cafeteria diet, as previously described [15], were the following: a) a dose of 500 mg GSPE/kg administered from the beginning of the cafeteria diet feeding until the end of the experiment, daily every other week (Simultaneous-Intermittent-Treatment-CAF; **SIT-CAF**), b) a dose of 500 mg GSPE/kg daily administered during the last 15 days of the cafeteria intervention as a corrective treatment (**CORR500-CAF**), or c) a dose of 100 mg GSPE/kg daily administered during the last 15 days of the cafeteria intervention as a corrective treatment (**CORR100-CAF**). To control gavage, every week in which the SIT-CAF group was receiving daily days gavage (7 days), the other groups (SD, CAF, CORR) received two separate days gavage with vehicle (tap water). The last week before sacrifice, in which SIT-CAF and CORR-CAF groups received daily GSPE gavage (see Figure 1), CAF group also received daily administration of vehicle gavage.

For the administration, at 17:00h food was removed and at 18:00h GSPE dissolved in water was orally gavaged in a volume of 500 µL. The animals not supplemented with GSPE received water as a vehicle. At 19:00 food was replaced.

The dose of 500 mg GSPE/kg was chosen due to its effects at modulation of enteroendocrine system observed after acute treatments and in standard-fed rats [18, 19]. This dose corresponds to 81 mg/kg bw in adult humans, when considering the body surface area according to Reagan-Shaw et al. [20]. This is a dose achievable through supplements. . The GSPE administration in the SIT-CAF group, was performed every other week so animals could recover from the daily oral gavage, and avoid that a prolonged exposure led to desensitizing effects on cell receptors. In addition,it reduced the total administered dose.

2.4 Blood and tissue collection

At the end of the study, the animals were fasted for 1-4 hours, anaesthetized with sodic pentobarbital (70 mg/kg body weight; Fagron Iberica, Barcelona, Spain) and exsanguinated from the abdominal aorta. The blood was collected using lithium heparin (Deltalab, Barcelona, Spain) as an anticoagulant. The blood was collected and aliquoted. Samples to measure GLP-1 were treated with a commercial Dipeptidyl peptidase-4 inhibitor (DPPIV, Millipore, Madrid, Spain) and a serine protease inhibitor (Pefabloc SC, Roche, Barcelona, Spain). The samples to be analysed for active ghrelin were treated with the serine protease inhibitor and 0.1 M HCl. All the samples were stored at −80ºC. Plasma was obtained by centrifugation (1500g, 15 minutes, 4°C) and stored at −80°C until analysis. The caecum was quickly weighed before and after caecal content removal. Intestinal segments were measured. The caecal content together with stomach and intestinal segments from the duodenum, jejunum, ileum and proximal colon were immediately frozen in liquid nitrogen and then stored at -80º C for further analysis.

2.5 Ussing chamber methodology

Intestinal segments of 0.28 cm^2 were mounted in Ussing chamber apparatus (Dipl.-Ing. K. Mussler Scientific Instruments, Aachen, Germany). Up to 6 segments from each animal were used. Mucosal compartments were filled with 1.5 ml KRB buffer (with D-Mannitol 10 mM) and the serosal compartments filled with KRB buffer (with D-Glucose 10 mM) [21]. The chambers were kept at 37 °C and continuously oxygenated, 95% O_2 /5% CO_2, with a circular gas flow. Before starting the measurements, tissues were equilibrated for 15 min in the chambers to achieve steady-state conditions in transepithelial potential differences.

The transmucosal potential difference was continuously monitored under open circuit conditions and recorded through 0.8 mm Ag/AgCl glass electrodes. The basal transepithelial electrical resistance (RT) was calculated according to Ohm's law from the voltage

deflections induced by bipolar constant current pulses of 50 mA (every 60 s) with a duration of 200 ms applied through platinum wires (Mussler Scientific Instruments, Aachen, Germany).

After the the equilibration period, measurements of the secretion of enterohormones of intestinal segments obtained from the treated animals were performed.

2.6 Plasma and tissue hormone analysis

Enterohormones were analysed using commercial ELISA kits for, GLP-1 7-37 amide (Millipore, Billerica, MA, USA), desulfated CCK8 (Peninsula Laboratories, San Carlos, CA, USA), PYY (Phoenix Pharmaceuticals, Burlingame, CA, USA), and specific octanoyl ghrelin (Phoenix Pharmaceuticals, Burlingame, CA, USA).

2.7 mRNA analysis.

Total RNA was extracted using Trizol (Ambion, USA) and trichloromethane-ethanol (Panreac, Barcelona, Spain), and purified using a Qiagen RNAeasy kit (Qiagen, Hilden, Germany). The cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA). Real time PCR amplification was performed using specific TaqMan probes (Applied Biosystems, Waltham, USA): Rn01460420_g1 for PYY, Rn00563215_m1 for CCK, Rn00562293_m1 for proglucagon and Rn00572319_m1 for ghrelin. The relative amount of mRNA transcripts was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with cyclophilin A, PPIA (Rn00690933_m1), as reference.

2.8 Short chain fatty acid quantification

The concentration of short chain fatty acid (SCFA) (formic, acetic, propionic, butyric, isovaleric, valeric, lactic and succinic) were assayed in cecal content thawed at 4ºC. Briefly, approximately 1 g of sample was added to a screw cap glass tube containing 1 ml ultrapure deionized water (1:1, w/w), and was vortexed vigorously. 100 microliters of the internal standard 4-methyl-valeric acid, 2 ml of ethylic ether and 0.5 ml of HCl 37% were added to this samples, and in parallel to 1 ml a reference solution containing different concentrations of the standard SCFA. The tube was vortex mixed for 1 min and centrifuged for 15 minutes at 3500 g. 65 µl of supernatant was used for the derivatization step, performed with 10 µl of MTBSTFA ((N-methyl-N (tert-butyldimethylsilyl) -trifluoroacetamide), Aldrich 375934) at 80ºC for 30 minutes. Derivatized SCFAs were analysed using gas chromatography (Agilent 6890-NT, Santa Clara, USA,) coupled with a 30 m × 0.25 mm i.d., column, with a film thickness of 0.25 micras (Agilent DB-23, Barcelona, Spain) and a flame ionization detector (FID) to determine SCFA concentrations [22]. The carrier gas used was helium. A constant flow mode was used (split 25:1; 30 ml/min split flow). The column temperature was programmed to gradually increase from 60°C to 220°C during the analysis. In addition, injector port and FID temperatures were fixed at 250°C. Injection volume was set to 1 µl, and analyses were performed in duplicate [23].

2.9 Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). A Student's T-test was used to compare the treatments with the CAF group. p-values < 0.05 were considered to be statistically significant. These calculations were performed using XL-Stat 2017 software.

1. Results

3.1 GSPE limits ghrelin secretion in the stomach

The effects of the different treatments with GSPE on the enterohormone profile was studied throughout the gastrointestinal tract. As a first site of action, ghrelin was measured in the stomach. 500 mg/kg bw of GSPE administered simultaneously and intermittently with the cafeteria diet daily every other week (SIT-CAF) significantly increased the amount of ghrelin in tissue (Table1). This seemed not to be due to an increased production or increased activation (mRNA of ghrelin O-acyltransferase (GOAT) was significantly reduced in SIT-CAF). A corrective treatment led to a higher increase in ghrelin content in stomach (doubling the effect of SIT-CAF, p≤0.05), either with 500 mg/kg (CORR500-CAF) but also at a lower dose of 100 mg/kg bw (CORR100-CAF). In this case the ghrelin-activating gene (GOAT) mRNA was not reduced as in SIT-CAF. This accumulation in the cells was accompanied by a reduction in plasma levels (30-40%), although this was not statistically significant. The effects of GSPE in the duodenum might not contribute to action on ghrelin since it was below detection limits in both quantity and mRNA.

3.2 In the duodenum GSPE limits basal CCK secretion.

Duodenal sections of treated animals were mounted in Ussing chambers in order to analyse basal CCK secretion. Table 2 shows that the SD group has a lower CCK secretion compared to CAF animals, and higherCCK tissue content. The SIT-CAF group also had a lower basolateral CCK levels compared to the CAF group (and no different than SD, t-test p>0.05), thus avoiding the stimulation of basal secretion exerted by the cafeteria diet. This reduced secreted CCK levels did not seem to reflect defects in CCK production, since mRNA levels or tissue content were not modified. The same dose applied as a corrective

treatment showed no significant effects on basolateral CCK secretion although tissue content was significantly increased compared to the CAF. A lower dose (CORR100-CAF) did reduce basolateral CCK levels compared to CAF (0.334 ± 0.01, p≤0.05). The effects were not translated to CCK plasma levels, where CCK values were not significantly modified by any of the GSPE treatments, while the SD animals showed a tendency to have lower plasma CCK levels compared to the CAF group.

3.3 GSPE treatments modulate GLP-1 and PYY .

Total GLP-1 and PYY were measured in the ileum and the colon in all the treatments. A corrective treatment with 500 mgGSPE /kg bw significantly decreased PYY gene mRNA in the ileum, opposite to the effects of the CAF that tended to increase it (Table 3). However, this treatment increased the mRNA of PYY and GLP-1 in the colon, while no stimulation was observed in PYY secretion of samples mounted in Ussing chambers or plasma levels at sacrifice. When the same dose of GSPE was administered in a simultaneous intermittent way for 17 weeks, the effects in the colon were somehow maintained, but they were different in the ileum, where PYY was not changed and GLP-1 tended to increase. When the corrective treatments were performed with a lower dose, a different profile was observed, since the mRNA increase in PYY and GLP-1 was found in the ileum, but GLP-1 in the colon was maintained at control levels. In this case, however, total plasma GLP-1 was significantly increased and PYY decreased by the CORR100-CAF treatment. Also, the corrective treatments showed a tendency to oppose the CAF PYY secretion profile in ex vivo samples, since PYY basolateral levels tended to be lower than in the CAF, and were no different (t-test, p<0.05) than the SD .

3.4 GSPE reduces cecal butyric acid.

A simultaneous intermittent GSPE treatment increased colon size compared to the CAF animals (15.42 ± 0.4 and 17.00 ± 0.6 cm in CAF and SIT-CAF groups respectively, p<0.05), while the corrective treatments did not modify it. No differences in small intestine size were observed in any group. Despite the fact that the standard and the CAF animals showed no statistically significant differences in the small or large intestine, the CAF animals showed a significantly higher small intestine/colon length ratio (Figure 2.a). Such increased ratio was not found in the simultaneous intermittent GSPE treated rats, which had it normalysed (there were no significant differences between SIT-CAF and SD, T-test p>0.05) .

The caecum was weighed and Figure 2.b shows that the empty caecum weight was significantly increased by GSPE when this was administered simultaneously with the cafeteria diet, daily every other week for the 17 weeks' duration of the experiment. Interestingly, a corrective treatment of 15 days with GSPE at the same dose at the end of the experiment induced a significant increase in caecum weight, similar to that of the simultaneous intermittent treatment. The corrective treatment with a lower dose, however, did not modify caecum weight.

Short chain fatty acids of caecal content were measured. As can be seen in Table 1 supplementary materials, the standard animals had significantly higher butyric acid content (73%) and a tendency to lower propionic and isobutyric acid content when compared to the CAF group. The CORR500-GSPE treatment significantly reduced the butyric acid content compared to CAF rats (10.5 ± 1.4 and 19.07 ± 3.6 in CORR500-GSPE and CAF, respectively, p<0.05). The simultaneous intermittent treatment led to butyric acid levels not different from the corrective treatment (12.21 ± 3.0), despite values that were not statistically different from the CAF values. However, if considering the % of butyric acid in the caecal content (% of the sum of all other measured SCFA), then simultaneous intermittent and corrective treatments led to a reduction in its % (30.15 ± 0.9, 12.22 ± 1.5, 7.81 ± 1.2, 4.80 ±

0.2, 7.61± 0.9 in STD, CAF, SIT, CORR500, and CORR 100 respectively, all of them p≤0.05 versus CAF).

3.5 GSPE corrective treatment reduces energy intake.

Since enterohormones regulate food intake, we evaluated the effects of the different treatments on this parameter. 20 hours' energy intake was measured at two different occasions. When administered as corrective treatment, i.e. after 15 weeks of cafereria feeding, 500 mg GSPE/kg bw reduced energy intake (Figure 3). Such reduction was not accompanied by a decrease in body weight gain, as previously published [15]. Instead, the synchronic treatment, which led to reduced body weight gain [15], showed no reduced food intake at these time points. The lower dose 100 mg /kg BW dose (CORR100-CAF) showed no significant effects either on on body weight or food intake.

4.Discussion

In the present paper we describe that an intermittent GSPE treatment alters the intestinal enteroendocrine system in CAF rats. Interestingly, several parameters that are modified by the simultaneous intermittent treatment are also altered in a corrective manner, suggesting that the effects on the gastrointestinal tract do not require long-term treatments, although their possible translation to body weight homeostasis modulation does require a more prolonged treatment.

We find changes in stomach acylated ghrelin content, suggesting an accumulation of ghrelin not due to increased production. Our resuts show that only 15 days are needed to exert such an effect and that, when a simultaneous intermittent treatment is performed, the effects are still found but to a lower extent. Ghrelin has previously been shown to be a target for GSPE

[14]. In fact, in standard diet-fed animals, an 8-day treatment with the same GSPE dose (500 mg/kg bw) led to a lower plasma ghrelin due to a reduced production in the stomach and secretion in the intestine, in fasted animals. Our present results suggest that a reduction in secretion by subchronic treatment with GSPE in the stomach also takes place in animals on an obesogenic diet. This effect is mitigated when the treatment is performed intermittently for 17 weeks, possibly due to a long-term effect of GSPE on the regulation of the ghrelin-activating enzyme (GOAT) mRNA expression. In the present sacrifice conditions, with only 4 hours of fasting, we found no significant change in ghrelin plasma levels, possibly because it usually peaks right before meal initiation. In fact ghrelin has been shown to act centrally, modulating not only food intake but also energy homeostasis, since when administered to different parts of the brain it increases food intake but also reduces energy expenditure and increases RQ [24, 25]. Thus the modulation of ghrelin secretion could be related to the previously observed effects of GSPE in favouring the use of lipids as substrate [15], although further studies are required to confirm this hypothesis.

Concerning other enterohormones,our results show that in the duodenum, a simultaneous intermittent treatment with GSPE leads to lower basal secretion of CCK compared to the CAF animals, which might not be linked to a lower production, counteracting the effects of the cafeteria diet. A role for GSPE acutely inhibiting CCK secretion in explants [26] and *in vivo* [13] has been previously shown. However, it is unlikely that the present reduction in secretion is related to acute effects, since the last GSPE dose was administered 36 h previous to sacrifice and the tissues were thoroughly washed. CCK is released after stimulus such as aminoacids, protein hydrolyzates or long chain fatty acids, although in our system there was no such stimulus. Instead we measured basal CCK secretion, although for the moment we do not know which mechanism is involved in it. Thus a better understanding of the basal secretory mechanisms and the pathways by which cafeteria diet modifies them will help in elucidating the role of GSPE in this.

We found that, as previously shown for animals on a standard diet sub-chronically (8 days) treated with GSPE [19], a sub-chronic GSPE treatment has satiating properties in rats that have been subjected to an obesogenic (cafeteria) diet. The role of GLP-1 as a mediator of GSPE's acute inhibition of food intake has previously been demonstrated [27], and we have previously shown that GSPE stimulates GLP-1 secretion in a glucose dependent manner [26].

Despite we did not find significant changes in plasma GLP-1 in most of GSPE treatments, our results show that GSPE regulates its gene expression. It has previously been shown that GSPE acutely modulates GLP-1 mRNA depending on the feeding state [26], and studies have reported an increase in intestinal GLP-1 gene expression after a chronic GSPE treatment with a lower (25 mg/kg bw) dose in rats fed a cafeteria diet [12]. Here we confirm that GSPE modulates intestinal GLP-1 mRNA depending on the treatment and tissue. The differential effects found on GLP-1 mRNA between the ileum and the colon might also derive from the different molecules that reach these tissues, since the microbiota in the caecum and the colon contributes greatly to the metabolism of ingested structures. We found that 500 mg/kg bw increased the caecum empty weight, and this was maintained when the treatment was prolonged intermittently. Caecum enlargement has previously been shown by treatment with prebiotic fibres, and it parallelled a reduction in adiposity in high-fat diet-fed [28] and standard-fed animals [29]. Gut hypertrophy and hyperplasia have been shown as an effect of fibre after only 8 days' treatment, attributed to the fermentation products of dietary fibre. Consumption of nopal (a prickly pear cactus with high fibre and polyphenol content) on an high fat diet for 6 weeks results in an enlarged caecum and increased caecal SCFA (propionate, acetate, isobutyrate, isovalerate and valerate, but not butyrate) while at the same time it counteracts high-fat induced adiposity [30]. Modulation of caecum weight was accompanied by small changes in SCFA composition, where the GSPE treatments led to a reduced % of butyric acid. Butyric acid has been claimed beneficial effects on intestinal homeostasis and energy metabolism, although its role in obesity remains controversial (reviewed in [31]). Our results do not support that modulation of SCFA are directly involved

in the antiobesity effects of GSPE, but these reflect changes in the microbiota that take place after only 15 days of GSPE treatment, as previously observed in standard-fed animals after 8 days of GSPE treatment [18], and that might be maintained after a simultaneous intermittent treatment. These changes may contribute to the increased colon length that we found in the simultaneous intermittent treatment.

Finally we also found that the mRNA of PYY partially parallelled that of GLP-1, since it was up-regulated in colon or ileum depending on the treatment. However regardless of whether there is more PYY available in the cells, our results also show that its secretion is dependent on stimulus, since basal secretion from ex-vivo explants was not significantly altered.

To conclude, we show here that GSPE treatment in the context of an obesity-inducing cafeteria diet modulates the enterohormone system throughout the gastrointestinal tract. Our results suggest that 15 days with GSPE treatment modulates ghrelin accumulation in the stomach, CCK secretion in the duodenum and GLP-1 and PYY mRNA in the ileum or the colon (depending on the dose). It also increases caecal hypertrophy and reduces butyrate content. These effects are maintained if the treatment is performed every other week during 17 weeks, and then lead to an increase in colon size. These intestinal effects might be related to reduced body weight gain, although further studies on the metabolic role of enterohormones are required.

5. References

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Accepted Article

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Figure captions

Figure 1. Schematic diagram of the experimental design.
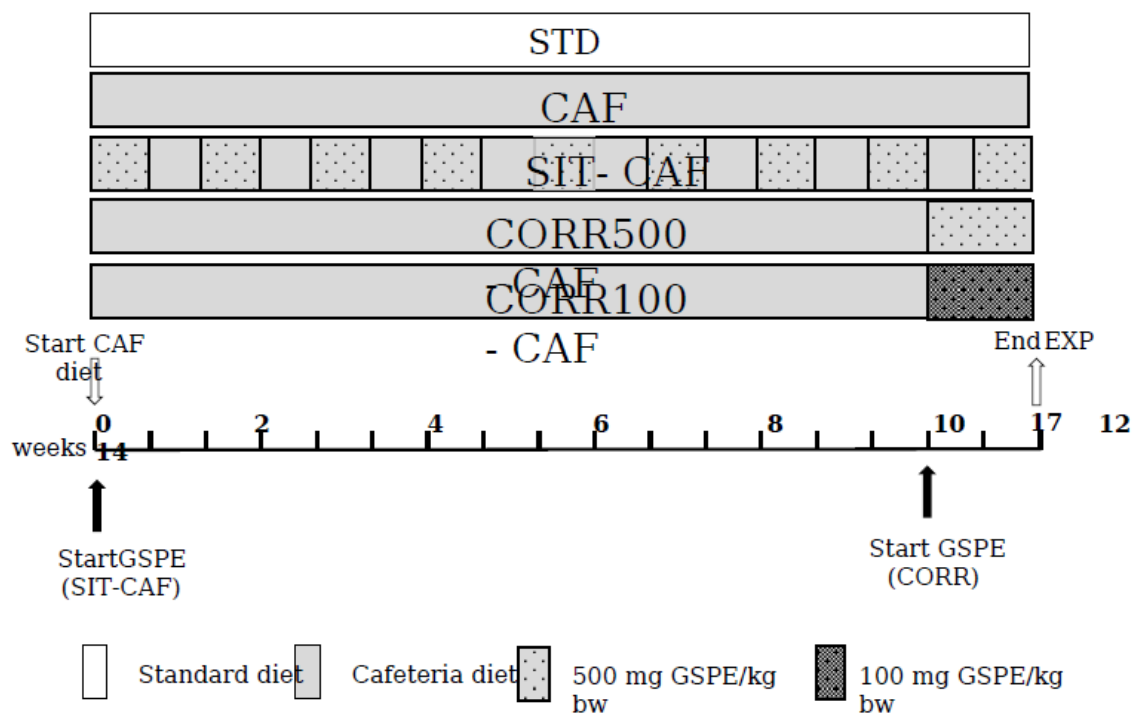
Figure 2. Effects of the different GSPE treatments on a) the length of the intestine (ratio between small intestine and colon length, measured in cm, and b) the weight of the empty caecum. STD: standard chow-fed controls, CAF: cafeteria-fed controls, SIT-CAF: rats fed cafeteria diet plus GSPE (500 mg/kg bw) daily every other week during 17 weeks, CORR500-CAF: 500 mg GSPE/kg daily administered during the last 15 days of cafeteria, CORR100-CAF: 100 mg GSPE/kg daily administered during the last 15 days of cafeteria. Values represent mean ± SEM of 6-10 animals per group. *$p \leq 0.05$ versus CAF, T-test.
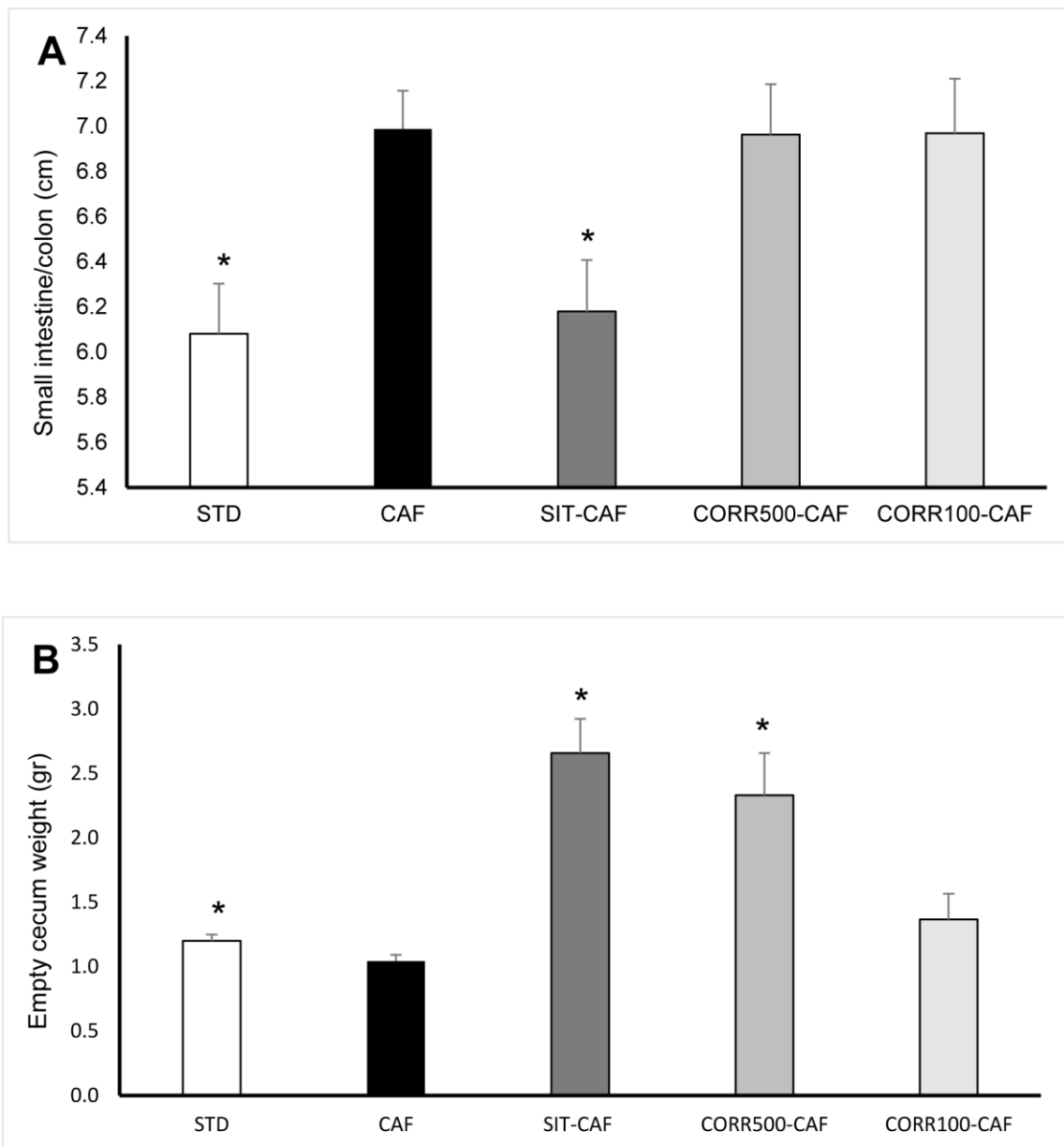
Figure 3. Effects of the different GSPE treatments on relative food intake between week 15 (before the corrective treatment) and week 17 (after the corrective treatment) in animals fed a cafeteria diet for 17 weeks. STD: standard chow-fed controls, CAF: cafeteria-fed controls, CORR500-CAF: 500 mg GSPE/kg daily administered during the last 15 days of cafeteria, CORR100-CAF: 100 mg GSPE/kg daily administered during the last 15 days of cafeteria. Values represent mean ± SEM of 5-9 animals per group. *p ≤0.05 versus CAF, T-test.
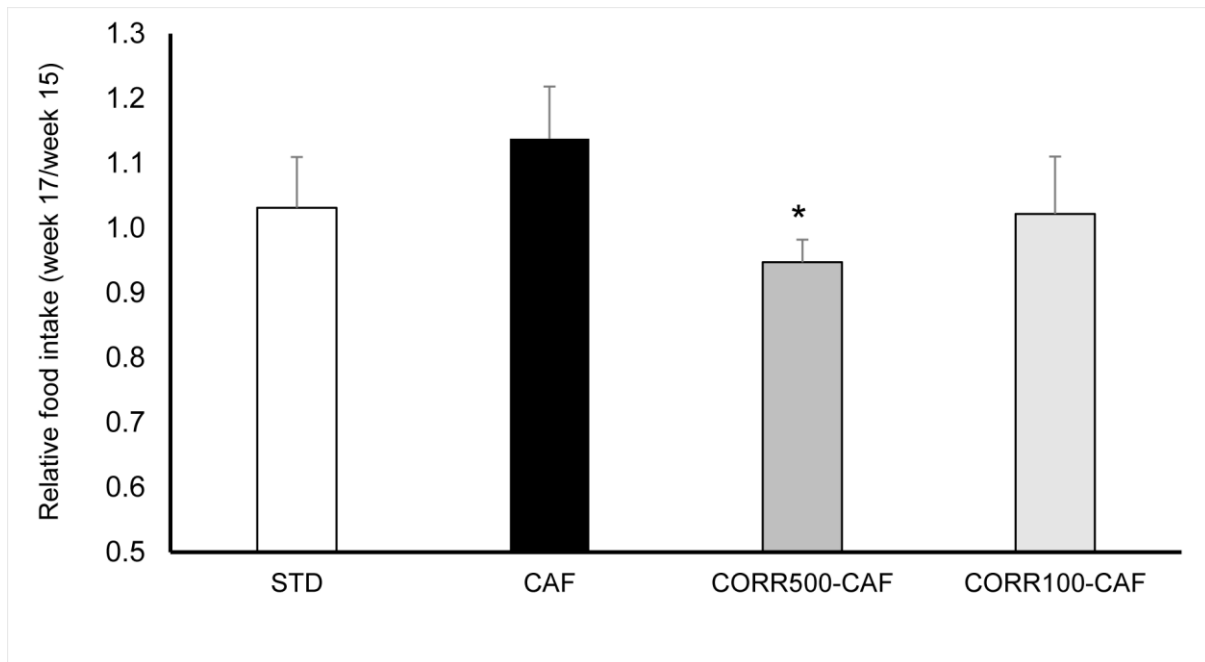
Tables

Table 1. GSPE effects on ghrelin.

	Ghrelin mRNA	GOAT mRNA	pg Ghrelin/µg tissue (stomach)	pg Ghrelin/ mL (plasma)
STD	0.74 ± 0.1 #	1.30 ± 0.17	1.90 ± 0.1	181.19± 21.10
CAF	**1.09 ± 0.2**	**1.01 ± 0.06**	**2.01 ± 0.2**	**240.30 ± 72.94**
SIT-CAF	1.44 ± 0.3	0.65 ± 0.06*	3.51 ± 0.5 *	162.38 ± 46.34
CORR500-CAF	1.38 ± 0.2	1.05 ± 0.08	6.76 ± 0.7 *	138.55 ± 31.75
CORR100-CAF	1.62 ± 0.5	0.96 ± 0.1	6.82 ± 1.3 *	NA

First column, ghrelin mRNA levels relative to cafeteria controls (CAF), in the stomach.
Second column, GOAT levels relative to cafeteria controls (CAF), in the stomach. Third
column, ghrelin content in stomach samples obtained at sacrifice. Last column, plasma
ghrelin concentration at sacrifice. Values represent mean ± SEM of 5-7 animals per group.
*$p \leq 0.05$ **versus CAF**; # $p \leq 0.1$ versus CAF.. NA: not analysed.

Table 2. Effects of GSPE treatments on CCK.

	CCK mRNA	ng CCK in basolateral medium	ng CCK/g tissue	CCK plasma levels (ng/ml)
STD	1.06 ± 0.2	0.316 ± 0.04 *	7.763 ± 1.00 *	0.38 ± 0.1 #
CAF	**1.07 ± 0.2**	**0.381 ± 0.04**	**3.041 ± 0.53**	**0.67 ± 0.2**
SIT-CAF	0.9 ± 0.3	0.321 ± 0.01 *	4.365 ± 0.91	0.89 ± 0.2
CORR500-CAF	1.5 ± 0.3	0.390 ± 0.04	8.071 ± 1.23 *	0.65 ± 0.1

First column, mRNA levels relative to cafeteria controls (CAF), in the duodenum. Second column, CCK levels found in the basolateral media of duodenum segments mounted in Ussing chambers. Third column, CCK content in duodenum samples obtained at sacrifice. Last column, plasma CCK concentration at sacrifice. Values represent mean ± SEM of 5-7 animals per group. *$p \leq 0.05$ versus CAF**; # $p \leq 0.1$ versus CAF.

Table 3. Effects of GSPE treatments on GLP-1 and PYY.

		ileum		colon		plasma
		mRNA	**pg /ml Ussing**	**mRNA**	**pg /ml Ussing**	**pg PYY/ml or pmol total GLP-1 /L**
STD	**PYY**	0.79 ± 0.09 #	55 ± 12.7	0.86 ± 0.15	104.49 ± 3.20 #	228.20 ± 66.3 #
	GLP-1	1.16 ± 0.32		1.25 ± 0.35		31.78 ± 3.99 #
CAF	**PYY**	**1.02 ± 0.09**	**67.33 ± 14.4**	**1.07 ± 0.17**	**145.10 ± 20.70**	**429.1 ± 106.7**
	GLP-1	**1.02 ± 0.09**		**0.91 ± 0.14**		**40.23 ± 3.52**
SIT-CAF	**PYY**	1.07 ± 0.11	60.53 ± 16.1	1.71 ± 0.25 #	113.99 ± 9.96	630.4 ± 170.02
	GLP-1	1.79 ± 0.46 #		2.20 ± 0.39 *		36.92 ± 4.25
CORR500-CAF	**PYY**	0.66 ± 0.08 *	91.71 ± 24.4	2.09 ± 0.21 *	105.56 ± 3.38 #	255.94 ± 48.7
	GLP-1	1.03 ± 0.23		2.28 ± 0.17 *		46.00 ± 5.05
CORR100-CAF	**PYY**	1.62 ± 0.07 *	78.35 ± 24.9	2.10 ± 0.54 #	109.82 ± 3.28 #	31.41 ± 8.22 *
	GLP-1	3.32 ± 0.92 *		1.06 ± 0.18		51.55 ± 2.64 *

First column, mRNA levels relative to cafeteria controls (CAF), in the ileum. Second column, enterohormone levels found in the basolateral media of ileal segments mounted in Ussing chambers. Third column, enterohormone mRNA levels relative to cafeteria controls (CAF), in the colon. Fourth column, enterohormone levels found in the basolateral media of colonic segments mounted in Ussing chambers. GLP-1 in basolateral medium was not detected. Last column, plasma enterohormones concentration at sacrifice. Values represent mean ± SEM of 5-7 animals per group. *$p \leq 0.05$ versus CAF*; # $p \leq 0.1$ versus CAF,   T-test.

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Text for graphical abstract:

In rats fed a cafeteria diet, GSPE modulates ghrelin accumulation, CCK secretion and GLP-1 and PYY gene expression. It also increases caecal hypertrophy, enlarges the colon and reduces butyrate content. 15-day GSPE limits food intake on an obesogenic diet, but a longer treatment does not.