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Differential effects of a cafeteria diet and GSPE preventive treatments on the enterohormone secretions of aged *vs.* young female rats

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Grape seed derived procyanidins (GSPE) have been shown to effectively prevent intestinal disarrangements induced by a cafeteria diet in young rats. However, little is known about the effects of procyanidins and cafeteria diet on enterohormone secretion in aged rats, as the ageing processes modify these effects. To study these effects in aged rats, we subjected 21-month-old and young 2-month-old female rats to two sub-chronic preventive GSPE treatments. After three months of cafeteria diet administration, we analysed the basal and stimulated secretion and mRNA expression of CCK, PYY and GLP-1, caecal SCFA and intestinal sizes. We found that the effects of a cafeteria diet on the basal duodenal CCK secretion are age dependent. GLP-1 in the ileum was not modified regardless of the rat's age, and GSPE preventive effects differed in the two age groups. GSPE pre-treatment reduced GLP-1, PYY and ChgA in mRNA in aged ileum tissue, while the cafeteria diet increased these in aged colon. The GSPE treatments only modified low-abundance SCFAs. The cafeteria diet in aged rats increases the caecum size differently from that in young rats and GSPE pre-treatment prevents this increase. Therefore, ageing modifies nutrient sensing, and the cafeteria diet acts mainly on the duodenum and colon, while procyanidins have a larger effect on the ileum.

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Introduction

Life expectancy is increasing in the world population, and obesity and diabetes are two major public health problems that are reaching pandemic proportions. Both dietary habits and food composition contribute to the onset of obesity and related diabetogenic situations. The aging process also contributes to their development, although there is no consensus on the main factors involved.[1] Aging is a biological process characterized by the progressive functional decline of many interrelated physiological systems at multiple levels of the biological organization, resulting in reduced metabolic flexibility.[2,3]

Grape seed proanthocyanidins (GSPE) have been shown to effectively protect against several pathologies. They are highly effective in improving the lipidic[4] and glucose metabolism,[5] and as anti-inflammatory agents,[6] among others. GSPE affect different tissues, acting on the glucose metabolism under obesogenic situations. In the adipose tissue they decrease insulin resistance,[7] and in the endocrine pancreas they protect from lipidic accumulation[8] and favour a higher incretin effect. It was found that GSPE treatment prevented the decrease in DPP4 produced by a cafeteria diet[9] and increased ileum GLP-1 secretion.[10] This GSPE treatment favours the endocrine pancreas having a higher sensitivity to incretins.[11] A GSPE dose with satiating effects clearly acts on the gastrointestinal wall where it modulates the secretion of the enterohormone, and thus has an impact on the food intake during the treatment.[12] Moreover, some effects also remained several months after the treatment.[10] These GSPE treatments were also effective under cafeteria diet feeding conditions in female rats, modifying enterohormone secretions.[13]

All these results and most of the studies have been carried out with young animals, which have a robust metabolic response. However, there is less information regarding the effects of GSPE or a hypercaloric diet on aged rats. Thorburn *et al.* showed that as animals age, they become insulin resistant, and a mild hyperglycaemia appears with the ageing process. Moreover, a high-fat diet plays a relevant role in initiating syndrome X.[14] Teixeira *et al.* observed that 3- and 18-month-old rats responded differently to a cafeteria diet.[15] Zhu *et al.* reported that alogliptin, an inhibitor of DPP4, improves the survival and health of mice on a high-fat diet.[16]

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Balaskó *et al.*[17] showed the differential efficacy of CCK injected intraperitoneally throughout life, with the strongest resistance in the middle age (12-month-old Wistar males). We showed that GSPE treatment improved some metabolic parameters of the ageing processes in 21-week-old female rats[18] and also in the same rats under a cafeteria diet challenge.[19] Aged animals had a standard response to satiating GSPE doses as in young animals.[18] They also reduced food intake during cafeteria treatment when administered GSPE.[19] Some of these effects could be related to the presence of some of the molecules of the extract reaching the target tissues, but other effects could be related to the ability of these compounds to modify enterohormone secretions.[13] This information is not available at present for aged rats.

The intestine plays an important role in metabolic homeostasis.[20] Gut alterations could be attributed to macronutrient intake in typically obesogenic "western diets".[21] In particular, in the small intestine it represents an early event that precedes and predisposes to obesity and insulin resistance.[22] Ageing processes may alter some of the intestinal functions, although there are some controversial results. It has been suggested that there are changes in the incretin profile,[23,24] which could affect the insulin release or impair the CCK satiating effect.[25]

Since there is very little information regarding the effects of procyanidins and a cafeteria diet on the enterohormone secretion in aged rats, here we aimed to analyse the response of the main enterohormones (GLP-1, PYY and CCK) to a cafeteria diet in 21-month-old female rats. We also studied the ability of two different GSPE preventive treatments to protect against metabolic disruptions induced by this diet. We compared our results in aged (21-month-old) rats to those obtained for a group of young (2-month-old) female rats treated under the same conditions.

Materials and methods

Proanthocyanidin extract

Grape-seed extracts rich in proanthocyanidins (GSPE) were provided by Les Dérivés Résiniques et Terpéniques (Dax, France). According to the manufacturer, the GSPE used in the study with aged animals (batch number: 207100) contained a total procyanidin content of 76.9% and consisted of a mixture of monomers (23.1%), dimers (21.7%), trimers (21.6%), tetramers (22.2%), and pentamers (11.4%) of flavan-3-ols. The batch number 124029 used with young animals contained a total procyanidin content of 75.0% and consisted of a mixture of 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.

Animal studies

Aged group. Female aged Wistar rats (21 months old), each weighing 300–350 g, were acquired from Envigo (Barcelona, Spain). After one week of adaptation, the rats were individually housed in the animal quarters at 22 °C with a 12 h light/12 h dark cycle and fed a standard chow diet (Teklad 2014 Envigo, Barcelona, Spain) *ad libitum* and tap water. Laboratory rats started reproductive senescence when approximately 20 months old.[21] According to the age correlation with humans at this period of life, our study model could be useful to understand alterations linked to obesity and ageing in 60-year-old humans. The rats were then randomly divided into four experimental groups (n = 14) and fed a standard chow diet *ad libitum*. The control group (AGED STD) only received the standard chow diet. In addition to the standard chow diet, the other groups received a cafeteria diet as a model of a high-fat/high-sucrose diet (AGED CAF groups). The cafeteria diet consisted of bacon, sausages, pâté and biscuits, carrots, muffins, and sugared milk, and induced voluntary hyperphagia.[22] This diet was offered freshly *ad libitum* every day for 75 days.

Two of the cafeteria-fed groups were supplemented with GSPE. An oral dose of 500 mg per kg BW (body weight) was administered (1) as a preventive treatment for 10 days prior to the cafeteria diet intervention (AGED CAF GSPE PRE) and (2) at the beginning of the cafeteria diet for 5 days. It was repeated once per month (AGED CAF GSPE MONTHLY). The GSPE was dissolved in tap water and administered by oral gavage to the animals at 6 p.m., three hours after all available food had been removed. Fresh food was given to the animals one hour after they received the GSPE dose. The animals that did not receive supplementation with GSPE received the same amount of water as a vehicle. At the end of the study, the animals were euthanized by decapitation after they had fasted for 12 hours. All procedures were approved by the Experimental Animal Ethics Committee of the autonomous government of Catalonia, Spain (Department of Territory and Sustainability, General Directorate for Environmental and Natural Policy, project authorization code: 10183).

Young group. Female Wistar rats (3 months old) weighing 240–270 g were purchased from Charles River Laboratories (Barcelona, Spain). After one week of adaptation, they were individually caged in animal quarters at 22 °C with a 12 h light/12 h dark cycle and fed *ad libitum* with a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. As previously described,[12] the rats were randomly distributed into experimental groups (n = 7–10 per group). The control group (STD) received only the standard chow diet. The other groups, in addition to this diet, received a cafeteria diet for 17 weeks. The cafeteria composition is described in the Aged group section. The STD group and the cafeteria group (CAF) received an oral gavage of tap water as a vehicle together with the chow diet and cafeteria diet, respectively. The preventive treatment group (PRE) received an oral dose of 500 mg GSPE per kg of BW for 10 days before starting the cafeteria diet, according to the details provided in the Aged group section. At the end of the study, the animals were fasted for 1–4 h, anaesthetized with sodic pentobarbital (70 mg per kg BW) provided by Fagron Iberica (Barcelona, Spain), and exsanguinated from the abdominal aorta. All procedures were approved by the Experimental Animal Ethics Committee of the Universitat Rovira i Virgili (Code: 0152S/4655/2015).

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Tissue and blood collection

The intestine was carefully removed, measured and weighed, and the different intestinal segments were excised. The caecum was quickly weighed before and after the caecal content removal. The caecal content, together with intestinal segments from the duodenum, ileum and proximal colon, was immediately frozen in liquid nitrogen and then stored at −80 °C for further analysis. The blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. The plasma was obtained by centrifugation (1500*g*, 15 min, 4 °C) and stored at −80 °C in different aliquots until analysis. All the samples were stored at −80 °C.

Ex vivo treatment of intestinal segments

We used Krebs–Ringer bicarbonate (KRB) buffer (Hepes 11.5 mM, $CaCl_2$ 2.6 mM, $MgCl_2$ 1.2 mM, KCl 5.5 mM, NaCl 138 mM, $NaHCO_3$ 4.2 mM, NaH_2PO_4 1.2 mM) pH 7.4, supplemented with either 10 mM D-glucose (KRB-D-glucose buffer) or 10 mM D-mannitol (KRB-D-mannitol buffer). For enterohormone secretion studies, KRB-D-glucose was supplemented with protease inhibitors amastatin 10 μM (Enzo Life Sciences, Madrid, Spain) and aprotinin 100 KIU (Sigma, Barcelona, Spain), and 0.1% fatty acid free-bovine serum albumin (BSA). We used samples from the proximal duodenum, distal ileum and proximal colon. The tissue was rinsed with ice-cold KRB-D-mannitol buffer and dissected into segments (0.5 cm diameter). The tissue segments were placed in prewarmed (37 °C) KRB-D-mannitol buffer for 15 minutes to stabilize the tissue. After this, the medium was replaced for the treatments: KRB-D-glucose buffer as a control and peptone from bovine meat, enzymatically digested (Cat. no: 70175, Sigma-Aldrich, Madrid, Spain), to stimulate enterohormone secretion. The tissue segments were incubated for 30 minutes in a humidified incubator at 37 °C, 95% O_2 and 5% CO_2. After this period the whole volume was aliquoted and stored at −80 °C for further measurements. Enterohormones were analysed using commercial ELISA kits for GLP-1 7-37 amide (Millipore, Billerica, MA, USA. Catalogue no. EGLP-35K), desulphated CCK8 (Peninsula Laboratories, San Carlos, CA, USA. Catalogue no. Bachem S1205), and PYY (Phoenix Pharmaceuticals, Burlingame, CA, USA. Catalogue no. FEK-059-03).

GLUTag cell studies

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). They were a stable immortalized relatively differentiated murine enteroendocrine cell line that has been defined as a good model for proximal L-cells.[26] The medium where the cells were cultured was composed of DMEM (Dulbecco's modified Eagle's medium) containing 1 g L^{-1} D-glucose, supplemented with 10% foetal bovine serum (FBS), 1% of 100 U mL^{-1}/100 mg L^{-1} penicillin/streptomycin and 1% of glutamine (final concentration: 2 mM). The cells were incubated under a 5% CO_2-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 per mL 24 h before the secretion study. The cells were then washed twice with PBS buffer and treated for 24 h with palmitate (0.4 mM) or glucose (10 mM). Both treatments were performed in low glucose DMEM supplemented with 1% FBS, 0.8% BSA and 0.08% dimethyl sulfoxide. All the treatments were performed in triplicate 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 CCK. Next, the cells were lysed with RIPA buffer and the lysates were stored at −80 °C. They were then used to analyse the total protein content with a BCA kit.

Gene expression analysis

Total RNA and cDNA were obtained as previously defined.[10] Quantitative PCR amplification was performed using specific TaqMan probes from Applied Biosystems (Waltham, USA): Rn01460420_g1 for PYY gene (*Pyy*), Rn00563215_m1 for CCK gene (*Ckk*), Rn00562293_m1 for proglucagon gene (*Gcg*), Rn00562406_m1 for GLP-1 receptor gene (*Glp1r*) and Rn00572200_m1 for chromogranin A gene (*Chga*). The relative expression of each gene was calculated against the control group using the $2^{-\Delta\Delta Ct}$ method, with PPIA (Rn00690933_m1) as the reference.

Short chain fatty acid quantification

The concentrations of short chain fatty acids (SCFAs) (formic, acetic, propionic, butyric, isovaleric, valeric, lactic and succinic acids) were assayed in cecal content thawed at 4 °C. Briefly, approximately 1 g of sample was added to a screw cap glass tube containing 1 ml of 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 37% HCl were added to this sample, in parallel to 1 ml of 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*. A total 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 80 °C for 30 minutes. Derivatized SCFAs were analysed using a gas chromatography system (Agilent 6890-NT, Santa Clara, USA) coupled with a 30 m × 0.25 mm i.d. column with a film thickness of 0.25 µm (Agilent DB-23, Barcelona, Spain) and a flame ionization detector (FID) to determine SCFA concentrations.[27] The carrier gas used was helium. A constant flow mode was used (split 25 : 1; 30 ml min^{-1} split flow). The column temperature was programmed to gradually increase from 60 °C to 220 °C during the analysis. In addition, the injector port and FID temperatures were fixed at 250 °C. The injection volume was set to 1 µl, and analyses were performed in duplicate.[28]

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Statistical analysis

Our results have been presented as the mean ± SEM. Data were analysed with the XLSTAT 2020.1 (Addinsoft, Barcelona, Spain) statistical software. An ANOVA test was used to compare experiments with more than two treatments. Tukey (HSD) test was used as a *post-hoc* test to identify differences between treatments. We used Student's *t*-test for the analysis of only two treatments. $P < 0.05$ was considered statistically significant.

Results and discussion

A cafeteria diet modified basal CCK secretion in the duodenum differently in aged *vs.* young rats; GSPE was only preventive in young animals

We analysed the enterohormone-secreting response of aged rats to a cafeteria diet and compared it with the response of young animals. We also studied the ability of GSPE pre-treatments to protect against the effects of the cafeteria diet.

Initially we analysed the effects on CCK, which in rats is mainly secreted in the duodenum. Table 1 shows that in young animals, cafeteria feeding increases the basal duodenal CCK secretion, as we had previously shown.[13] We used GLUTag cells to further characterize the diet components responsible for this effect. Fig. 1 shows that a chronic (24 hours) palmitate treatment effectively reproduced this effect *in vitro* and that, at a concentration of 10 mM, glucose showed a trend towards it (when analysed by *T*-test). This suggests that the components of the cafeteria diet, whose presence in obesogenic diets has been defined in the literature,[29] could participate in the increased basal CCK secretion. There are very few studies that have analysed *in vitro* chronic treatments for enterohormone secretions. Similar to our results, increasing GLP-1 secretion was observed after 48 hours of palmitate treatment, at half our dose, in GLUTag cells.[30]

Table 1 shows that GSPE administered for 10-days previously to a cafeteria diet prevented basal CCK increase in young animals. The basal CCK response differed in the 21-month-old rats. In old animals (Table 2), the cafeteria diet decreased the *ex vivo* basal CCK secretion, when analysed by the *T*-test, and this was not statistically prevented by either of the two GSPE treatments assayed: a 10-day GSPE previous to the cafeteria diet (AGED CAF GSPE PRE), identical to that performed in young rats and a simultaneous 5-day GSPE treatment administered each month during the cafeteria treatment (AGED CAF GSPE MONTHLY). Thus, cafeteria effects on basal CCK secretion were age-dependent: in young cells basal CCK secretion increased but in aged cells it did not. Our results show that GSPE works only in a preventive way, on being administered previously to a cafeteria diet in young rats, where there is metabolic flexibility. It does not work in aged animals, where the system's flexibility has been lost.[2,3]

Another relevant aspect in addition to basal CCK is the ability of the tissue to secrete CCK after stimulation. There are controversial results regarding obesity effects on CCK levels in

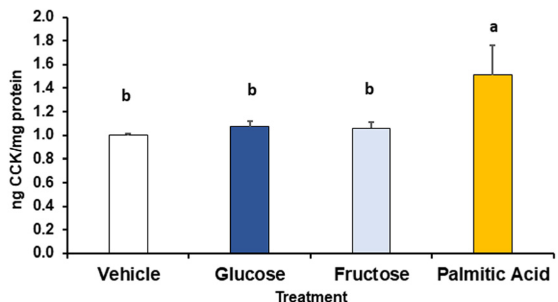

Fig. 1 CCK secretion by GLUTag cells after 24 hours of treatment. GLUTag cells were plated at a density of 200 000 cells per mL 24 h before the secretion study. Cells were then washed twice with PBS buffer and treated for 24 h with palmitate (0.4 mM), glucose (10 mM) or fructose (10 mM). All the treatments were performed in triplicate in each cell plate and repeated for three passages. After the treatment, the medium of each well was collected. Results are expressed as the mean ± SEM. Different superscripts indicate statistically different groups by the ANOVA test ($p < 0.05$).

Table 1 Preventive effects of GSPE against the cafeteria diet in young rats on CCK secretion

	CCK in the medium (ng mL^{-1})	CCK plasma (ng mL^{-1})
STD	0.316 ± 0.040^{ab}	0.38 ± 0.10
CAF	**0.381 ± 0.040^{a}**	**0.67 ± 0.19**
PRE	0.275 ± 0.020^{b}	0.49 ± 0.11

Left column, CCK levels secreted *ex vivo* to the basolateral media by duodenum segments mounted in Ussing chambers. Right column, plasma CCK concentration at sacrifice. Values represent the mean ± SEM of five to seven animals per group. STD, control group; CAF, cafeteria group; PRE, GSPE preventively treated group. Different superscripts indicate statistically different groups by the ANOVA test ($p < 0.05$).

Table 2 Preventive effects of GSPE against the cafeteria diet in aged rats on CCK secretion

	CCK in the medium (ng mL^{-1})		CCK in plasma (ng mL^{-1})
	Baseline	Stimulated	
AGED STD	0.295 ± 0.039	0.722 ± 0.089	0.637 ± 0.031^{ab}
AGED CAF	**0.178 ± 0.020**	**0.572 ± 0.051**	**0.710 ± 0.049^{a}**
AGED CAF GSPE PRE	0.28 ± 0.08	0.59 ± 0.07	0.699 ± 0.032^{a}
AGED CAF GSPE MONTHLY	0.183 ± 0.019	0.461 ± 0.050	0.526 ± 0.041^{b}

CCK levels secreted *ex vivo* to the media by duodenum segments under no stimulation (baseline) and after peptone stimulation with 50 mg mL^{-1} for 30 minutes (stimulated). Right column, plasma CCK concentration at sacrifice. Values represent the mean ± SEM of five to ten animals per group. AGED STD, control group; AGED CAF, cafeteria group; AGED CAF GSPE PRE, GSPE preventively treated group; AGED CAF GSPE MONTHLY, GSPE once per month treated group. Different superscripts indicate statistically different groups by the ANOVA test ($p < 0.05$).

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young subjects.[31] To analyse this situation, we treated duodenum segments of 21-month-old aged rats with peptone, a well-known secretagogue. Peptone-stimulated CCK secretion was not modified by the cafeteria diet in young rats (results not shown), nor was it modified in aged animals (Table 2). Therefore, ageing does not modify the duodenum's ability to respond to CCK-secreting stimuli under the cafeteria diet. Furthermore, the GSPE treatment did not change the tissue's ability to respond to stimulation in aged rats (Table 2). Most studies on CCK and ageing analysed the organism's response to this enterohormone.[17] However, there is very little information available regarding the secretory response of the enteroendocrine cells in ageing.

The plasma levels reflected the equilibrium between CCK production and CCK removal. In young rats, the cafeteria diet-increased basal secretion is reflected in a tendency to increase the plasma levels, as previously shown.[13] Here, in aged rats, there is also a trend toward higher levels in plasma CCK but that does not agree with the changes in CCK secretion (Table 2). Similarly, the GSPE-induced reduction in basal secretion does not lead to lower CCK plasma levels in young rats (Table 1). These results could suggest that the body adapts to reduced basal CCK secretion to maintain the enterohormone plasma levels both in young and aged rats. The decrease found in the plasma levels after GSPE treatment, in the group of old rats that were GSPE administered monthly (Table 2), suggests that since we did not find changes in the duodenum, this more prolonged GSPE treatment affected other areas of the body, probably related to CCK removal from plasma. The kidney is a tissue highly implicated in CCK clearance.[32] Other authors[33] have clearly shown that it is an important target tissue for GSPE doses similar to those assayed in our study. We did not find any change in the urea/creatinine ratio, indicative of renal function, in these aged rats in any of the treatments;[19] therefore, further studies are required to elucidate this point.

Ileum enteroendocrine cells showed the highest sensitivity to a GSPE treatment previous to the cafeteria diet in aged rats (21 months old)

We next analysed the effects on GLP-1 and PYY, which are more abundantly expressed in the ileum in rats. The cafeteria diet in young rats did not change the mRNA levels of GLP-1 in the ileum or colon, its basal secretion in the ileum[13] or the total amount of GLP-1 protein in the ileum (results not shown). This lack of effect of the cafeteria diet on GLP-1 differs from the previously mentioned results of Thombare *et al.*[30] in GLUTag cells, in which they describe that palmitate increases the GLP-1 secretion. One of the reasons for the difference could be the *in vivo* intestinal location *vs. in vitro* studies. Here we show the results derived from *in vivo* studies with ileum segments that were very far from the high concentrations of nutrients derived from the diet; a situation that differs from upper gastrointestinal locations or *in vitro* cell cultures. In 21-month-old rats, the cafeteria diet did not change the basal secretion, and did not stimulate peptone active GLP-1 secretion (Fig. 2) or GLP-1 mRNA levels in the ileum (Table 3).

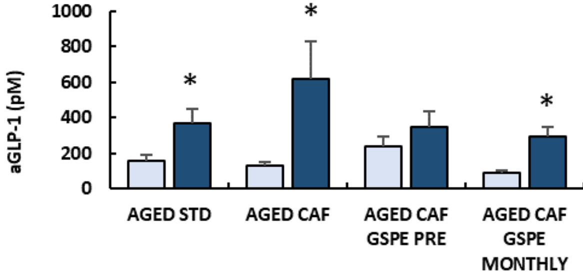

Fig. 2 Active GLP-1 secreted to the medium by ileum explants in aged rats. Segments of the ileum were obtained at sacrifice from all the groups of aged rats. Treatment with peptone (50 mg mL^{-1}) was conducted for 30 minutes. Light blue columns reflect the baseline (vehicle treated) and dark blue columns reflect stimulated conditions. Results are expressed as the mean ± SEM of 5 to 7 animals. * Indicates p values <0.05 *vs.* baseline situation for each group, by Student's T test.

Table 3 Preventive effects of GSPE against the cafeteria diet in aged rats on GLP-1 in the ileum and pancreas

	mRNA levels		
	GLP-1 (A.U.) ileum	GLP-1 R (A.U.) ileum	GLP-1 R (A.U.) pancreas
AGED STD	1.12 ± 0.20	1.09 ± 0.11	0.91 ± 0.22
AGED CAF	**1.29 ± 0.20**	**1.19 ± 0.18**	**1.55 ± 0.41**
AGED CAF GSPE PRE	0.86 ± 0.18	1.09 ± 0.10	0.68 ± 0.22
AGED CAF GSPE MONTHLY	1.38 ± 0.31	1.31 ± 0.10	0.87 ± 0.09

Columns from left to right: mRNA levels of GLP-1 in the ileum; mRNA levels of GLP-1R in the ileum; mRNA levels of GLP-1R in the pancreas. Values represent the mean ± SEM of five to seven animals per group. AGED STD, the control group; AGED CAF, the cafeteria group (bold row); AGED CAF GSPE PRE, GSPE preventively treated group; AGED CAF GSPE MONTHLY, GSPE once per month treated group; A.U.: arbitrary units.

Therefore, regardless of the rat's age, the cafeteria diet does not affect ileum GLP-1. This can help explain the fact that GSPE, which showed an anorectic effect through GLP-1 signalling,[19] maintains this effect in both ages, in young rats[12] and aged animals[18] fed a cafeteria diet.[19] Balaskó *et al.*[17] showed there is a change in sensitivity to agonisms with ageing. It decreased in animals of middle age (12 months old) but was restored in ageing models (22 months old). They also reported that diet-induced obesity accelerates this entire process. These changes can be the explanation for the difference in our results compared to the results of Okada *et al.*, working with a one-day cafeteria diet in 14-month-old mice. These authors showed an attenuated anorectic effect of GLP-1 after this short cafeteria diet.[34]

The GSPE treatment before the cafeteria diet was very effective in young rats, increasing ileum GLP-1 mRNA, together with GLP-1R,[10] as well as pancreas GLP-1R,[11] giving these animals a higher pancreatic sensitivity to GLP-1. However, we found a different situation in 21-month-old rats. GSPE-treated rats showed a trend towards a decreased ileal GLP-1 mRNA

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expression (Table 3) and higher basal aGLP-1 secretion (Fig. 2) when analysed by the *T*-test *vs.* the cafeteria aged group. This situation could be an explanation for the limited ability of the tissue to react against peptone stimulation (Fig. 2). This situation differs from the effects observed in young animals,[11] independently of the fed/fasted situation (unpublished results).

GSPE PRE treatment showed different trends between aged and young animals. But when GSPE was administered concomitantly to the cafeteria diet in aged rats (GSPE MONTHLY) it did not produce effects on the GLP-1 parameters of the ileum of the 21-month-old rats (Fig. 2 and Table 3). It also did not change the colon mRNA for GLP-1 or GLP-1R in the pancreas (Tables 6 and 3). This suggests a stronger effect of the cafeteria diet that masks GSPE effects on these GLP-1 producing cells, which were obtained in the pre-treatment with GSPE before the cafeteria diet.

As the ileum showed sensitivity to GSPE pre-treatment we also analysed the PYY enterohormone in the ileum. In young rats, GSPE treatment did not affect it (Table 4). In the 21-month-old rats, there was a reduction in the PYY mRNA expression by GSPE (aged caf: 1.092 ± 0.064; aged caf GSPE pre: 0.809 ± 0.055; A. U.; $p < 0.05$ ANOVA test) and there was no effect of GSPE on the PYY secretion (Table 5).

Table 4 Preventive effects of GSPE against the cafeteria diet in young rats on PYY and ChgA in the ileum

	mRNA levels	
	PYY (A.U.)	ChgA (A.U.)
STD	0.79 ± 0.09	0.92 ± 0.08^{ab}
CAF	**1.02** ± 0.09	**1.00** ± 0.03^{a}
PRE	1.29 ± 0.20	0.72 ± 0.08 ^{b}

mRNA levels of PYY and ChgA in the ileum. Values represent the mean ± SEM of five to seven animals per group. STD, control group; CAF, cafeteria group (bold row); PRE, GSPE preventively treated group; A.U. Different superscripts indicate statistically different groups by the ANOVA test ($p < 0.05$).

Table 5 Preventive effects of GSPE against the cafeteria diet in aged rats *ex vivo* on PYY secretion in the ileum

	PYY (pg mL^{-1})	
	Baseline	Stimulated
AGED STD	25.6 ± 4.9	107.54 ± 15.69
AGED CAF	**23.4 ± 6.9**	**82.44 ± 10.99**
AGED CAF GSPE PRE	30.8 ± 3.6	91.06 ± 8.43
AGED CAF GSPE MONTHLY	37.0 ± 0.7	82.21 ± 2.50

PYY secreted *ex vivo* to the media by ileum segments under no stimulation (baseline) and after peptone stimulation with 50 mg mL^{-1} for 30 minutes (stimulated). Values represent the mean ± SEM of five to seven animals per group. AGED STD, control group; AGED CAF, cafeteria group (bold row); AGED CAF GSPE PRE, GSPE preventively treated group; AGED CAF GSPE MONTHLY, GSPE once per month treated group.

Since we found that PYY and GLP-1 mRNA expression levels decreased with GSPE pre-treatment, we also measured the gene expression of a marker of endocrine cells, chromogranin A (ChgA), which also showed a reduction due to GSPE pre-treatment (aged caf: 1.127 ± 0.05; aged caf gspe pre: 0.823 ± 0.07; $p < 0.05$ ANOVA test). This suggests that an effect of this GSPE treatment is limiting the enteroendocrine cell differentiation in the ileum tissue. This effect was not found in young rats, where GLP-1 mRNA was increased,[10] PYY was not modified and ChgA was also decreased by this pre-treatment (Table 4). This is a surprising result because GSPE had been shown to promote enterohormone cell differentiation. In ileum organoids a GSPE treatment for 24 hours upregulated GLP-1 and PYY markers, as well as the endocrine marker ChgA.[35] Also *in vivo*, a treatment with GSPE upregulated ChgA in the colon.[9,36] Therefore, these effects of GSPE, observed twelve weeks after the treatment, must be exclusive to the enteroendocrine population present in aged ileum.

Aged colonic tissue was not sensitive to the GSPE treatments assayed, despite some effects on SCFAs

Table 6 summarizes the effects on mRNA levels on colon segments in aged animals. ANOVA tests of all these treatments showed p values higher than 0.05, but very close to it. Then we checked the cafeteria diet effect on the control group by the *T*-test in all these mRNA levels. There was a clear effect of the cafeteria diet as reflected by the increase in the expression of GLP-1, PYY and ChgA in mRNA. GSPE did not change also the basal tGLP-1 secretion (aged caf: 29.5 ± 6.03; aged caf GSPE pre: 28.43 ± 6.89; aged caf GSPE monthly: 18.13 ± 2.94; pM).

Since the effects of the cafeteria diet suggest an increase in the enteroendocrine cell number in the colon, we then analysed the caecal SCFA profile. The two more abundant SCFAs, butyric acid and propionic acid, have been shown to be stimulators of differentiation of EE cells.[37] Fig. 3 shows that the levels of propionic and butyric acids were decreased by the cafeteria diet in aged rats, with butyric acid being the only statistically significant species as inferred from the ANOVA test. Thus, changes in butyric and propionic acid levels could not explain the trends observed in the amount of differentiated cells because the levels were reduced by the cafeteria diet. A reason could be the loss of sensitivity of the colonic segment

Table 6 Effects of cafeteria diet and GSPE treatments on the mRNA levels of GLP-1, PYY and ChgA in the colon of aged rats

	GLP-1	PYY	ChgA
AGED STD	1.01 ± 0.07	1.02 ± 0.10	1.04 ± 0.12
AGED CAF	**1.51 ± 0.23**	**3.48 ± 0.85**	**1.92 ± 0.40**
AGED CAF GSPE PRE	1.58 ± 0.27	3.16 ± 0.68	1.98 ± 0.53
AGED CAF GSPE MONTHLY	1.60 ± 0.20	2.02 ± 0.22	1.61 ± 0.21

Values represent the mean ± SEM of five to seven animals per group. Data are provided as A.U. *vs.* AGED STD. AGED STD, control group; AGED CAF, cafeteria group (bold row); AGED CAF GSPE PRE, GSPE preventively treated group; AGED CAF GSPE MONTHLY, GSPE once per month treated group.

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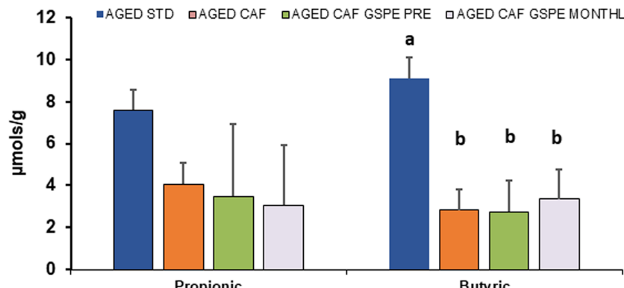

Fig. 3 The caecal propionic and butyric content altered by the cafeteria diet and GSPE treatments. At sacrifice, the caecal content was collected and SCFA was measured. Blue columns represent the AGED STD group, orange columns represent the AGED CAF group, green columns represent the AGED CAF GSPE PRE group and light pink columns represent the AGED CAF GSPE MONTHLY group as detailed in the legend of the picture. Values represent the mean ± SEM of six to ten animals per group. Different superscripts indicate statistically different groups by the ANOVA test ($p < 0.05$).

Table 7 Effects of cafeteria diet and GSPE treatments on the intestinal size, caecum weight and SCFA caecal content of aged rats

	Ratio small intestine/colon (cm)	Empty caecum (g)	Total caecal SCFA (µmol g^{-1})
AGED STD	5.26 ± 0.15^{b}	0.88 ± 0.05^{b}	100.4 ± 19.3
AGED CAF	$\mathbf{6.06 \pm 0.16^{a}}$	$\mathbf{1.14 \pm 0.07^{a}}$	**74.5 ± 21.0**
AGED CAF GSPE PRE	6.03 ± 0.19^{a}	0.89 ± 0.08^{b}	57.9 ± 9.1
AGED CAF GSPE MONTHLY	5.77 ± 0.14^{ab}	1.10 ± 0.04^{ab}	76.5 ± 19.9

Total caecal SCFAs measured were corrected by the total caecal content. Values represent the mean ± SEM of eight to twelve animals per group. AGED STD, control group; AGED CAF, cafeteria group (bold row); AGED CAF GSPE PRE, GSPE preventively treated group; AGED CAF GSPE MONTHLY, GSPE once per month treated group. Different superscripts indicate statistically different groups by the ANOVA test ($p < 0.05$).

to adjust enteroendocrine expression to these fatty acids.[38] These results also differed from those obtained in young rats, where there was no clear effect of the cafeteria diet on enteroendocrine cell abundance, and the only SCFA that decreased due to the cafeteria diet was butyric acid. Propionic acid showed a trend towards increasing levels.[13]

GSPE did not modify the levels of these two more abundant SCFAs (propionic and butyric acids) (Fig. 3) but it did modify other SCFAs that are less abundant. Valeric acid was also decreased by the cafeteria diet, and its levels were indeed lower with both GSPE treatments (aged std: 0.62 ± 0.22(a); aged caf: 0.34 ± 0.31(b); aged caf GSPE pre: 0.015 ± 0.02(c); aged caf GSPE monthly: 0.04 ± 0.03(b, c); µmol g^{-1}; $p < 0.05$ by ANOVA test). Succinic acid showed a completely different profile: it increased with the GSPE administered monthly (aged std: 0.55 ± 0.52(b); aged caf: 1.33 ± 1.20(b); aged caf GSPE pre: 1.26 ± 0.80(b); aged caf GSPE monthly: 4.87 ± 4.21(a); µmol g^{-1}; $p < 0.05$ by the ANOVA test). Thus, SCFAs of aged animals were more sensitive to GSPE treatments than SCFAs of young rats, as the GSPE treatment assayed did not modify the SCFA profiles produced by cafeteria diets in young animals.[10]

Effects on the whole gastrointestinal tract

Ageing is associated with many changes in the form and function of the gastrointestinal tract, some of which may have nutritional and metabolic consequences. We showed that the cafeteria diet in young animals increased the ratio of small intestine/colon and decreased the colon size, which was only prevented by a high GSPE treatment administered every other week during the cafeteria diet.[13] Here we show that the cafeteria diet reproduces these effects in aged rats (Table 7), and only the concomitant GSPE treatment with the cafeteria diet prevented the effects in aged animals. There was a clear difference between the caecum size of the aged rats and young rats. This was increased by the cafeteria diet in aged animals (Table 7) unlike what was observed in young animals, which had a smaller empty caecum.[13] Caecum enlargement is usually associated with indigestible fibres and prebiotics that reach this location.[39] This is not the reason for the enlargement of the caecum in aged rats, which can be explained rather by the larger amount of undigested food that reaches it. Mathers *et al.* showed that in rats, as in humans, there is a decline in the digestive capacity of the small intestine, which causes a distal shift at the site of digestion. Therefore, a higher proportion of digestion occurs in the large bowel in aged rats compared to young rats.[39] This higher development could be limited in the colonic tissue due to the lower production of butyric acid (needed for colonic growth), characteristic of aged animals. In fact, we observed a trend towards a stimulation of the presence of enterohormone cells due to cafeteria treatment, which could be related to this larger amount of nutrients reaching it in obesogenic diets, as has been defined by other authors.[40]

An interesting effect was found on the caecum size by the GSPE treatment administered 11 weeks before we measured it. This treatment limits the organ size (Table 7). In the search for an explanation for this GSPE effect on the caecum size, the small differences found in the SCFA make it difficult to justify it as an effect related to SCFA. However, it must be highlighted that the GSPE treatment was carried out 11 weeks before the caecal size was measured. The explanation could be related to the ability of GSPE to modulate some epigenetic mechanisms.[10,41] However, more work is needed to check this hypothesis.

Conclusions

GSPE has previously been shown to be protective against a cafeteria diet challenge in young animals. Here we showed that the aged enteroendocrine system had a different sensitivity to these two dietary challenges compared with the enteroendocrine system of young animals. The duodenum and colon were the main segments sensitive to the cafeteria diet

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challenge, with no protection from GSPE treatments in aged rats. The ileum and caecum were more sensitive to being modified by the GSPE treatment before a cafeteria diet was administered in the 21-month-old rats. Therefore, it is important to be careful when the results obtained for the enteroendocrine system with young animals are extrapolated to old animals as there are very large differences. This, and the importance of the intestinal segment, must be considered when a treatment targeted at the gastrointestinal tract is defined. The duodenum and colon showed a higher sensitivity to the bulk of the diet, while GSPE showed more effectiveness in the ileum and caecum. All these results were obtained in an animal model with a metabolism very close to humans. To translate it to humans, it must be considered that there are some differences, clearly defined, on the relative abundance of enteroendocrine cells in the different intestinal segments.

Author contributions

AMG and MSC: data curation, formal analysis, and investigation; AMPV: investigation; ERG and RBD: methodology; XT: data curation; AA and MP: conceptualization and supervision. AA: project administration and original draft; MP: resources. All the authors participated in funding acquisition and revised and edited the final document before submission.

Conflicts of interest

There are no conflicts to declare.

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