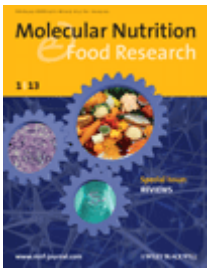

Long term exposure to a grape seed proanthocyanidin extract enhances L-cell differentiation in intestinal organoids

Journal:	*Molecular Nutrition and Food Research*
Manuscript ID	mnfr.202000303.R2
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
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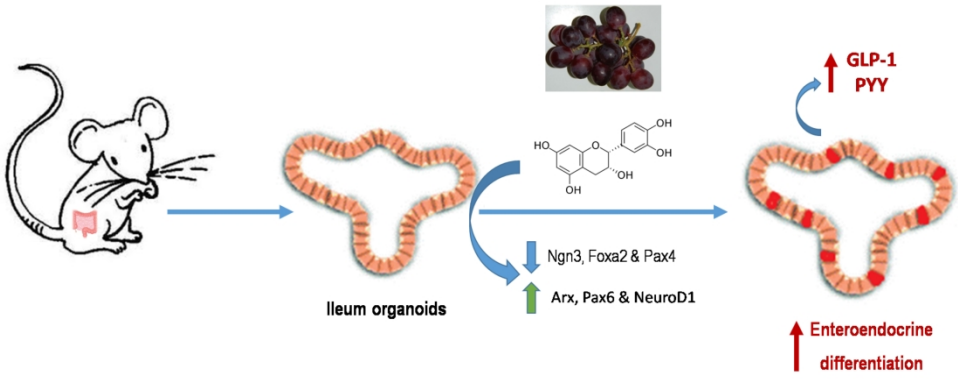
Keywords:	Proanthocyanidins, Enteroendocrine, Incretin, GLP-1, PYY

This study shows that a grape-seed proanthocyanidin extract increases L-cell numbers in ileum organoids through the modulation of early transcription factors involved in enteroendocrine differentiation, thus inducing a higher release of glucagon-like peptide-1 and peptide YY. Due to the key role of this hormones in appetite regulation and glucose homeostasis, this extract appears as a potential therapeutic strategy for obesity and T2DM treatment.



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Long term exposure to a grape seed proanthocyanidin extract enhances L-cell differentiation in intestinal organoids

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Keywords

Proanthocyanidins, enteroendocrine, incretin, GLP-1

Abbreviations

GSPE, grape seed proanthocyanidin extract; **GLP-1**, glucagon-like peptide-1; **PYY**, peptide YY; **T2DM**, Type-2 Diabetes Mellitus; **EC**, epicatechin; **GA**, gallic acid; **EEC**, enteroendocrine cell; **DPP4**, dipeptidyl-peptidase 4; **ChgA**, chromogranin A; **CGC**, glucagon; **Muc2**, mucin 2; **Lyz1**, lysozyme 1; **Lgr5**, Leucine-rich repeat containing G protein-coupled receptor 5; **SIS**, Sucraseisomaltase; **Ngn3**, neurogenin 3; **NeuroD1**, neurogenic differentiation factor 1; **Pax**, paired box; **Foxa**, Forkhead box; **Arx**, Aristaless related homeobox factor; **bHLH**, basic helix-loop-helix.

ABSTRACT

Scope: A grape-seed proanthocyanidin extract (GSPE) interacts at the intestinal level, enhancing glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) release, which modulate appetite and glucose homeostasis. Thus, enhancing L-cell numbers could be a strategy to promote hormone production, providing a potential strategy for obesity and type-2 diabetes mellitus treatment.

Methods & Results: Mice ileum organoids were used to evaluate the long-term effects of GSPE and two of its main components, epicatechin (EC) and gallic acid (GA), on intestinal differentiation. Hormone levels were determined using RIA and ELISA kits, and gene expression of transcription factors involved in intestinal cell differentiation, as well as markers of different cell types, were assessed by real-time qPCR. GSPE up-regulated enterohormone gene expression and content, as well as the pan-endocrine marker ChgA. GSPE also modulated the temporal gene expression profile of early and late transcription factors involved in L-cell differentiation. Furthermore, GSPE up-regulated goblet cell (Muc2) and enterocyte (SIS) markers, while down-regulating stem cell markers (Lgr5+). Although EC and GA modified enterohormone release, they did not reproduce GSPE effects on transcription factor's profile.

Conclusions: This study shows the potential role of GSPE in promoting enteroendocrine differentiation, effect that is not mediated by EC or GA.

1. INTRODUCTION

The increase in lifestyle-related diseases such as obesity and type 2 diabetes mellitus (T2DM) in developed societies highlights the need to search for new therapeutic strategies.[1] The gastrointestinal tract has a key role in short-term appetite signals and glucose homeostasis through the release of the anorectic hormones, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY). GLP-1 and PYY are secreted by the L-cells, one type of enteroendocrine cells (EECs), whose presence increases progressively towards the distal ileum and colon.[2] These hormones have been extensively reported to suppress appetite and improve glycemic control in animals and humans (reviewed in [3,4]), making them an interesting target for the development of anti-obesity/anti-diabetic therapies. Therefore, one potential therapeutic strategy could be to increase the L-cell numbers to enhance the release of anorexigenic hormones.

Proanthocyanidins are a group of phenolic compounds that are widely distributed in vegetables, fruits, plants and their derived foods like red wine, green tea and chocolate. Much attention has been given to proanthocyanidins in recent years because of their beneficial effects on health, being reported to act as cardioprotectants, antioxidants, hypolipidemic or anti-hyperglycemic agents (reviewed in [5]). A grape-seed procyanidin extract (GSPE) has been found to improve glucose homeostasis and reduce food intake partly through the modulation of circulating active GLP-1 levels.[6,7] This modulation could be explained by the previous reported inhibitory effect on dypeptidyl-peptidase 4 (DPP4), the GLP-1 inactivating enzyme,[8] but also by a direct stimulation of its release.[6,9] Moreover, our research group has shown that a chronic GSPE treatment counteracts the effects induced by a cafeteria diet, increasing colonic GLP-1 and PYY gene expression, as well as the endocrine marker chromogranin A (ChgA),[10,11] suggesting a potential effect on enteroendocrine differentiation. Previous studies also found that proanthocyanidins can modulate differentiation of several cell types;[12–14] however, the effects of those phenolic compounds on EEC differentiation is not fully understood yet. Therefore, using intestinal organoids, we aimed to investigate the effects of GSPE and two of its main monomers, epicatechin (EC) and gallic acid (GA), on intestinal L-cell differentiation and hormone release.

2. MATERIALS AND METHODS

2.1 Compounds and dosage information

GSPE was obtained from Les Dérivés Résiniques et Terpéniques (Dax, France), using the same lot (#124029) in all studies. The small molecules were previously characterized by liquid chromatography-

tandem mass spectrometry.[15] (-)-Epicatechin (EC) and gallic acid (GA) were obtained from Sigma (Poole, UK).

Doses were chosen according to previous studies [16] and treatment toxicity was analysed using a LDH kit from Cell Biolabs Inc. (Cambridge Bioscience, UK). LDH release was calculated as a fraction of the total LDH activity (from supernatant and organoid lysates).

2.2 Crypt isolation

Small intestines were harvested from 6-12-week-old C57BL/6 mice (Charles River, Margate, UK). An incision was made in the ileocecal junction and the distal 8 cm of intestine from the ileocecal junction was excised to acquire the ileum segment. Ileum crypts were isolated as previously described,[16,17] with slightly modifications. Briefly, tissue fragments were further washed with cold 10% FBS in PBS and incubated with 12.5mM EDTA in DPBS on a rotator for 30 min at 4°C. After removal of EDTA, tissue fragments were suspended in DPBS and vigorously shaken to release the crypts. The supernatant fraction containing the crypts was passed through a 70-µm cell strainer (BD Bioscience, VWR, UK) and centrifuged at 800 rpm for 5 min. All animal procedures undertaken were approved by the UK Home Office Animals (Scientific Procedures) Act 1986 (Project License Number: 70/8068).

2.3 Organoids culture

Mice ileal crypts were grown into organoids as described previously,[16,17] with slightly modifications. Isolated crypts were embedded in Matrigel (BD Biosciences) diluted 1:1 in Complete Growth Medium (CGM) and seeded in a 48-well plate. CGM contained Advanced DMEM/F12 (Invitrogen, Paisley (UK)) supplemented with 100µg/ml Primocin (Invivogen, France), 10mM HEPES, 1x Glutamax, 1x N2, 1x B27, 50ng/ml murine EGF (all from Invitrogen), 100ng/ml murine Noggin (Peprotech, London (UK)), 1.25mM N-acetylcysteine, 3µM CHIR99021 (both from Sigma) and 10% human R-spondin-1 conditioned medium.[18] Matrigel was polymerized for 30 minutes at 37°C and overlaid with 300µl of CGM. Medium was replaced every 2-3 days and CHIR99021 removed three days after seeding to promote stem cell differentiation. Organoids were passaged every 4-6 days in a 1:3 ratio using Gentle Dissociation Reagent (STEMCELL Technologies, Cambridge (UK)) and mechanical disruption.

2.4 Enterohormone secretion, content and gene expression studies

For one experimental replicate, freshly passaged organoids were pooled and seeded into three 48-well plates (100 organoids/well), one for each 24h time point. Three days after seeding, CHIR99021 was withdrawn and organoids were incubated in CGM containing EC (1 or 10µM), GA (0.5 or 5µM), GSPE

(0.05, 0.5, 5mg/L), or 0.05% DMSO as vehicle control. Medium was collected and refreshed every 24h for a period of 72h. Following treatments, organoids were lysed in TRIzol (Invitrogen) or cell lysis buffer (95mM NaCl, 30mM Tris-HCl, 0.63% IGEPAL Ca-630, 7mM sodium deoxycholic acid, glass-distilled water and a tablet of EDTA-free protease inhibitor cocktail (all from Sigma)). All lysates and supernatants were stored at -20°C until further analysis.

All treatments were tested in at least three independent experiments, containing 2-3 wells per condition. Data was normalized to the control (untreated) well of their respective time point in the same experiment.

2.5 Enterohormone quantification

Total PYY and total GLP-1 content, as well as total GLP-1 release were quantified using sensitive and specific in-house radioimmunoassays, as previously described.[19,20] PYY release was measured using a fluorescent immunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA (USA)).

2.6 Flow cytometry

Flow cytometry was used to quantify L-cell numbers. After 72h of treatment with 5mg/L of GSPE, organoids were incubated in TrypLE Express (Invitrogen) for 10 min at 37ºC and further dissociated into single cells by mechanical disruption. Single cells were fixed and permeabilized using FIX & PERM™ Cell Permeabilization Kit (Thermo Fisher, Loughborough (UK)), and incubated overnight with a primary antibody against PYY (ab22663; Abcam, Cambridge (UK)) at 4ºC. Cells were then incubated with Alexa Fluor 488 goat anti-rabbit (Invitrogen) as secondary antibody for 1h at RT and % of stained cells was determined using a FACS Calibur flow cytometer (BD Biosciences). Data was obtained from at least three independent experiments (with 3 wells per condition) and normalized to the control wells in the same experiment.

2.7 Quantitative PCR analysis

Total RNA and DNA were extracted from organoids using TRIzol and chloroform (Panreac, Barcelona (Spain)). Total RNA was purified using a Qiagen RNAeasy kit (Hilden, Germany) and cDNA generated using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham (USA)). Quantitative PCR amplification was performed using specific TaqMan probes (Applied Biosystems) (**Supporting Information Table S1**) and the relative expression of each gene was calculated against the control group using the 2-ΔΔCt method, with cyclophilin A (PPIA) as reference.

2.8 Statistical Analysis

Hormone release and content, and L-cell number data were analysed using Student's t-test or one-way ANOVA with Dunnett's test, as appropriate. Gene expression data at different time-points were analysed by independent Student's t-tests. Overall gene expression kinetics were analysed using two-way ANOVA, followed by an appropriate multiple comparison test. $P < 0.05$ was considered statistically significant. All analysis was carried out using Graphpad Prism 8.2.0 (GraphPad Software Inc., San Diego, CA, USA).

3. RESULTS

3.1 GSPE and its pure compounds modify enterohormone secretion and production in mouse ileum organoids

In order to understand the effect of chronic exposure to polyphenols in basal enterohormone release, we evaluated the accumulation of GLP-1 in the medium of organoids treated with GSPE up to 72 hours. 5mg/L of GSPE induced a 2.6-fold increase on basal GLP-1 release after 24h, effect that was lost after 48h. After 72h, this effect seemed to be recovered (*p=0.037*, unpaired t-test) although it was not significant when analysed by one-way ANOVA (*p=0.176*). Lower doses of GSPE did not show any significant increase in total GLP-1 at any of the studied time points (**Figure 1a**). We also tested the effects on GLP-1 release of EC, a monomer of flavan-3-ols that represents 21% of the total GSPE extract, and GA, a main phenolic acid that represents 7%,[15] in order to understand whether they are the responsible of the effect of GSPE. GA did not modify GLP-1 secretion at any time point or concentration (**Figure 1c**). However, 10µM of EC enhanced GLP-1 release following the same pattern than GSPE, increasing after 24h and again after 72h (*p=0.046*, unpaired t-test) (**Figure 1e**).

PYY has been found to be expressed in the same subset of cells as GLP-1,[21] thus we also analysed the release of PYY after exposure to the higher doses, which were found to be the most effective on GLP-1 release. As shown in **Figure 1b**, 5mg/L of GSPE did not modify PYY release over the course of 72h. Conversely, 5µM GA and 10µM EC significantly reduced PYY secretion during the first 24h, returning to normal levels after 48h (**Figure 1d,f**).

As our previous studies showed changes on L-cell production,[11] we also analysed enterohormone content in ileum organoids after chronic exposure to GSPE. GLP-1 content was increased after 24h of GSPE treatment (**Figure 2a**), followed by a significant and steady increase up to 72h when compared to the 24h

time point (1.00 ± *0.03* (24h) vs. 1.999 ± *0.16*** (48h) and 2.452 ± *0.38*** (72h)). PYY intracellular levels were also increased from 48h onwards (**Figure 2b**).

3.2 GSPE promotes L-cell development

To test whether the effects seen on L-cell production are due to an increase on L-cell numbers, ileum organoids exposed to 5mg/L of GSPE for 72h were stained for PYY, as a marker of L-cells, and analysed by flow cytometry. A significant 1.5-fold increase in the number of PYY-expressing cells was found when comparing to the untreated wells (**Figure 2c**). This effect on L-cell numbers was accompanied by a 2-fold increase on the gene expression of the endocrine marker ChgA from 48h onwards (**Figure 3a**). In agreement, gene expression of the specific L-cell markers, GCG and PYY, was also significantly higher after 48h, effect that was maintained until 72h, although showing only a trend for GCG at this last time point (*p=0.10*) (**Figure 3b,c**).

To further understand the role of GSPE on L-cell development, we also analysed the gene expression of several transcription factors involved in L-cell differentiation. Our results show a strong modulation of the transcription factor's profile by 5mg/L GSPE (**Figure 3**). GSPE significantly down-regulated Ngn3, Foxa2 and Pax4 gene expression, considered early/intermediate transcription factors, at all-time points (**Figure d-f**). Conversely, the late transcription factors analysed, Arx, Pax6 and NeuroD1, were up-regulated but only after 48h exposure to GSPE. At this time point, GSPE caused a significant up-regulation in Arx and Pax6 (*p<0.01*), and a trend (*p=0.058*) to increase in NeuroD1 gene expression (**Figure 3g-k**).

We also tested the effects of GA and EC on L-cell markers and found no differences on the gene expression of either ChgA, GCG or PYY. Regarding to the gene expression of transcription factors involved in L-cell differentiation, the only change observed after 5μM GA treatment was a down-regulation of Pax4, although to a lower extent than GSPE and only at 48h. The rest of the genes and time points were not affected by GA treatment (**Supporting Information Figure S1**). 10μM EC down-regulated Foxa2 after 24h and NeuroD1 after 48h, effects that were not maintained in the following time points. No other significant changes were observed after EC treatment (**Supporting Information Figure S1**).

3.3 GSPE promotes cellular differentiation in mouse ileum organoids

Finally, to evaluate whether GSPE had an effect on cell differentiation of other intestinal cell lineages, gene expression of markers of goblet cells (Muc2), enterocytes (SIS), paneth cells (Lyz1) and stem cells

(Lgr5+) were analysed after 72 hours of treatment initiation. GSPE up-regulated SIS and Muc2 gene expression (**Figure 4a,b**). Lyz1 gene expression was slightly reduced (**Figure 4c**) while Lgr5+ gene expression was strongly down-regulated (**Figure 4d**).

We also analysed the effect of EC and GA on those cell lineages, finding a slight decrease on SIS gene expression after exposure to GA for 72h. No effects were found on any of the other cell markers (**Supporting Information Figure S2**).

4. DISCUSSION

GSPE has been previously found to increase enterohormone production in colon, as well as the pan-endocrine marker ChgA,[9–11] suggesting a potential effect of GSPE on L-cell development. EECs are classified according to their specific secretory products, which have been reported to be based on tissue location. Specifically, the anorectic hormones GLP-1 and PYY, are released from L-cells, a subset of enteroendocrine cells that are more abundant in the distal ileum and colon.[22] Therefore, in the present study, we used intestinal organoids, a 3D *in vitro* model that recapitulates the *in vivo* intestinal architecture better that the usual cell lines,[16,23] in order to further understand the potential role of GSPE in intestinal cell differentiation.

In ileum organoids, exposure to GSPE for 24h significantly increased GLP-1 release, in parallel to an increase on its content but without affecting its gene expression. This is in line with previous studies showing increased GLP-1 levels after an acute treatment, both in *in vivo* and *ex vivo* models, without increasing GCG gene expression. Therefore, it is likely that GSPE directly increases GLP-1 secretion in ileum organoids, as it has been previously shown in other models.[6,7,9] The mechanisms involved in such regulation are still unknown, although the agonistic effect of flavonoids on the bitter taste receptors,[24–27] as well as their potential involvement in gut hormone release,[27–29] has been previously suggested. In any case, a more prolonged exposure to GSPE induced a significant and steady increase on GLP-1 content, which was accompanied by an increased GLP-1 release at 72h. Moreover, chronic exposure to GSPE up-regulated the gene expression of the endocrine cell marker ChgA, as well as the specific L-cell markers GCG and PYY, from 48h onwards. Although the effects of GSPE on GCG gene expression fainted after 48h, this is in agreement with Gehart et al. who recently reported that GCG expression peaks at 60h while retaining GLP-1 protein for a significant time afterwards.[30] Therefore, these results might suggest that long-term exposure to GSPE enhances basal GLP-1 levels by increasing L-cell population.

Although PYY gene expression and content were significantly up-regulated after chronic exposure to GSPE, no effects were found on PYY release in ileum organoids. Accordingly, we have previously shown that acute GSPE stimulation induces PYY secretion to a much lower extent than GLP-1,[31] and that the different compounds found in GSPE might act through different mechanisms, leading to a differential enterohormone release.[32] In fact, GLP-1 and PYY have been found to be stored in different storage vesicles in L-cells,[33,34] suggesting the existence of different response mechanisms for the regulation of the release of each hormone. On the other hand, it has recently been described that L-cells transition towards an I- or N-cell identity after around 70h of differentiation, being N-cells the ones showing a higher PYY gene expression,[30] which might explain the delayed increase in PYY content when compared to GLP-1, thus inducing a delayed secretory response. In this light, the higher numbers of PYY-expressing cells found in GSPE-treated organoids further suggests a potential effect of this extract on L-cell numbers. However, further studies are still required to clarify the differential effects of long-term exposure to GSPE on PYY and GLP-1 release.

The present results suggest a potential effect of long-term GSPE exposure on L-cell development, so we next analysed gene expression of different transcription factors involved in L-cell differentiation. While it is widely known that the basic helix-loop-helix (bHLH) transcription factor Ngn3 is required for the specification of the enteroendocrine cell fate,[35] its gene expression was found to be down-regulated by GSPE in ileum organoids. However, Ngn3 has a specific temporal expression pattern; it is essential in early differentiation stages and switches off before terminal differentiation (reviewed in [36]), having its peak during the first 24h of differentiation. Therefore, we analysed NeuroD1, which is a later bHLH transcription factor expressed sequentially downstream of Ngn3,[35] finding a trend to increase after 48h of exposure to GSPE. Moreover, two-way ANOVA analysis of temporal NeuroD1 gene expression in the control group revealed a peak at 72h, suggesting that GSPE might be able to induce an earlier NeuroD1 peak. Accordingly, the previously observed down-regulation in Nng3 expression might be due to the induction of an earlier differentiation by GSPE, therefore inducing an earlier peak of Ngn3 and, consequently, an earlier disappearance.

Downstream of Ngn3, several late-appearing transcription factors have been reported to control differentiation of a specific EEC type (reviewed in [36]). Among them, Arx is expressed during the crypt-villus migration in a subset of progenitor cells, maintained in nascent hormone-expressing cells and subsequently switched off. It has been reported that Arx and Pax4 play antagonistic roles, promoting the

differentiation of L- and D-cell lineages, respectively.[37] Accordingly, a peak on Arx was achieved after 48 hours in GSPE-treated organoids, resuming to control levels 24 hours later, which was coupled to a gradual down-regulation of Pax4. Arx has also been suggested to repress the winged-helix transcription factors Foxa1/a2,[37] which is consistent with the down-regulation of Foxa2 gene expression observed in GSPE-treated organoids.

The paired box transcription factor Pax6 has been shown to induce proglucagon gene transcription by binding to the G1 and G3 elements in the proglucagon promoter,[38] and to be activated by NeuroD1 during pancreatic differentiation.[39] Moreover, Gehart et al. recently reported that Pax6 expression peaks between 50 and 70h after starting differentiation in L- and N- cells.[30] Therefore, we also analysed Pax6 gene expression in ileum organoids after chronic exposure to GSPE, finding an up-regulation at 48h. These results further suggest an enhanced earlier differentiation towards L-cell due to the chronic exposure to GSPE, which correlates with the earlier peak found in NeuroD1 gene expression. In summary, even though enteroendocrine cell differentiation involves a complex network of transcription factors that is still unclear, our present data strongly suggest that GSPE might be able to induce L-cell differentiation in ileum organoids by regulating the gene expression of early transcription factors.

In order to understand whether the effects exerted by GSPE on cell differentiation were restricted to the L-cell lineage or also affected other intestinal cell types, we analysed gene expression of other cell type markers. Chronic GSPE treatment in ileum organoids enhanced Muc2 gene expression, marker of goblet cells, which is consistent with previous studies showing an increase on the density of goblet cells and Muc2 expression *in vivo* after chronic supplementation with a grape seed extract.[14,40] The green tea polyphenol (-)-epigallocatechin-3-gallate has also been reported to down-regulate β-catenin, the central player in the Wnt signalling cascade,[41–43] which regulates the proliferative state and is essential for the maintenance of the intestinal stem cell niche.[44] Despite differences in the structure of the polyphenols, the increase found in markers of the secretory lineages (ChgA and Muc2) after chronic GSPE treatment might be explained by an inhibition of the β-catenin signalling. Moreover, Paneth cells and stem cells undergo active Wnt pathway, inducing their maturation and proliferation, respectively;[45] and a down-regulation of both cell markers (Lyz1 and Lgr5+, respectively) was found after chronic exposure to GSPE, which is consistent with a potential inhibition of the β-catenin pathway. On the other hand, the Notch pathway is also essential for the maintenance of intestinal homeostasis, as it controls cell fate decisions (absorptive lineage versus secretory lineage) and patterning in the intestinal epithelium, based

on cell-cell communication pathways.[46] In parallel to an increase in the secretory lineages, chronic GSPE supplementation also enhanced SIS expression (marker of enterocytes), which might be due to an attempt to maintain the secretory:absorptive ratio by Notch-mediated lateral inhibition between adjacent cells in order to prevent the neighbouring cells adopting the same cell fate.[47] Taking altogether, our results suggest that GSPE might promote cell differentiation while suppressing cell proliferation, which is in agreement with previous reports showing an increased ileal villus height:crypt depth ratio after chronic grape seed extract supplementation *in vivo*.[40]

GSPE is a mixture of several molecules with different bioavailabilities and bioactivities, and EC and GA represent two of the most abundant compounds in this extract.[15] Therefore, they were also tested in an attempt to identify the molecules responsible for the above mentioned effects. Both EC and GA slightly modified gut hormone release although following a distinct pattern than GSPE. Furthermore, they did not show changes on gene expression of enteroendocrine markers and, although they also modulated the gene expression of a few transcription factors, those changes did not follow the same patterns than GSPE either. Therefore, it seems that these compounds are not the main responsible for the GSPE effects on EEC differentiation. These results are in line with previous *in vitro* studies showing that low molecular weight forms of flavonoids contained in GSPE do not mimic its acute effect on GLP-1 secretion,[9] suggesting that the GSPE-induced changes on L-cell development might be exerted by high molecular weight polymeric forms of polyphenols.

In conclusion, the present study demonstrates that GSPE is able to increase the production of the anorectic hormones PYY and GLP-1, which might be due in part to an enhanced L-cell differentiation. Although further studies are necessary to fully understand the mechanisms involved in GSPE-induced intestinal differentiation, this extract appears as a potential therapeutic strategy for obesity and T2DM treatment.

5. ACKNOWLEDGEMENTS

This work was supported by a grant (AGL2014-55347-R, AGL2017-83477-R) from the Spanish government. Àngela Casanova-Martí received a grant for Ph.D. students from the Universitat Rovira i Virgili. Joan Serrano received a pre-doctoral fellowship from the Government of Catalonia. Montserrat Pinent is a Serra Húnter fellow. We would like to thank Niurka Llopiz and Ingrid Pino for their technical support.

A.C-M., N.G-A., MT.B., and M.P., designed research; A.C-M., N.G-A., and J.S., performed experiments and collect data; A.C-M., N.G-A., X.T., G.F., and M.P., analysed data and interpreted results; A.C-M., N.G-A., G.F., M.P., and A.A., wrote manuscript.

The authors declare no conflict of interest.

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FIGURE CAPTIONS

Figure 1. Effects of GSPE, GA and EC on basal gut hormone release. GLP-1 and PYY levels were determined into the medium and normalized to DNA content after 24-72h of exposure to 0.05, 0.5 or 5mg/L of GSPE (**a,b**), 0.5 or 5µM GA (**c,d**), and 1 or 10µM EC (**e,f**). Data was normalized to thecorresponding control for each time point in the same experiment and presented as mean ± *SEM*. n=2 wells per condition collected across at least 3 independent experiments. One-way ANOVA with Dunnett's post-hoc test (a,c,e) or unpaired t-test (with Welch's correction when necessary) (b,d,f); **P<0.05,* ***P<0.005* vs control.

Figure 2. GSPE enhances enterohormone production and L-cell numbers. GLP-1 content (**a**), PYY content (**b**), and L-cell number (**c,**) in ileum organoids exposed to 5mg/L of GSPE for 72h. Data was normalized to the corresponding control for each time point in the same experiment and presented as mean ± *SEM*. n=3 wells per condition collected across at least 3 independent experiments. Unpaired t-test (with Welch's correction when necessary); **P<0.05,* ***P<0.01,* ****P<0.005,* *****P<0.0005,* vs control.

Figure 3. GSPE promotes L-cell development. Gene expression of the generic endocrine maker chromogranin A (ChgA) (**a**); specific L-cell markers, glucagon (GCG) and PYY (**b,c**); and transcription factors involved in L-cell differentiation: Ngn3, Foxa2, Pax4, NeuroD1, Arx, Pax6 (**d-i**), in ileum organoids exposed to 5mg/L of GSPE for 72h. Data are relative gene expression determined by the ΔΔCT method and presented as mean ± *SEM*. n=2 wells per condition collected across at least 3 independent experiments. Unpaired t-test (with Welch's correction when necessary); *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001, vs control.

Figure 4. GSPE promotes stem cell differentiation. Gene expression of the intestinal cell makers: Enterocytes (SIS) (**a**); Goblet cells (Muc2) (**b**); Paneth cells (Lyz1) (**c**); and stem cells (Lgr5+) (**d**), in ileum organoids exposed to 5mg/L of GSPE for 72h. Data are relative gene expression determined by the ΔΔCT method and presented as mean ± *SEM*. n=2 wells per condition collected across at least 3 independent experiments. Unpaired t-test (with Welch's correction when necessary); *P<0.05, **P<0.005, ****P<0.0001, vs control.

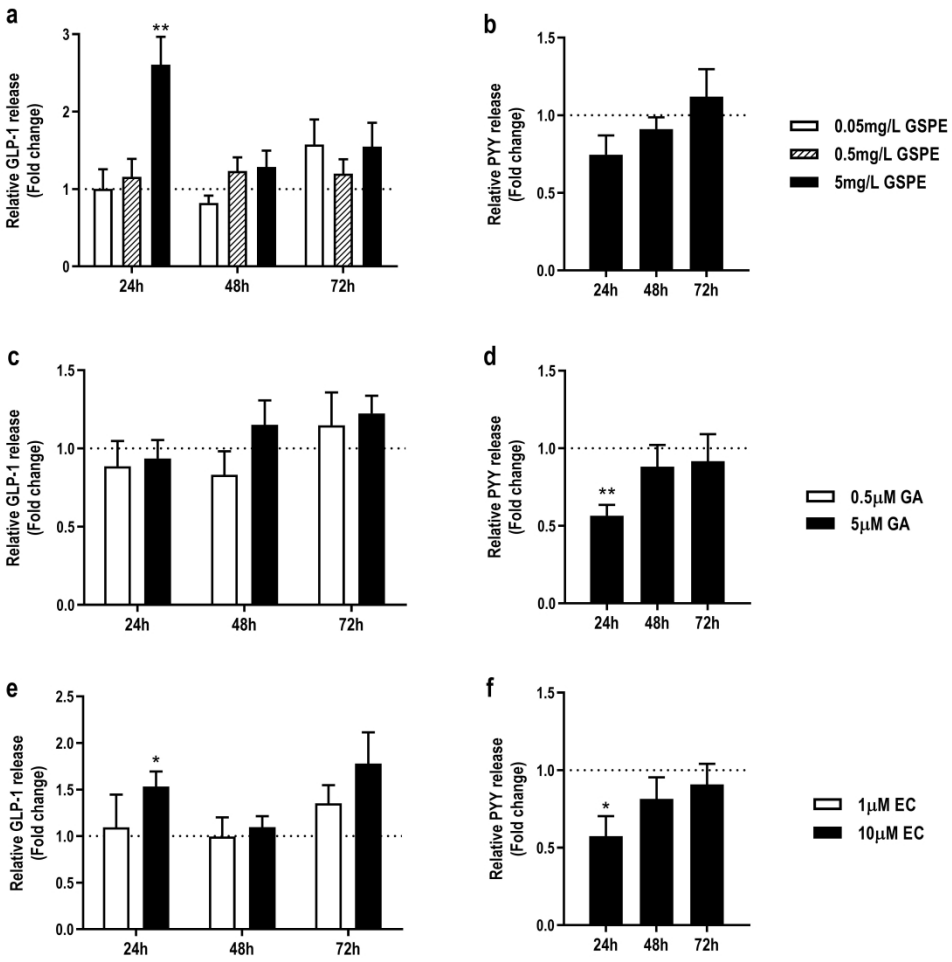

Figure 1

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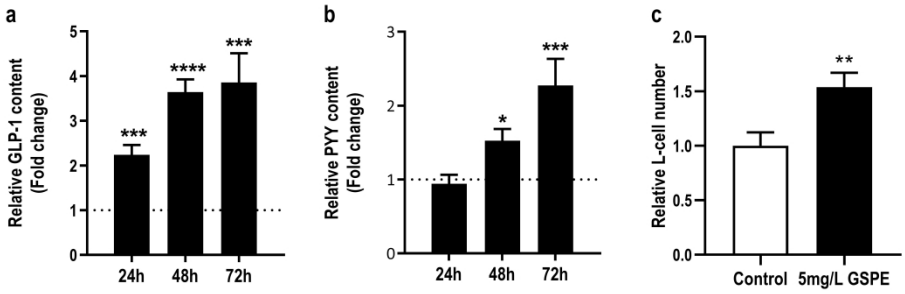

Figure 2

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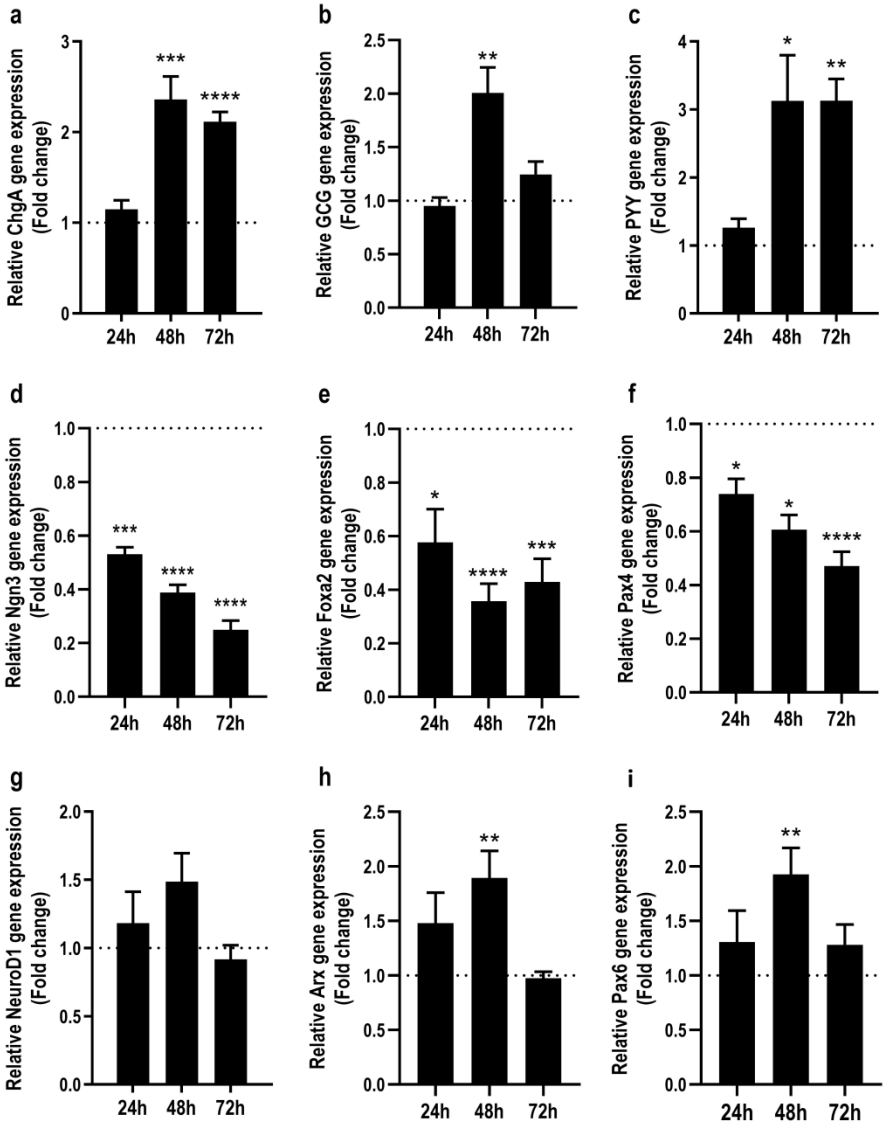

Figure 3

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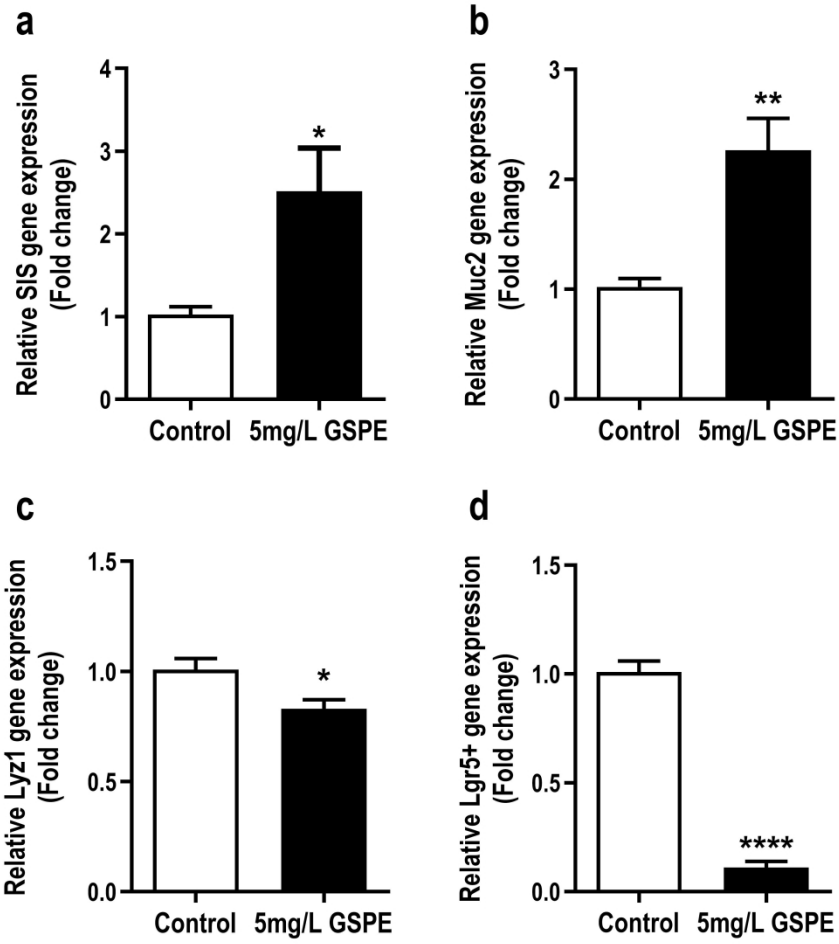

Figure 4

80x83mm (1200 x 1200 DPI)