The co-administration of proanthocyanidins and an obesogenic diet prevents the increase in intestinal permeability and metabolic endotoxemia derived to the diet

Katherine Gil-Cardoso, Iris Ginés, Montserrat Pinent, Anna Ardévol, Mayte Blay, Ximena Terra

PII: S0955-2863(18)30241-9
DOI: doi:10.1016/j.jnutbio.2018.07.012
Reference: JNB 8023

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 13 March 2018
Revised date: 14 June 2018
Accepted date: 25 July 2018

Please cite this article as: Katherine Gil-Cardoso, Iris Ginés, Montserrat Pinent, Anna Ardévol, Mayte Blay, Ximena Terra , The co-administration of proanthocyanidins and an obesogenic diet prevents the increase in intestinal permeability and metabolic endotoxemia derived to the diet. Jnb (2018), doi:10.1016/j.jnutbio.2018.07.012

The co-administration of proanthocyanidins and an obesogenic diet prevents the increase in intestinal permeability and metabolic endotoxemia derived to the diet

Gil-Cardoso, Katherine; Ginés, Iris; Pinent, Montserrat; Ardévol, Anna; Blay, Mayte; Terra, Ximena.

MoBioFood Research Group. Department of Biochemistry and Biotechnology, Rovira i Virgili University. Marcel·lí Domingo 1. PC 43007. Tarragona. Spain.

Running title: Effect of proanthocyanidins on intestinal barrier function

Keywords: cafeteria diet; gut dysfunction; permeability; barrier integrity; obesity; proanthocyanidins

Correspondence to:

Mayte Blay

Department of Biochemistry and Biotechnology, Rovira i Virgili University, Sescelades Campus, Marcel·li Domingo, 1

43007 Tarragona, Spain

E-mail: mteresa.blay@urv.cat

Phone: +34 977 55 8497

Abstract

The consumption of Westernized diets leads to hyperphagia and obesity, as well as intestinal alterations. In the present study, we evaluated the effect of the administration of a grape seed proanthocyanidin extract (GSPE) at different time points on the modulation of intestinal barrier function (intestinal permeability and metabolic endotoxemia), in rats with high-fat/high-carbohydrate diet-induced obesity. Animals were fed a cafeteria diet (CAF) supplemented with a preventive (PRE-CAF) or simultaneously intermittent (SIT-CAF) GSPE treatment (500 mg/Kg bw). Changes in the plasma levels of an orally administered marker of intestinal permeability (ovalbumin, OVA), lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) were analysed after animals were fed the obesogenic diet for 8, 12 and 17 weeks. In addition, *ex vivo* variations in transepithelial electrical resistance (TEER), the expression of tight junction (TJ) genes and the activity of myeloperoxidase (MPO) in the small and large intestines were monitored at the end of the experiment. The CAF diet increased OVA, LPS, MPO and TNF-α levels, accompanied by decreased TEER values in the small and large intestines. Interestingly, both GSPE treatments prevented these detrimental effects of the CAF diet, being the SIT-CAF group the most effective after 17 weeks of diet intervention. For the first time, this study provides evidence of the ameliorative effect of a proanthocyanidin extract, administered before or together with an obesogenic diet, on barrier dysfunction, as measured by intestinal permeability and metabolic endotoxemia.

Introduction

The intestinal epithelium is the first site where diet, microbes and host interact [1]. Intestinal epithelial cells are distributed in a single layer lining the intestinal lumen and have two critical functions: a selective filter allowing the translocation of water, electrolytes and molecules from the intestinal lumen into the circulation [2–4] and a barrier that prevents the passage of harmful intraluminal entities, including foreign antigens and their toxins [5,6]. The transport of molecules from the intestinal lumen to the lamina propria occurs through two recognized routes: transepithelial/transcellular and paracellular pathways. Transcellular permeability is associated with solute transport through the epithelial cells and is regulated by selective transporters for electrolytes, amino acids, short chain fatty acids and sugars [2–4]. On the other hand, paracellular permeability consists of the transport of molecules in the space between epithelial cells, a mechanism regulated by intercellular complexes located at the apical-lateral membrane junction [7]. TJs are the most important shield of the intestine and prevent the paracellular permeation of macromolecules into the circulation [8]. TJs are formed by the interaction of transmembrane proteins, such as occludin (OCLN) [9], claudins [10], and junctional adhesion molecules (JAMs) [11], with intracellular plaque-forming zonulin/zonula occludens (ZO) proteins [12].

According to previous results from our group, rodents fed a cafeteria (CAF) diet for extended periods, as a model of high-fat/high-carbohydrate diet, exhibit diet-induced obesity (DIO) [13,14], which has been proposed to disrupt the intestinal barrier function by altering TJs [15]. This fact was associated with increased bacteria-derived lipopolysaccharide (LPS) absorption across the intestinal barrier, increasing its plasma levels by two to three times, process referred to as metabolic endotoxemia [13,15–18]. Due to the link between intestinal alterations and the detrimental consequences of obesity, finding modulators of the physiological functions in the intestine with anti-obesity properties, is of great interest. We have previously reported that a corrective treatment with a grape-seed extract rich in proanthocyanidins (GSPE) at a dietary dose of 25 mg/kg of body weight was effective ameliorating obesity-associated intestinal alterations by limiting TJs gene expression modulation and reducing intestinal inflammation in rats [15]. However, this nutritional dose was not effective at decreasing body weight. In contrast,

we have previously reported that a 500 mg/kg of body weight sub-chronic treatment was effective at reducing body weight by limiting food intake and activating energy expenditure in rats fed standard chow diet [19]. Additionally, from a functional food perspective, the most appropriate way for a compound to be effective as an anti-obesity agent is to prevent and/or approach the problem at the initial stages, and the same hypothesis might be applied for intestinal health.

To measure intestinal permeability, TJs gene expression and protein localization studies provide important data, but limited at the same time, on how paracellular pathway might be altered [20]. In a more approximate way, *in vivo* intestinal permeability tests have been useful to show impaired intestinal permeability in presence of the obesity phenotype [21,22]. However, more in-sight studies are needed to identify the site of the defect. In this work, *ex vivo* studies, based on Ussing Chamber technic, were performed to compare changes derived from nutritional interventions in the different sections of the small and large intestine.

To prove the protective effectiveness of proanthocyanidins before an obesogenic situation, in the present study, we evaluate the effects of a high pharmacological dose of GSPE (500 mg/kg) administered at different time points with respect to the start of the obesogenic challenge. With this experimental design, we aimed: i) to compare the effectiveness of different dosing strategies against intestinal disarrangements associated with obesity, ii) to investigate whether a pharmacological dose is more or less effective than lower doses, and iii) finally, to investigate the duration of the potential protective effect.

Materials and Methods

Proanthocyanidin extract

Grape-seed proanthocyanidins extract (GSPE) was provided by Les Dérives Résiniques et Terpéniques (Dax, France). This proanthocyanidin extract contained monomeric (21.3%), dimeric (17.4 %), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 units, 31.7%) proanthocyanidins. The GSPE composition used in this study is described in **Table 1**.

Animal model

Forty 7-week-old, female Wistar rats, each weighing 240-270g, were purchased from Charles River Laboratories (Barcelona, Spain). Rats were individually caged in the animal quarters at 22°C with a 12-hour light/12-hour dark cycle and were fed ad libitum a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. After a period of acclimation, animals were randomly distributed into 4 experimental groups (n=10) and were fed ad libitum a standard chow diet. One group of animals (PRE-CAF group) received a preventive treatment with proanthocyanidins (500 mg GSPE/Kg bw) for 10 days, whereas the rest of animals received just the vehicle (water). When the pre-treatment finished one group of animals was fed ad libitum a standard chow diet for the rest of the entire experimental procedure (STANDARD group). The PRE-CAF and a second group (CAF group) received the CAF diet as a model of high fat/high carbohydrate diet for 17 weeks combined with the standard chow diet. The last group received the standard chow diet and the CAF diet simultaneously with the treatment of proanthocyanidins (500 mg GSPE/Kg bw) every other week (Simultaneous-Intermittent-Treatment-CAF; SIT-CAF) for the 17 weeks. This diet was freshly offered ad libitum per day to the animals in a plate with enough quantities. The CAF intervention consisted of bacon, sausages, biscuits with paté, carrots, muffins, and sugared milk. The CAF diet was a high-energy diet and it had additional amounts of fat and sucrose, being a highly palatable diet that induces voluntary hyperphagia. The daily content of nutrients in the CAF and the standard diets is summarised in Table 2. The GSPE was dissolved in water and was orally gavage administered to the animals for each treatment in a volume of 500 μL. The experimental design is represented in **Figure 1**.

Blood and tissue collection

At the end of the study, animals were fasted for 4 hours, were anesthetized with sodium pentobarbital (70 mg/kg body weight; Fagron Iberica, Barcelona, Spain) and exsanguinated from the abdominal aorta. The blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500g, 15 minutes, 4°C) and stored at −80°C until analysis. After opening the abdomen, the different white adipose tissue depots (retroperitoneal (RWAT), mesenteric (MWAT) and periovaric (PWAT)) were rapidly removed, weighed, frozen in liquid nitrogen and stored at −80°C until later analysis. Subsequently, the small intestine, defined as the part

of the gastrointestinal tract between the pylorus and the ileocecal valve was dissected and its length was measured, the same process was followed to the colon dissection. A 5 cm segment of duodenum, ileum and colon were taken for Ussing chamber measurements. The whole jejunum together with the leftover segments of the other areas of the small intestine and the colon were stored at −80°C pending gene expression posterior analysis. All the experimental procedures were performed according to the national and institutional guidelines for animal care and use that are in place at our university. The Animal Ethics Committee of our university approved all the procedures.

Morphometric variables

Body weight was monitored weekly, and the percentage of weight gain was calculated. Adiposity was expressed with an adiposity index, which was computed for each rat as previously described [23]. These variables together with the MWAT depot weight were evaluated as physiological markers of the degree of obesity in these animals.

Oral intestinal permeability test

The intestinal permeability was assessed *in vivo* by the oral ovalbumin (OVA) test at the week 12 and 17 [24]. For OVA tests, animals were previously fasted four hours. OVA (Sigma-Aldrich, Madrid, Spain) was administered to the rats by orally gavage at a concentration of 250 mg/Kg bw diluted in 500 µl of PBS. One hour after, blood was collected from the saphenous vein, was heparinized and centrifuged (10 min, 12,000 *g*, 4 °C). Plasma OVA levels were determined by ELISA with detection range of 16-10,000pg/mL (MyBioSource, Madrid, Spain).

Ussing chamber experiments: TEER and evaluation of the intestinal mucosa integrity by LY

Intestinal permeability was also evaluated *ex vivo* by the measurement of transepithelial electrical resistance (TEER). At the end of the experiment, freshly intestinal tissues (duodenum, ileum and colon) were immediately placed in cold oxygenated Krebs buffer, dissected to remove serosal and muscular layers, and mounted on 0.237-cm^2 aperture Ussing chambers (Dipl.-Ing. Muβler Scientific Instruments, Aachen, Germany). Tissues were mounted within 10 min following euthanasia and were bathed on the mucosal and serosal sides with Krebs buffer (KRB buffer). The serosal bathing solution, which contained 10 mM glucose (Panreac, Barcelona, Spain) was osmotically balanced on the

mucosal side with 10 mM mannitol (Sigma, Madrid, Spain). Bathing solutions were oxygenated and circulated in water-jacketed reservoirs maintained at 37 °C. The spontaneous potential difference (PD) was short circuited through Ag-AgCl electrodes with a voltage clamp that corrected for fluid resistance. TEER ($\Omega \cdot cm^2$) was calculated from the spontaneous PD and short-circuit current.

After 20-minutes equilibration period, the KRB buffer in the donor compartment (apical: representing the intestinal lumen) and in the receiver compartment (basolateral: representing the blood circulation) were replaced by fresh KRB buffer containing 10 mM glucose, proteases inhibitors: 10 μM amastatin (Enzo Life Sciences, Madrid, Spain), 500 KIU aprotinin (Sigma, Madrid, Spain) and 0.1 % of bovine serum albumin (BSA) fatty acid free. The unidirectional permeability of paracellular marker lucifer yellow (LY) was determined to ascertain the integrity of the intestinal mucosa. LY (VWR, Barcelona, Spain) in a concentration of 50 μM was added to the donor compartment and samples collected from receiver compartment were measured by fluorescence (excitation wavelength 485 nm and emission wavelength 535 nm) at time 0 and 30 minutes.

LPS and TNF-α plasma determinations

Plasma LPS levels were determined using Pyrochrome Lysate Mix (Associates of Cape Cod, E. Falmouth, MA) diluted in Glucashield buffer (Associates of Cape Cod, E. Falmouth, MA), which inhibits cross-reactivity with (1→3)-β-d-glucans. ELISA Kit was used to measure the plasma concentrations of tumour necrosis factor-α (TNF-α) (Merck Millipore, Madrid, Spain). LPS and TNF-α were monitored during the experiment the weeks 8, 12 and 17 following the manufacturer's protocol.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was analysed in the ileum following the protocol described previously [13], based on a modification of the 2-*o*-methoxyphenol (guaiacol) peroxidation assay.

Tissue RNA extraction and gene expression analysis by qRT-PCR

RNA was isolated from 50 mg of duodenum, ileum and colon using Trizol (AMbion, USA) following the instructions as we described previously [13]. RNA concentration and purity were assessed using NanoDrop 1000 Spectrophotometer (Thermo Fisher

Scientific, Barcelona, Spain). Sample absorbance was measured at 260 nm and 280 nm, and the 260/280 ratio was used to assess RNA purity. RNA purity was considered adequate when the 260/280 ratio was ≥1.9. cDNA was obtained using the High capacity cDNA Reverse Transcription kit (Applied Biosystems, Madrid, Spain) following manufacturer's instructions. For quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR), amplification and detection were performed using TaqMan Universal PCR Master Mix and Taqman probes (Applied Biosystems, Madrid, Spain). The references of Taqman probes are the following: Rn02116071_s1 for rat *Zo-1*, Rn01420322_g1 for rat *Ocln*, Rn00587389_m1 for rat *Jam-A*, Rn00581740_m1 for rat *Claudin-1*, Rn02063575_s1 for rat *Claudin-2*, Rn00581751_s1 for rat *Claudin-3*. All the results were normalized respect to cyclophilin E (*Ppia*) (Rn00690933_m1) and *Gapdh* (Rn01775763_g1). For the rest of the genes, cDNA (5 ng/mL) was subjected to qRT-PCR amplification using SYBR Green PCR Master Mix (Applied Biosystems, Madrid, Spain). The forward (Fw) and reverse (Rv) primer sequences used are: *Ppia*, Fw: 5-CTTCGAGCTGTTTGCAGACAA-3, Rv: 5-AAGTCACCACCCTGGCACATG-3; *Gapdh,* Fw: 5-TCTTGTGCAGTCCCAGCCT-3, Rv: 5-CAATATGGCCAAATCCGTTCA-3; *Mlck*, Fw: 5-CCCTTCCTTCTCTAGTGTTCTGA-3, Rv: 5-AGCCTCACAGATGGATCGAG-3. Reactions were run on a qRT-PCR system (Applied Biosystems, Madrid, Spain) where the thermal profile settings were 50 °C for 2 minutes, 95 °C for 2 minutes and then 40 cycles of 95 °C for 15 seconds and 60 °C for 2 minutes. The relative mRNA expression levels were calculated with the $2\text{-}\Delta\Delta C_t$ method, where ΔC_t = Ct gene of interest - C_t cyclophilin and $\Delta\Delta C_t = \Delta C_t$ treated samples - the mean of ΔC_t control samples.

Statistical analysis

The results are expressed as the mean value ± standard error of the mean (SEM). The effects were assessed using ANOVA test. P-values < 0.05 were considered to be statistically significant. Pearson's correlation coefficient was evaluated to assess relationships between TEER values, plasma OVA, TNF-α and LPS levels, compared with morphometric variables and compared with intestinal permeability and systemic inflammation markers. These results were obtained using XLSTAT 2015.5 (Addinsoft) software.

Results

Effects of GSPE on morphometric variables

We evaluated morphometric parameters as physiological markers of the degree of obesity recorded after the diet intervention. After 17 weeks of feeding on the CAF diet, the CAF group showed significant increases in weight gain and final body weight, corresponding to higher percentages of adiposity and MWAT weight compared to the STD group (**Table 3**). In addition, the SIT-CAF supplementation with 500 mg/Kg bw GSPE significantly decreased the body weight, MWAT and adiposity index; however, no changes were observed in the PRE-CAF group at the end of the experiment (**Table 3**). A complete analysis of the effects of GSPE on morphometric variables has been previously published [14].

Oral intestinal permeability test

We used the oral OVA test as a physiological marker of possible alterations in the intestinal barrier permeability at weeks 12 and 17. After 12 weeks of feeding on the CAF diet, animals showed a higher plasma OVA level, which translates to a higher intestinal permeability. Interestingly, both the PRE-CAF and SIT-CAF GSPE supplements reduced these values at this time point. After 17 weeks, the CAF group showed a higher plasma OVA concentration than the 12-week measurement, and the SIT-CAF supplemented maintained its protective effect on intestinal barrier disruption. Interestingly, lower OVA levels were detected in the PRE-CAF group than in the CAF group, then the pre-treatment with GSPE was able to maintain its early protective effect after 17 weeks of diet intervention (**Figure 2**).

Pearson's correlation test was used to identify whether intestinal permeability was associated with the degree of obesity observed at different time points. OVA levels at week 12 were positively associated with final weight gain ($r=0.475$, $p=0.044$), MWAT weight ($r=0.418$, $p=0.028$) and the final body weight ($r=0.500$, $p=0.041$). After 17 weeks, these correlations were stronger and a new positive correlation with the adiposity index was established (**Table 4**). We also correlated the plasmatic variables measured at week 8 and 12 with the percentage of weight gain obtained at the weeks 8 and 12, respectively, but no significant relationships appeared (data not shown).

Integrity of the intestinal mucosa and intestinal TEER values

Duodenum, ileum and colon sections were collected for *ex vivo* assessments. LY was evaluated to ascertain the integrity of the intestinal mucosa using an Ussing chamber assembly. No changes in any of the intestinal sections or the treatment groups were observed (**Figure 3A**). These results confirm the correct operation of the assembly and the mechanism of action of the Ussing chamber system.

Regarding the TEER results, we obtained similar TEER values for both the small and large intestines in the STD group, ranging between 20-25 Ωxcm^2. However, the CAF diet intervention significantly reduced TEER values by more than the 50% (between 8-11 Ωxcm^2) compared with the STD group in all the sections analysed. Both GSPE treatments (PRE-CAF and SIT-CAF) clearly protected against intestinal barrier dysfunction in the duodenum induced by the CAF diet, without any difference between treatments. In the ileum and colon, the GSPE treatment also reversed the effects of the CAF diet, although this recovery was not as effective as the level observed in the duodenum (**Figure 3B**).

We then evaluated the potential correlations between TEER values and morphometric parameters. In general, intestinal TEER values exhibited a strong negative correlation with the degree of obesity observed at 17 weeks. The ileal TEER value was negatively correlated with MWAT, adiposity, and final weight. The TEER values in the duodenum were negatively correlated with MWAT and adiposity, and finally, the colon TEER was correlated with adiposity (**Table 4**). Additionally, small and large intestinal TEER values were negatively correlated with the results of the OVA permeability test at the end of the experiment, and a correlation between the ileal TEER and OVA levels began to be observed at week 12 (**Table 5**).

In order to determine the inflammatory status of the intestine myeloperoxidase (MPO) activity was evaluated in ileum samples. CAF group showed increased MPO activity (7528±1236 mU/g) compared to the STD group (1991±227 mU/g, $p<0.001$). However, PRE-CAF and SIT-CAF treatments were able to normalize MPO activity (2070±516, $p<0.001$, and 1872±383 mU/g, $p<0.001$, respectively).

Effects of GSPE on plasma TNF-α and LPS levels

We monitored plasma LPS levels throughout the experiment to investigate the correlations between obesity, barrier dysfunction and metabolic endotoxemia. From

week 8 until the end of the experiment, statistically significantly higher plasma LPS levels were detected in the CAF group than in the STD group. Additionally, both GSPE treatments were effective at normalizing LPS levels after rats had consumed the CAF diet for 17 weeks (**Figure 4A**).

We measured plasma TNF-α levels at different time points during the experiment to monitor the degree of inflammation associated with DIO in the animals. After 8 on the CAF diet, no changes were observed compared to the STD group. Beginning at week 12, the CAF group started to show increased plasma TNF-α concentrations, and this state of inflammation persisted until the end of the diet intervention. The SIT-CAF supplement with GSPE was effective at reducing TNF-α levels at 12 and 17 weeks of feeding on the CAF diet. The PRE-CAF treatment also reduced TNF-α levels, although this difference did not reach the statistical significance (**Figure 4B**).

LPS levels at week 8 were positively correlated with final adiposity (r=0.389, p=0.028). Interestingly, weight gain, final weight and adiposity at the end of the CAF diet intervention were also related to LPS levels (Table 4). In addition, 17 week LPS levels were positively correlated with 12 and 17 weeks OVA levels (Table 5). As expected, TNF-α levels at 12 weeks were positively correlated with final adiposity (r=0.327, p=0.047) and with MWAT and adiposity at the end point (Table 4). In addition, TNF-α levels at 12 and 17 weeks were positively correlated with 12 weeks OVA (r=0.497, p=0.012; r=0.367, p=0.049, respectively). *Ex vivo* permeability tests revealed strong correlations between TEER values in all sections and TNF-α levels (**Table 5**).

Expression of TJ and MLCK genes in the small and large intestines

The paracellular pathway is constituted by TJs, consisting of zonulins, occludin, claudins, and JAMs proteins. In the present study, we evaluated the effects of the CAF diet and the supplementation with proanthocyanidins on the expression of TJ proteins in the small (duodenum and ileum) and large intestine (colon). *Claudin-1* gene expression in the ileum was decreased by the CAF intervention, and the SIT-CAF treatment increased its expression. We did not observe any changes in the expression pattern of TJ genes in the duodenum and colon (**Table 6**).

In addition, myosin light-chain kinase (MLCK) is another molecule that plays a central role in regulating intestinal TJ permeability. Cytokine-mediated barrier dysfunction might be regulated by the expression of the *Mlck* gene and protein. Therefore, the

expression of the *Mlck* gene was evaluated in the present study. However, diet- or GSPE-induced changes in *Mlck* expression throughout the intestine were not observed (**Table 6**).

Discussion

In the present work, animals were fed an unhealthy, highly palatable, and energy-dense human CAF *ad libitum*. Compared to traditional lard-based high-fat diets, the CAF diet is considered a more robust model of obesity and other metabolic disruptions, as metabolic syndrome [25]. This diet induces voluntary hyperphagia, resulting in rapid weight gain and an increase in the fat pad mass. In our experiment, rats fed a CAF diet showed a 27% increase in body weight, a 136% increase in the adiposity index and a 169% increase in MWAT weight compared to animals fed a STD diet. These results support the conclusions obtained by Sampey *et al.*, reaffirming the CAF diet as a solid model of obesity [25]. In addition, the SIT-CAF supplementation with GSPE drastically reduced the body weight and adiposity index, suggesting that at this dose proanthocyanidins act as potent anti-obesity agents. As reflected in our previous publication, these results were at least partially derived from the inhibition of energy intake and increase in energy expenditure [14] and are consistent with the results obtained by Serrano *et al.* at using the same dose of GSPE administered to healthy rats [19].

Once we achieved a strong obesity model induced by the CAF diet intervention, markers of intestinal barrier function were analysed and monitored at different time points of the intervention. According to our results, the CAF group showed a significant and time-dependent increase in plasma OVA levels, indicating an increase in the intestinal permeability caused by a negative effect of the diet. In addition, lower TEER values were observed both in the small and the large intestines of the CAF group than in the STD group. When the effects of proanthocyanidins were evaluated, we detected a protective effect at week 12, with a reduction in the intestinal permeability in response to both the SIT-CAF and PRE-CAF treatments, but only the SIT-CAF treatment still maintained this beneficial effect throughout the intervention. To our knowledge, no other study has described the effect of proanthocyanidins on the intestinal permeability throughout the intestine of an obesity model *ex vivo.* For the first time, we show that both PRE-CAF and

SIT-CAF proanthocyanidin treatments were able to normalize TEER values in the intestine. On the other hand, an early negative correlation was observed between ileal TEER values and OVA levels at week 12, but not in values obtained in duodenum or colon sections. In this sense, the ileum is more closely related to morphometric parameters and therefore the global state of diet-induced obesity. Based on these results, the ileum is the most sensitive region of the intestine to early changes in barrier function, consistent with the findings reported by Hamilton *et al.* [26]. These results might be explained by the observation that the immune sensors of the intestine, Peyer's patches (PPs) are mainly located in the ileal region of the small intestine. In addition, several studies have revealed the critical roles of the ileum and the PPs in the pathogenesis of chronic intestinal diseases associated with increased intestinal permeability and intestinal inflammation [27,28]. Once the beneficial role of proanthocyanidins in the regulation of barrier function has been described, myeloperoxidase (MPO) activity was evaluated in the ileum as a marker of intestinal inflammation and an index of neutrophil infiltration in the intestinal mucosa [29,30]. Results show that in the CAF group, MPO activity was increased by 278% in comparison with the standard group, whereas both treatments of GSPE normalized MPO activity in the ileum. These results confirm intestinal permeability is directly associated with intestinal inflammation, not only in the pathogenesis of chronic intestinal diseases, but also in metabolic diseases. More importantly, the results reveal the effectiveness of proanthocyanidins on promoting intestinal health.

Small changes in TJ gene expression were observed along the different sections analysed in this study in response to the diet or the proanthocyanidin administration. *Claudin-1* expression was reduced in the ileum in response to the CAF diet, and the SIT GSPE treatment normalized its expression. As shown in our recent study of Wistar rats with similar characteristics as the rats used in the present study, the administration of dietary doses of GSPE for 21 days after 15 weeks of feeding on a CAF diet exerted a beneficial effects on diet-induced *Zo-1* alterations, but not *Claudin-1* alterations [15]. Depending on the dose administered and the time of administration, proanthocyanidins could be modulating in a different way transcription factors that control the expression of TJs, as well as inducing different epigenetic modifications. Transcription factors and epigenetic modifications play crucial roles in the regulation of gene expression. Although their mechanism of action is not fully defined, flavonoids were found to act

mostly as site-directed small molecule inhibitors or promoters on signalling [31,32]. In addition, flavonoids are postulated as potent epigenetically active compounds, affecting the modulation of the DNA methylation status and histone acetylation [33–35], that could explain the variability observed in terms of TJs gene expression modulation by proanthocyanidins.

Increased intestinal permeability has been closely linked to systemic endotoxemia [16–18]. At week 8, increased LPS levels were observed in the plasma of the animals fed the CAF diet compared to the control group, and these values continued to increase over the course of the nutritional intervention. The LPS concentration at the end of the experiment was positively correlated with intestinal permeability at weeks 12 and 17 and with morphometric measurements, supporting the hypothesis that the disruption of intestinal integrity occurs before metabolic endotoxemia and in parallel with the degree of obesity of these animals. Both GSPE treatments prevented the CAF diet-induced increase in the plasma LPS levels at the end of the experiment. Metabolic endotoxemia is also related to systemic inflammation [18,36]. In the present study, the CAF diet increased TNF-α levels from the 12th week to the end of the experiment and the SIT GSPE treatment normalized these values. Because LPS levels began to increase at the 8th week, we conclude that metabolic endotoxemia precedes a global stage of systemic inflammation. Simultaneously, the positive correlation between TNF-α levels and both OVA and TEER throughout intestine reaffirm the crosstalk between barrier physiology and function and systemic inflammation.

In summary, this study describes the temporal relationship between obesity and impaired intestinal barrier dysfunction as well as the protective effects of proanthocyanidin supplementation. Consumption of a CAF diet as a model of a high-fat/high-carbohydrate diet leads to excessive energy intake and storage, as well as increases intestinal permeability and metabolic endotoxemia, which together generally contribute to a state of systemic inflammation associated with obesity. In addition, the positive correlations between all markers of intestinal health and adiposity index suggest a key role of the intestine-adipose tissue axis in the development of obesity and metabolic disorders. Additionally, our results demonstrated the consumption of proanthocyanidins improves intestinal health, including intestinal permeability and metabolic endotoxemia, and also provides fundamental data regarding possible

nutritional treatments for obesity, demonstrating the effectiveness of proanthocyanidins consumed in a SIT way. Considering that this product is available for human consumption, proanthocyanidins applications could be directly translated to the human population. However, the timing of the treatment and the optimal dose need to be adjusted. Although, the intermittent has been proved to be the most effective strategy, the PRE-CAF treatment interestingly maintains a positive effect until the 12th week of the obesogenic diet intervention. Margalef et al. demonstrated long-term GSPE intake did not trigger a flavanol tissue accumulation [37], indicating a clearance of products at each daily dosage, and suggesting that polyphenol benefits in a disease state, like obesity, would be due to a daily pulsatile effect. This fact creates more doubts about the protective effect of the PRE-CAF treatment, which could suggest a memory effect of proanthocyanidins in the organism, however, more studies are needed that elucidate the molecular mechanisms involved in this effect. It is necessary to point out that the levels of proanthocyanidins in the diet are not correlated with their body levels, considering that proanthocyanidins are known to be poorly absorbed in the intestine. Therefore, the beneficial health effects of flavonoids are mainly attributed to compounds derived from their metabolism [38].

Based on these findings, nutritional and therapeutic interventions based on intestinal health and modulation of the intestinal barrier function should be extensively explored in obese subjects. Future investigations are necessary to elucidate the particular mechanisms underlying the effects of proanthocyanidins on these obesity-induced intestinal alterations. Progress in this field of investigation may lead to novel nutritional therapeutic modalities to reduce the impact of the Western lifestyle on whole-body homeostasis.

Author contributions

K. G.-C. performed the laboratory work, initiated the literature search and was in charge of drafting the manuscript. Both K. Gil-Cardoso. and I. Ginés designed the figures. M. Blay. revised the first drafts. X. Terra. was responsible for the final editing and content. All of the authors critically reviewed the manuscript and approved the final version.

Acknowledgments

K. Gil-Cardoso and I. Ginés are student fellows from the Martí i Franquès program of Rovira i Virgili University. M. Pinent is a Serra Húnter fellow.

Conflict of Interest

None.

References

[1] Natividad JMM, Verdu EF. Modulation of intestinal barrier by intestinal microbiota: Pathological and therapeutic implications. Pharmacol Res 2013;69:42–51.

[2] Kunzelmann K, Mall M. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. Physiol Rev 2002;82:245–89.

[3] Bröer S. Amino acid transport across mammalian intestinal and renal epithelia. Physiol Rev 2008;88:249–86.

[4] Ferraris RP, Diamond J. Regulation of intestinal sugar transport. Physiol Rev 1997;77:257–302.

[5] Blikslager AT, Moeser AJ, Gookin JL, Jones SL, Odle J. Restoration of barrier function in injured intestinal mucosa. Physiol Rev 2007;87:545–64.

[6] Podolsky DK, Babyatsky MW, DeBeaumont M, Thim L, Podolsky DK, Banan A, et al. Mucosal immunity and inflammation. V. Innate mechanisms of mucosal defense and repair: the best offense is a good defense. Am J Physiol 1999;277:G495-9.

[7] Van Itallie CM, Anderson JM. CLAUDINS AND EPITHELIAL PARACELLULAR TRANSPORT. Annu Rev Physiol 2006;68:403–29.

[8] Suzuki T, Hara H. Role of flavonoids in intestinal tight junction regulation. J Nutr Biochem 2011;22:401–8.

[9] Saitou M, Furuse M, Sasaki H, Schulzke JD, Fromm M, Takano H, et al. Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol Biol Cell 2000;11:4131–42.

[10] Krause G, Winkler L, Mueller SL, Haseloff RF, Piontek J, Blasig IE. Structure and function of claudins. Biochim Biophys Acta 2008;1778:631–45.

[11] Garrido-Urbani S, Bradfield PF, Imhof BA. Tight junction dynamics: the role of junctional adhesion molecules (JAMs). Cell Tissue Res 2014;355:701–15.

[12] Bauer H, Zweimueller-Mayer J, Steinbacher P, Lametschwandtner A, Bauer HC. The dual role of zonula occludens (ZO) proteins. J Biomed Biotechnol 2010;2010:402593.

[13] Gil-Cardoso K, Gines I, Pinent M, Ardevol A, Terra X, Blay M. A cafeteria diet triggers intestinal inflammation and oxidative stress in obese rats. Br J Nutr 2017:1–12.

[14] Ginés I, Gil-Cardoso K, Serrano J, Casanova-Martí À, Blay Mt, Pinent M, et al. Effects of an Intermittent Grape-Seed Proanthocyanidin (GSPE) Treatment on a Cafeteria Diet Obesogenic Challenge in Rats. Nutrients 2018;10:315.

[15] Gil-Cardoso K, Ginés I, Pinent M, Ardévol A, Arola L, Blay M, et al. Chronic supplementation with dietary proanthocyanidins protects from diet-induced intestinal alterations in obese rats. Mol Nutr Food Res 2017;61:1601039.

[16] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates

obesity and insulin resistance. Diabetes 2007;56:1761–72.

[17] Kaliannan K, Hamarneh SR, Economopoulos KP, Nasrin Alam S, Moaven O, Patel P, et al. Intestinal alkaline phosphatase prevents metabolic syndrome in mice. Proc Natl Acad Sci U S A 2013;110:7003–8.

[18] Kim K-A, Gu W, Lee I-A, Joh E-H, Kim D-H, Faulds M, et al. High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. PLoS One 2012;7:e47713.

[19] Serrano J, Casanova-Martí À, Gual A, Pérez-Vendrell AMAM, Blay MTT, Terra X, et al. A specific dose of grape seed-derived proanthocyanidins to inhibit body weight gain limits food intake and increases energy expenditure in rats. Eur J Nutr 2017;56:1629–36.

[20] Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009;58:1091–103.

[21] De La Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, Raybould HE. Propensity to High Fat Diet-Induced Obesity in Rats Is Associated with Changes in the Gut Microbiota and Gut Inflammation. Am J Physiol Gastrointest Liver Physiol 2010;299:G440-8.

[22] Teixeira TFS, Souza NCS, Chiarello PG, Franceschini SCC, Bressan J, Ferreira CLLF, et al. Intestinal permeability parameters in obese patients are correlated with metabolic syndrome risk factors. Clin Nutr 2012;31:735–40.

[23] Baselga-Escudero L, Pascual-Serrano A, Ribas-Latre A, Casanova E, Salvadó MJ, Arola L, et al. Long-term supplementation with a low dose of proanthocyanidins normalized liver miR-33a and miR-122 levels in high-fat diet–induced obese rats. Nutr Res 2015;35:337–45.

[24] Volynets V, Reichold A, Bárdos G, Rings A, Bleich A, Bischoff SC. Assessment of the Intestinal Barrier with Five Different Permeability Tests in Healthy C57BL/6J and BALB/cJ Mice. Dig Dis Sci 2016;61:737–46.

[25] Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: comparison to high-fat diet. Obesity (Silver Spring) 2011;19:1109–17.

[26] Hamilton MK, Boudry G, Lemay DG, Raybould HE. Changes in intestinal barrier function and gut microbiota in high-fat diet fed rats are dynamic and region-dependent. Am J Physiol Gastrointest Liver Physiol 2015;308:G840-51.

[27] D'Incà R, Annese V, Di Leo V, Latiano A, Quaino V, Abazia C, et al. Increased intestinal permeability and NOD2 variants in familial and sporadic Crohn's disease. Aliment Pharmacol Ther 2006;23:1455–61.

[28] Barreau F, Meinzer U, Chareyre F, Berrebi D, Niwa-Kawakita M, Dussaillant M, et al. CARD15/NOD2 Is Required for Peyer's Patches Homeostasis in Mice. PLoS One 2007;2.

[29] Ahn BO, Ko KH, Oh TY, Cho H, Kim WB, Lee KJ, et al. Efficacy of use of colonoscopy in dextran sulfate sodium induced ulcerative colitis in rats: The evaluation of the effects of antioxidant by colonoscopy. Int J Colorectal Dis 2001;16:174–81.

[30] Kim JJ, Shajib MS, Manocha MM, Khan WI. Investigating Intestinal Inflammation in DSS-induced Model of IBD. J Vis Exp 2012; 60: pii: 3678.

[31] Pallauf K, Duckstein N, Hasler M, Klotz LO, Rimbach G. Flavonoids as Putative Inducers of the Transcription Factors Nrf2, FoxO, and PPAR γ. Oxid Med Cell Longev 2017; 4397340.

[32] Park S. Polyphenol compound as a transcription factor inhibitor. Nutrients 2015; 7:8987-9004.

[33] Mukherjee N, Kumar AP, Ghosh R. DNA Methylation and Flavonoids in Genitourinary Cancers. Curr Pharmacol Reports 2015; 1:112-120.

[34] Ayissi VBO, Ebrahimi A, Schluesenner H. Epigenetic effects of natural polyphenols: A focus on SIRT1-mediated mechanisms. Mol Nutr Food Res 2014; 58:22-32.

[35] Gilbert ER, Liu D. Flavonoids influence epigenetic-modifying enzyme activity: structure - function relationships and the therapeutic potential for cancer. Curr Med Chem 2010; 17:1756-68.

[36] Cani PD, Bibiloni R, Knauf C, Neyrinck AM, Delzenne NM. Changes in Gut Microbiota Control Metabolic Diet – Induced Obesity and Diabetes in Mice. Diabetes 2008;57:1470–81.

[37] Margalef M, Pons Z, Iglesias-Carres L, Bravo FI, Muguerza B, Arola-Arnal A. Lack of Tissue Accumulation of Grape Seed Flavanols after Daily Long-Term Administration in Healthy and Cafeteria-Diet Obese Rats. J Agric Food Chem 2015;63:9996–10003.

[38] Del Rio D, Rodriguez-Mateos A, Spencer JPE, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. Antioxid Redox Signal 2013;18:1818–92.

Tables

Table 1. Concentration of flavan-3-ols and phenolic acids in the GSPE used in the study.

Compound	Amount (mg compound/g extract)
Gallic acid	17.7 ± 2.0
Protocatechuic acid	1.0 ± 0.1
Vanillic acid	0.1 ± 0.0
Proanthocyanidin dimer *	144.2 ± 32.2
Catechin	90.7 ± 7.6
Epicatechin	55.0 ± 0.8
p-Coumaric acid	0.1 ± 0.0
Dimer gallate *	39.7 ± 7.1
Epigallocatechin gallate	0.4 ± 0.1
Proanthocyanidin trimer *	28.4 ± 2.0
Proanthocyanidin tetramer *	2.0 ± 0.2
Epicatechin gallate †	55.3 ± 1.5
Quercetin-3-O-galactoside	0.2 ± 0.0
Naringenin-7-glucoside	0.1 ± 0.0
Kaempferol-3-glucoside	0.1 ± 0.0
Quercetin	0.3 ± 0.0

Adapted from Baselga et al. [23]. Phenolic components were determined by reverse-phase highperformance liquid chromatography–mass spectrometry. The results are expressed as means ± SD (n = 3). * Quantified using the calibration curve of proanthocyanidin B2. † Quantified using the calibration curve of epigallocatechin gallate.

Table 2. Energy content of the diets

	KJ/g	Energy CH %	Energy protein %	Energy lipid %
Standard	12.1	72.5	19.3	8.2
CAF	20.7	52.0	14.1	33.9

STD: standard chow provide to the control group; CAF: cafeteria diet provide to the rats in the CAF, PRE-CAF and the SIT-CAF groups.

Table 3. Morphometric parameters of rats fed a STD diet or a CAF diet, either with or without GSPE supplementation.

Variables	STD	CAF	PRE-CAF	SIT-CAF
Weight gain (%)	24.6 ± 2.3	59.5 ± 7.0*	48.1 ± 4.3	39.9 ± 5.0
MWAT weight (g)	4.2 ± 0.4	11.3 ± 1.3*	9.5 ± 0.9	6.1 ± 0.7#
Adiposity index (%)	5.0 ± 0.7	11.8 ± 0.8*	11.0 ± 0.6	6.5 ± 1.3#
Final body weight (g)	273.7 ± 7.8	346.2 ± 12*	316.8 ± 9.2	297.3 ± 9.8#

STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a 500 mg/kg GSPE pre-treatment; SIT-CAF: rats fed a cafeteria diet simultaneously with a 500 mg/kg GSPE treatment every other week; GSPE: grape seed proanthocyanidin extract; MWAT: mesenteric white adipose tissue. Values are presented as the means ± SEM. *$P < 0.05$ compared to the STD group. # $P < 0.05$ compared to the CAF group.

Table 4. Correlation coefficients for TEER values and plasma OVA, TNF-α and LPS levels compared with the morphometric variables.

	Morphometric variables			
Variables	**Weight gain (%)**	**MWAT weight (g)**	**Adiposity index (%)**	**Final body weight (g)**
17 weeks of diet intervention				
OVA (ng/mL)	**0.620***	**0.601***	**0.678***	**0.595***
LPS (EU/mL)	**0.432***	0.324	**0.387***	**0.479***
TNF-α (pg/mL)	0.323	**0.429***	**0.556***	0.313
TEER duodenum (Ωxcm^2)	-0.377	**-0.485***	**-0.597***	-0.379
TEER ileum (Ωxcm^2)	-0.338	**-0.421***	**-0.488***	**-0.468***
TEER colon (Ωxcm^2)	-0.223	-0.301	**-0.420***	-0.241

OVA: ovalbumin; TNF-α: tumor necrosis factor-α; LPS: lipopolysaccharide; TEER: transepithelial electrical resistance. n = 40 animals. *: *p-values< 0.05 are considered statistically significant*

Table 5. Correlation coefficients of TEER values and plasma OVA, TNF-α and LPS levels compared with intestinal permeability and systemic inflammation markers.

	Intestinal permeability		**Systemic inflammation**		
	OVA 12w (ng/mL)	**OVA 17w** (ng/mL)	**TNFα 8w** (pg/mL)	**TNF-α 12w** (pg/mL)	**TNF-α 17w** (pg/mL)
LPS (EU/mL)					
8w	0.051	0.064	-0.032	0.142	0.232
12w	0.117	0.342	0.324	0.389	0.301
17w	**0.398***	**0.415***	0.179	0.205	0.120
TEER (Ωxcm²)					
duodenum	-0.283	**-0.794***	-0.164	-0.316	**-0.686***
ileum	**-0.493***	**-0.627***	-0.118	0.015	**-0.495***
colon	-0.413	**-0.479***	-0.097	-0.121	**-0.496***

OVA: ovalbumin; TNF-α: tumor necrosis factor-α; LPS: lipopolysaccharide; TEER: transepithelial electrical resistance. n = 40 animals. *: *p-values< 0.05 are considered statistically significant.*

Table 6. Expression of TJ-related mRNAs in the ileum of rats fed a STD diet or a CAF diet either with or without GSPE supplementation.

	Ileum						
	Claudin-1	***Claudin-2***	***Claudin-3***	***Zo-1***	***Ocln***	***Jam-A***	***Mlck***
STD	1.25±0.23	0.96±0.14	1.14±0.25	1.12±0.19	1.02±0.12	0.97±0.19	1.08±0.15
CAF	**0.72±0.12***	0.88±0.11	1.32±0.20	1.34±0.17	1.22±0.12	1.09±0.08	1.17±0.16
PRE-CAF	0.97±0.13	1.14±0.15	0.99±0.09	1.40±0.20	1.27±0.13	1.06±0.06	1.20±0.14
SIT-CAF	**1.25±0.16#**	1.01±0.36	0.96±0.18	0.97±0.21	1.32±0.19	0.89±0.19	1.85±0.93

STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a 500 mg/kg GSPE pre-treatment; SIT-CAF: rats fed a cafeteria diet simultaneously with a 500 mg/kg GSPE treatment every other week; GSPE: grape seed proanthocyanidin extract; *Ocln*: occludin; *Jam-A*: junctional adhesion molecule-A; *Zo-1*: zonulin/zonula occludens-1. Values are presented as the means ± SEM. *$P < 0.05$ compared to the STD group. # $P < 0.05$ compared to the CAF group.

Figures

Figure 1. Schematic diagram of the experimental design. STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a pre-treatment with 500 mg/kg GSPE; SIT-CAF: rats fed a cafeteria diet simultaneously with a 500 mg/kg GSPE treatment every other week; GSPE: grape seed proanthocyanidin extract; OVA: ovalbumin; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor-α.

Figure 2. OVA intestinal permeability test at weeks 12 and 17 of CAF diet consumption. STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a pre-treatment with 500 mg/kg GSPE; SIT-CAF: rats fed a cafeteria diet simultaneously with a 500 mg/kg GSPE treatment every other week; GSPE: grape seed proanthocyanidin extract; OVA: ovalbumin. Values are presented as the means ± SEM. *P < 0.05 compared to the STD group. # P < 0.05 compared to the CAF group.

Figure 3. Measurement of the integrity of the intestinal mucosa. (A) Quantification of LY transport across the intestinal wall. (B) Evaluation of TEER values in each intestinal section. STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a 500 mg/kg GSPE pre-treatment; SIT-CAF: rats fed a cafeteria diet simultaneously with a 500 mg/kg GSPE treatment every other week; GSPE: grape seed proanthocyanidin extract; LY: Lucifer yellow; TEER: transepithelial electrical resistance. Values are presented as the means ± SEM. *P < 0.05 compared to the STD group. # P < 0.05 compared to the CAF group.

Figure 4. LPS (A) and TNF-α plasma levels (B) at weeks 8, 12 and 17 of CAF diet consumption. STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a 500 mg/kg GSPE pre-treatment of; SIT-CAF: rats fed a cafeteria diet simultaneously with a 500 mg/kg GSPE treatment every other week; GSPE: grape seed proanthocyanidin extract; LPS: lipopolysaccharide; TNF-α**:** tumor necrosis factor-α. Values are presented as the means ± SEM. *P < 0.05 compared to the STD group. # P < 0.05 compared to the CAF group.

Highlights:

- Cafeteria diet increases intestinal permeability and induces metabolic endotoxemia
- The pharmacological doses of proanthocyanidins ameliorate barrier dysfunction
- Proanthocyanidins improve intestinal permeability and prevent metabolic endotoxemia
- The intermittent treatment is the most effective after 17 weeks of diet intervention

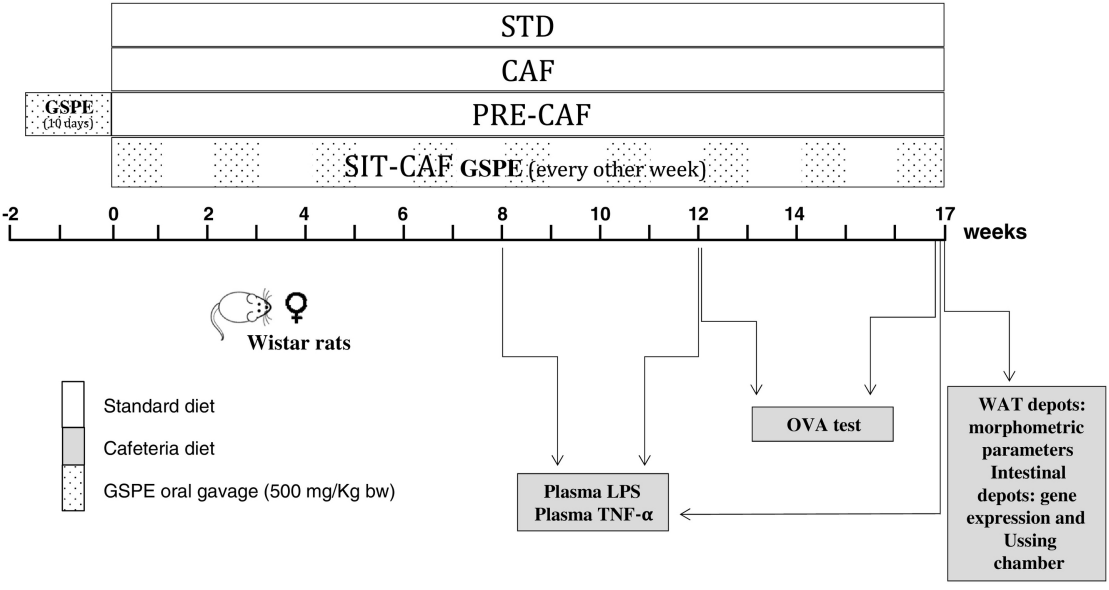
STD
CAF
GSPE
(10 days)
PRE-CAF
SIT-CAF GSPE (every other week)
-2
0
2
4
6
8
10
12
14
17
weeks
Wistar rats
Standard diet
Cafeteria diet
GSPE oral gavage (500 mg/Kg bw)
Plasma LPS
Plasma TNF-α
OVA test
WAT depots: morphometric parameters
Intestinal depots: gene expression and Ussing chamber

Figure 1

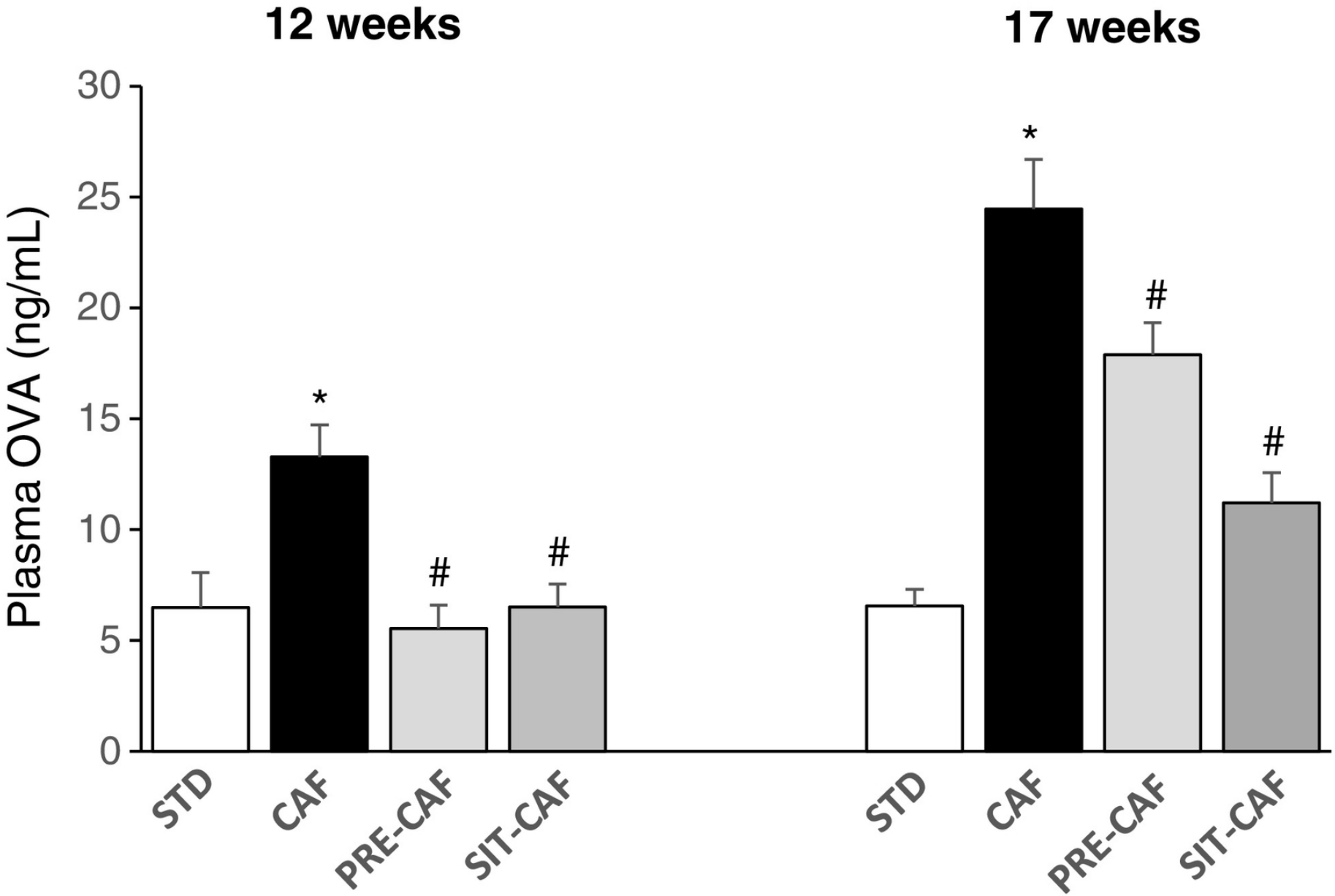
12 weeks
17 weeks
Plasma OVA (ng/mL)
30
25
20
15
10
5
0
*
#
#
*
#
#
STD
CAF
PRE-CAF
SIT-CAF
STD
CAF
PRE-CAF
SIT-CAF

Figure 2

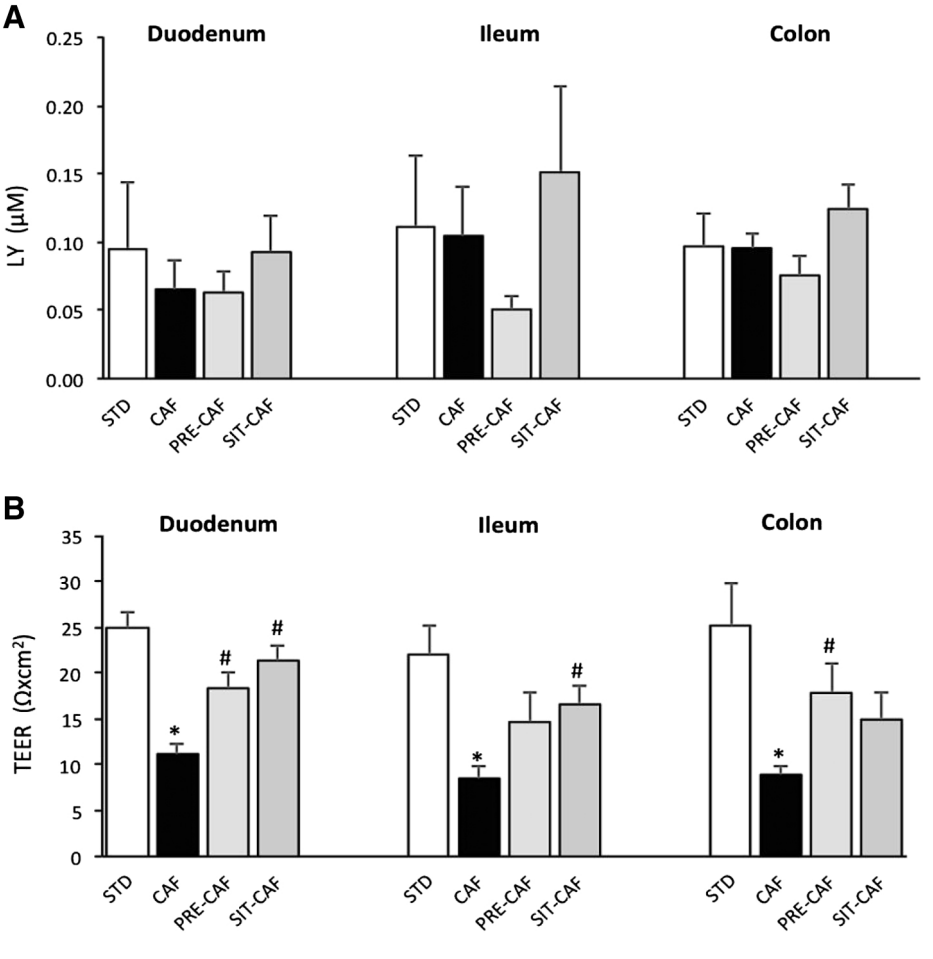
A
Duodenum
Ileum
Colon
LY (μM)
0.25
0.20
0.15
0.10
0.05
0.00
STD
CAF
PRE-CAF
SIT-CAF
B
Duodenum
Ileum
Colon
TEER (Ωxcm²)
35
30
25
20
15
10
5
0
*
#

Figure 3

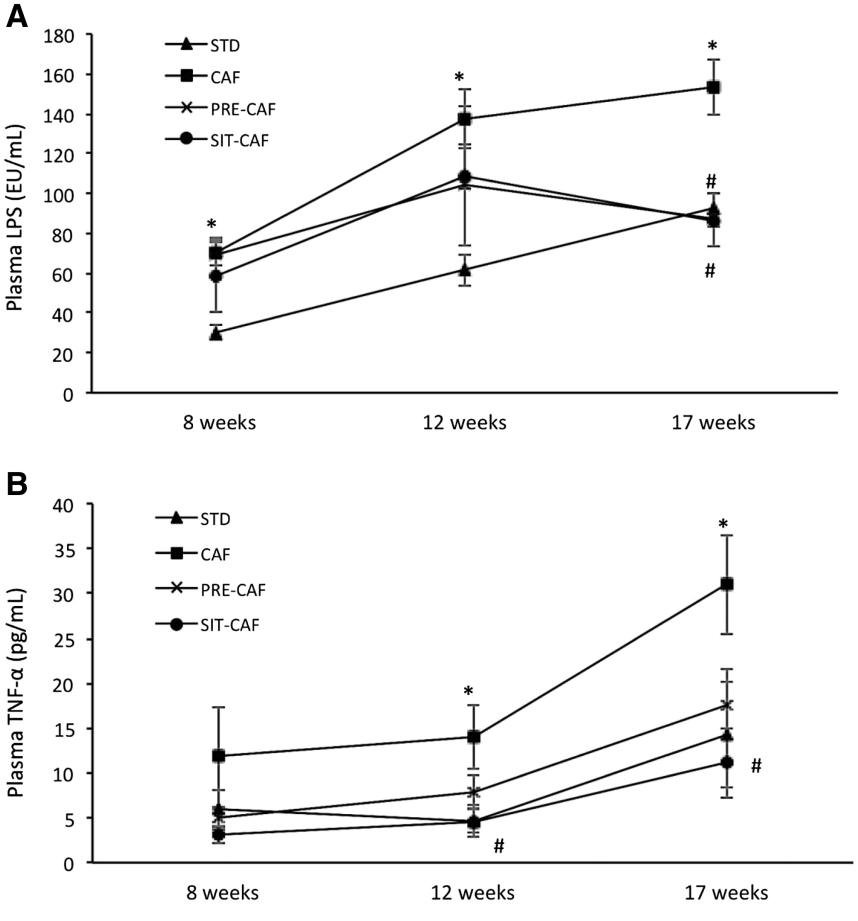
A
Plasma LPS (EU/mL)
180
160
140
120
100
80
60
40
20
0
STD
CAF
PRE-CAF
SIT-CAF
*
*
*
#
#
8 weeks
12 weeks
17 weeks
B
Plasma TNF-α (pg/mL)
40
35
30
25
20
15
10
5
0
STD
CAF
PRE-CAF
SIT-CAF
*
*
#
#
8 weeks
12 weeks
17 weeks

Figure 4