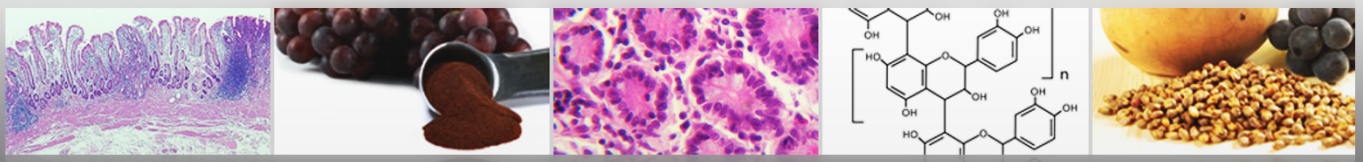
GRAPE SEED PROANTHOCYANIDINS AS MODULATORS OF THE INFLAMMATORY RESPONSE AND BARRIER FUNCTION IN THE INTESTINE

Katherine Gil Cardoso



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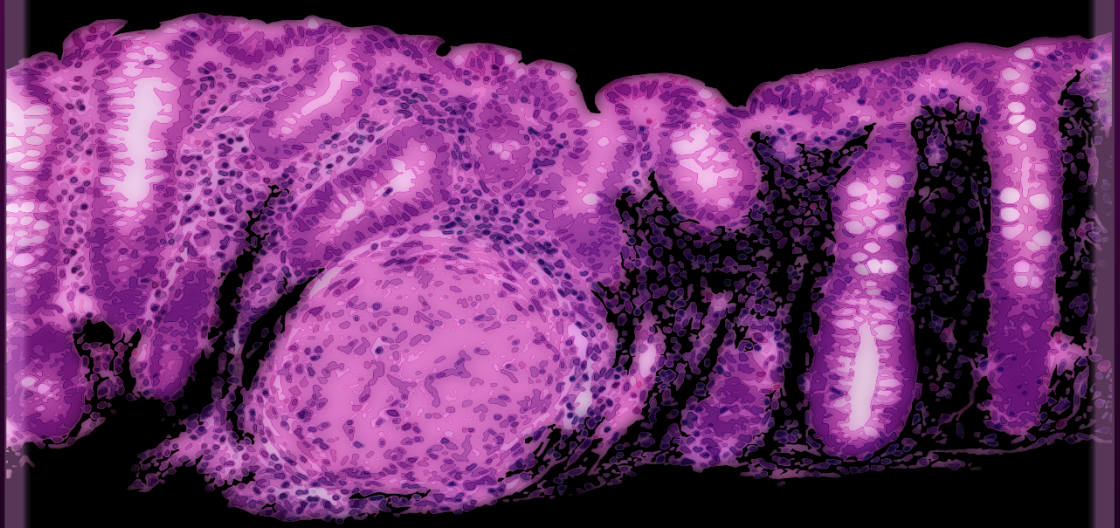

Katherine Gil Cardoso

Doctoral Thesis
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Grape seed proanthocyanidins as modulators of the inflammatory response and barrier function in the intestine

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Tarragona, 2018

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WE STATE that the present thesis, entitled **Grape seed proanthocyanidins as modulators of the inflammatory response and barrier function in the intestine**, presented by **Katherine Gil Cardoso** to obtain the award of Doctor, has been carried out under our supervision in the MoBioFood Research Group at the Department of Biochemistry and Biotechnology of this university, and fulfil the demanded requirements to get the European Mention.

FEM CONSTAR que la present tesi, titulada **Grape seed proanthocyanidins as modulators of the inflammatory response and barrier function in the intestine**, presentada per **Katherine Gil Cardoso** per a l'obtenció del títol de Doctor, ha estat realitzada sota la nostra direcció al Grup de Recerca MoBioFood al departament de Bioquímica i Biotecnologia d'aquesta universitat, i que acompleix els requeriments per optar a la Menció Europea.

Tarragona, 10th of January of 2018

Doctoral thesis supervisors/ Els directors de la tesi doctoral:

Dr. M. Teresa Blay Olivé

Dr. Ximena Terra Barbadora

"From our fears our courages are born, and in our doubts our certainties live.

The dreams announce another possible reality, and the deliriums another reason.

In the wanderings the findings are waiting for us, because it is necessary to get lost to find yourself again."

Eduardo Galeano (1940-2015).
Uruguayan journalist, writer and novelist.

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SUMMARY

Intestinal dysfunction is based on a pro-inflammatory state in the intestine and on a defective barrier function, both considered common features of intestinal chronic diseases. However, intestinal dysfunction has also been associated with obesity and other metabolic diet-related pathologies. In this regard, proanthocyanidins are natural bioactive compounds from the flavonoid family with anti-inflammatory and antioxidant properties that might have significant effects on the intestinal environment. In this framework, the present thesis was designed to elucidate the role of proanthocyanidins in the modulation of the intestinal inflammatory response and barrier function in complementary animal models of intestinal dysfunction.

To accomplish this global objective, firstly we examined the impact of an obesogenic diet on intestinal health status over time to then analyse the protective effect of a grape seed proanthocyanidin extract and compare the effectiveness of different doses and times of administration to protect against intestinal dysfunction. Secondly, the role of the same extract of proanthocyanidins was analysed in an animal model of acute intestinal inflammation and impaired intestinal permeability induced by lipopolysaccharides injection.

To sum up, the present thesis revealed that diet induced obesity and acute lipopolysaccharides exposition trigger similar degree of intestinal inflammation and impaired barrier function state, and more importantly, that the oral administration of proanthocyanidins improves intestinal inflammation and barrier function.

RESUM

La disfunció intestinal es basa en un estat pro-inflamatori en l'intestí i en una funció de barrera defectuosa, característiques comunes de diverses malalties cròniques intestinals, però també associades amb patologies metabòliques relacionades amb la dieta, com és l'obesitat. En aquest sentit, les proantocianidines són compostos bioactius naturals de la família dels flavonoides que han mostrat posseir propietats antiinflamatòries i antioxidants, que podrien tenir efectes significatius en l'entorn intestinal. En aquest marc, la present tesi va ser dissenyada per aclarir el possible paper de les proantocianidines en la modulació de la resposta inflamatòria intestinal i la funció de barrera en diferents models animals complementaris de disfunció intestinal.

Per assolir aquest objectiu global, primer vam examinar l'impacte d'una dieta obesogènica en l'estat de salut intestinal al llarg del temps i posteriorment vam analitzar l'efecte protector d'un extracte de proantocianidines de la llavor de raïm, comparant l'eficàcia de les diferents dosis i dels temps d'administració a l'hora de protegir de la disfunció intestinal. En segon lloc, vam analitzar el paper del mateix extracte de proantocianidines en un model animal d'inflamació intestinal aguda i d'alteració de la permeabilitat intestinal induïda per injecció amb lipopolisacàrids.

En resum, la present tesi va revelar que l'obesitat induïda per la dieta i l'exposició aguda a lipopolisacàrids desencadenen un grau similar d'inflamació intestinal i deteriorament de l'estat funcional de la barrera, i més important, que l'administració oral de les proantocianidines millora la inflamació intestinal i la funció de barrera.

ABBREVIATIONS

Ag antigen

AJ adherens junction

APC antigen-presenting cell

ATP adenosine triphosphate

CAF cafeteria

CD cluster differentiation

COX cyclooxygenase

DAMP damage-associated molecular pattern

DC dendritic cell

DIO diet induced obesity

DSS dextran sodium sulphate

FAE follicle-associated epithelium

FITC fluorescein isothiocyanate

GALT gut-associated lymphoid tissue

GI gastrointestinal

GSPE grape seed proanthocyanidin extract

HC high-carbohydrate

HF high-fat

IBD inflammatory bowel disease

IEL intraepithelial lymphocyte

IFN-γ interferon-γ

Ig immunoglobulin

IKK IκB kinase

IL interleukin

ILC innate lymphoid cell

ILF isolated lymphoid follicle

IP intraperitoneal

JAM juntional adhesion molecule

LBP lipopolysaccharides binding protein

LepR leptin receptor

LPS lipopolysaccharides

M cell microfold cells

MALT mucosa-associated lymphoid tissue

MAMP microbe-associated molecular pattern

MIS mucosal immune system

MLCK myosin light chain kinase

mLN mesenteric lymph node

MPO myeloperoxidase

MUC 2 mucin 2

NF-κB nuclear factor-κB

NO nitric oxide

NOS nitric oxide synthase

OVA ovalbumin

pIgR polymeric Ig receptor

PMN polymorphonuclear neutrophil

PP Peyer's patch

PRR pattern recognition receptor

ROS reactive oxygen species

TEER transepithelial electrical resistance

TGF-β transforming growth factor-β

Th T helper cells

TJ tight junction

TLR toll-like receptor

TNBS trinitrobenzene sulfonic acid

TNF-α tumor necrosis factor-α

Treg regulatory T cells

ZO zonula occludens

I. INTRODUCTION

1. Opening

Intestinal dysfunction is a clinical sign characterized by a pro-inflammatory state in the intestine accompanied by defects in the intestinal barrier function, which are common features of several intestinal chronic diseases [1]. Although intestinal dysfunction is a novel term and, therefore is not fully extended in the clinical vocabulary, we have adopted it during the entire manuscript to refer these two alterations developed in the intestine.

In the last years, some studies have been conducted to associate intestinal dysfunction with metabolic- and/or diet-related pathologies, especially obesity [2]. In addition, experimental exposure to low doses of endotoxins has also been shown to induce a similar state of intestinal dysfunction to that observed in obese humans [3]. However, both models are rarely used to examine intestinal dysfunction and the phenotypes have not yet been completely defined, suggesting the need for a more in-depth exploration.

Recent research has described the significant effects of some dietary natural compounds on the intestinal environment [4], which may have an important influence on intestinal health. The effects of these dietary constituents on the intestine and subsequent health outcomes in the host represent an interesting and novel area of inquiry. Therefore, progress in this field of investigation may lead to a search for novel animal models, as well as nutritional/therapeutic candidates to approach intestine-body homeostasis disruptions.

2. The intestine

The word intestine derives from a Latin root meaning “internal,” and, indeed, refers the two organs that together nearly fill the interior of the abdominal cavity: the small and the large intestines, also called bowels, or colloquially the “guts”. These organs represent the greatest mass and length of the gastrointestinal (GI) tract, performing all digestive system functions, with the exception of ingestion [5]. Additionally, the intestinal epithelium has a conditioning effect on intestinal homeostasis, controlling the mucosal immune response and the metabolic activity of the intestine and the entire organism [6].

The intestine is an important metabolic organ that has gained attention in recent years for its newly identified role in the pathophysiology of metabolic disorders, including obesity [7, 8]. The currently existing knowledge about the cellular effects exerted by nutrients through the intestine supports the possibility of the deregulation of intestinal homeostasis by some dietary components, such as fat and carbohydrates, and the possible positive modulation by other components of the diet.

The first step in the identification and interpretation of the mechanisms by the nutritional status and dietary habits influence intestinal health by promoting and/or restoring intestinal homeostasis is understanding of the anatomy, histology, and physiology of the intestine.

2.1. Anatomy of the intestine

The GI tract is the large, muscular tube that extends from the mouth to the anus, serving as an interface between the body and the external environment. It is a highly specialized organ system that includes the mouth, the oesophagus and the stomach, as well as the small and large intestines [9]. The system is connected to the vascular, lymphatic and nervous systems, facilitating the

regulation of food intake and the digestive function, and delivery of absorbed compounds to organs throughout the body [10]. Although the GI tract consists on diverse organs, the following sections will be specially focused on the small and large intestines.

The small intestine is the longest segment of the GI tract (approximately 35 cm in mice, 170 cm in rats and 700 cm in humans). It comprises three regions (the duodenum, jejunum and the ileum), which contain the same layers found throughout the upper GI tract. [11]. The duodenum is the first and shortest segment of the small intestine. The remainder of the small intestine is divided between the jejunum (proximal two-fifths) and ileum (distal three-fifths) [12] **(Figure 1)**.

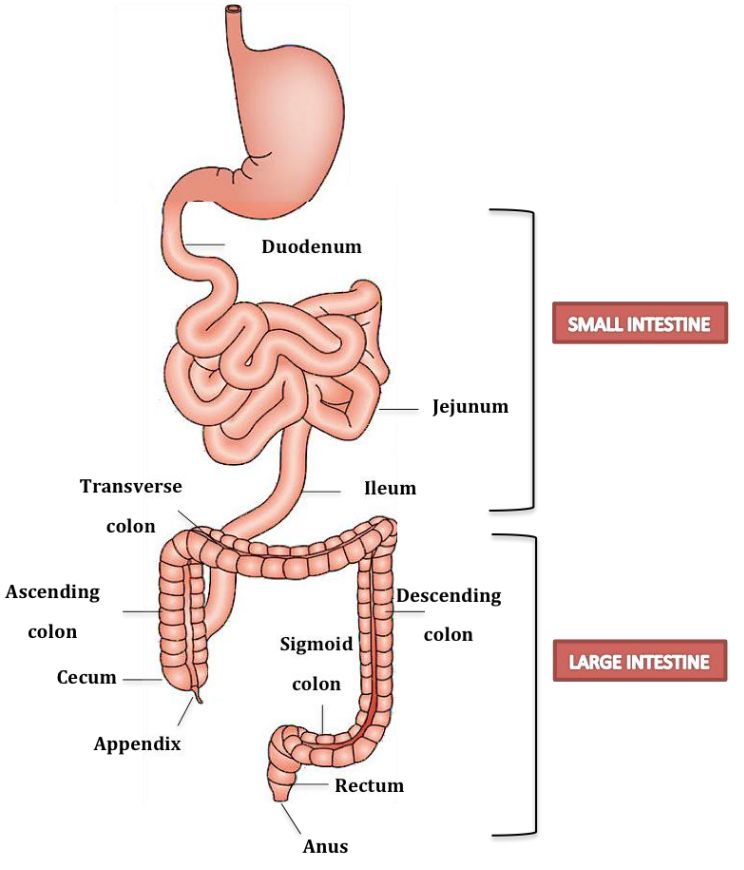

Figure 1. Anatomy of the human small and large intestines.

The large intestine is the final section of the GI tract that extends from the ileum of the small intestine to the anus. It commonly divided into the cecum, the appendix (humans only), colon, rectum and anus. In humans, the colon is divided into the right and the left colon. The right colon is composed of the appendix, cecum, ascending colon, and transverse colon, corresponding to the cecum and proximal colon in rodents. In contrast, the left colon includes the descending colon, sigmoid colon and the rectum, corresponding to the mid and distal colon in rodents [13, 14].

2.2. Histology of the intestine

The small and large intestines share certain histological characteristics. The wall of the entire intestine generally comprises four layers: the mucosa (or mucous membrane), submucosa, *muscularis* (or *muscularis propria*), and *adventitia* (or serosa) **(Figure 2)**.

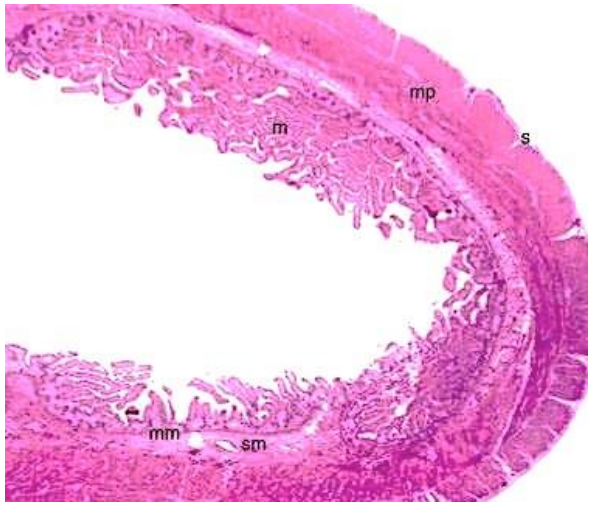

Figure 2. General microscopic architecture of the small intestine. m, mucosa; sm, submucosa; mm, *muscularis mucosae*; mp, *muscularis propria*; s, serosa. Haematoxylin and eosin, ×25. Adapted from [15].

The *lamina propia*, *muscularis mucosae* and epithelium constitute the mucosa, which is the innermost layer of the intestine. The *lamina propria* is a layer of reticular connective tissue formed by elastin, reticulin, collagen fibres, lymphocytes, plasma cells, granulocytes, lymphatics and capillaries. In contrast, the *muscularis mucosae* is a thin layer of smooth muscle between the mucosa and submucosa. Both layers, the *lamina propria* and *muscularis mucosae*, offer support and articulate the epithelium layer.

Various structures are recognizable in the epithelium [16]. Villi are projections into the lumen that are predominantly covered with mature absorptive cells, along with occasional mucus-secreting cells that serve to increase the mucosal surface area. Crypts of Lieberkuhn (crypts) are invaginations of the epithelium around the villi that are predominantly lined with younger epithelial cells. Microvilli are microscopic (100 nm in diameter) finger-like projections that cover the apical surface of the enterocytes, increasing the absorptive surface area. Microvilli form an undulated brush border covered with glycocalyx constituted of oligosaccharides attached to membrane glycolipids and glycoproteins.

Stem cells are pluripotent cells located at the base of the crypts that participate in renewing the intestinal epithelium by continuously dividing to provide all the epithelial cells in the crypts and on the villi [17]. Different cell types are produced by the stem cells. Each type matures according to its specific functionality and migrates up and out of the crypt throughout the intestine; however, the distribution of each cell type varies according to the function of each region [18, 19].

Enterocytes are the most abundant cell type in the intestinal epithelium and are responsible for nutrient absorption among other specific functions. Globet cells are oval or round cells with flattened basal nuclei that secrete the mucus layer that protects the epithelium from the luminal contents [20]. Paneth cells are

flask-shaped cells with an eosinophilic granular cytoplasm and a broad base positioned against the basement membrane that produce antimicrobial peptides and growth factors involved in maintaining the neighbouring stem cells [21]. In addition, enteroendocrine cells are specialized endocrine cells that secrete various GI hormones [22]. M cells are specialized epithelial cells overlying lymphoid follicles in the small intestine and colon and act an interface between the luminal content and the underlying immune cells [23]. In contrast, the precise functions of the last two cell types, cup and tuft cells, are still unknown [24] **(Figure 3)**.

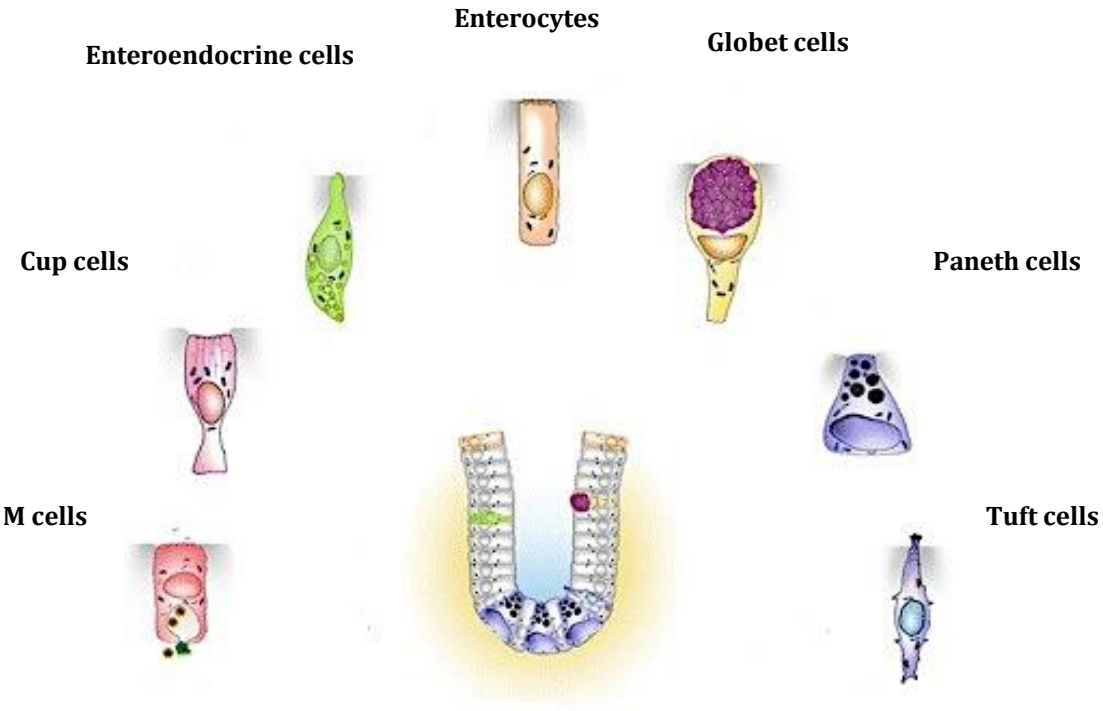

Figure 3. Representation of the epithelial cell types generated from stem cells. Adapted from [24].

The submucosa is a fibrous connective tissue layer compressed between the *muscularis mucosae* and the *muscularis propria*. Fibroblasts, mast cells, blood and lymphatic vessels, and a nerve fibre plexus form the submucosa. In

contrast, the *muscularis propria* consists of two layers of smooth muscle and it is mainly responsible for contractility. On the other hand, the adventitia is the outermost layer of connective tissue [11].

2.3. Physiology of the intestine

The intestinal epithelium consists of a single-cell layer that represents the largest and the principal barrier protecting the tissue from the external environment. It is a complex epithelial and mucosal multilayer system that integrates an external physical barrier and an inner immunological barrier [25]. On one hand, the physical barrier allows the exchange of molecules between the host and the environment and nutrient absorption from the diet [26–29], while preventing the entry of antigens (Ags) and microorganisms into the body [26, 30]. On the other hand, the inner immunological barrier allows a peaceful coexistence with intestinal symbionts without eliciting chronic inflammation, providing a measured inflammatory and defensive response to threats from pathogens [31, 32].

Given the importance of this complex dual role of the intestinal epithelium in intestinal health, its components and functions and the processes underlying its interactions will be further explored in the next sections.

2.3.1. Intestinal epithelial barrier function

The intestinal epithelium acts as external physical barrier permitting the translocation and absorption of essential dietary nutrients, electrolytes and water from the intestinal lumen into the circulation [26–29], but not the passage of intraluminal toxins, Ags and enteric microbiota [26, 30]. This feature enables an equilibrated permeability to be maintained [33]. The selective permeability of the intestinal epithelium is mediated by two major routes: transepithelial/transcellular [34] and paracellular pathways **(Figure 4)** [35].

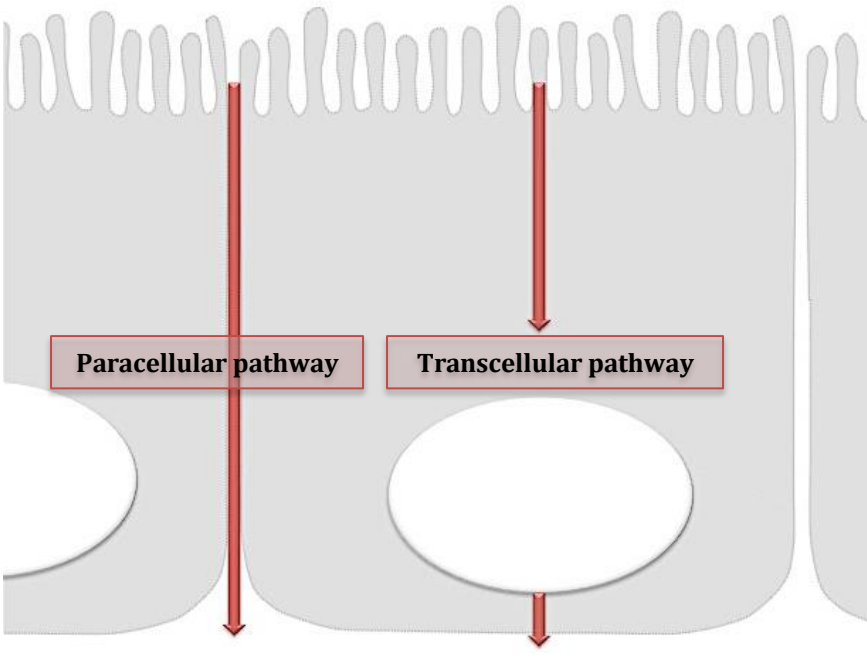

Figure 4. Schematic representation of the intestinal epithelia and transport pathways across the monolayer.

2.3.1.1. Transcellular transport

The transcellular permeability pathway is responsible for the transport of molecules through enterocytes by crossing the apical and basolateral membranes. Enterocytes have an apical-basal polarity defined by the orientation of the apical membrane toward the intestinal lumen and the basolateral membrane away from the lumen. The basolateral membrane is thinner and more permeable than the apical membrane due to a lower protein:lipid ratio [34]. The transcellular pathway is the specific mechanism for transporting low-molecular weight hydrophobic substances, which are able to diffuse through the membrane, either on their own or through interactions with a specific membrane transporter [36]. In both cases, this transport mechanism is determined by the concentration gradient across the intestinal membrane [37].

Facilitated diffusion consists of molecules or ions transport across intestinal membrane via specific transmembrane integral proteins, process that does not require chemical energy from adenosine triphosphate (ATP) [38]. Active transport is the transport of molecules against the concentration/gradient using the energy produced by the ATP [39]. In contrast, peptides and proteins mainly use endocytosis mechanisms of transport, which is classified into pinocytosis and receptor-mediated endocytosis or transcytosis. Pinocytosis is a fluid-phase endocytosis energy-dependent saturable mechanism in which the molecule travels inside membrane vesicle. Alternatively, molecules may be triggered by its binding to a specific receptor on the cell surface during endocytosis or transcytosis [40].

2.3.1.2. Paracellular transport

The paracellular pathway refers to transport through the space between epithelial cells. It depends on intercellular complexes localized at the apical-lateral membrane junction and along the lateral membrane that mechanically link adjacent cells and seal the intercellular space [41]. Three components that are responsible for the contact between intestinal epithelial cells have been distinguished at the ultrastructural level: adherens junctions (AJs), tight junctions (TJs) and desmosomes **(Figure 5)** [42, 43].

AJs and TJs consist of transmembrane proteins that are intracellularly linked to the actin cytoskeleton via adaptor proteins, providing different cell-cell adhesion functions [44].

Adherens junction

AJs, also known as zonula adherens, are involved in multiple cellular functions, including the initiation and stabilization of cell-cell adhesion, modulation of the actin cytoskeleton, intracellular signalling and transcriptional regulation [42, 45].

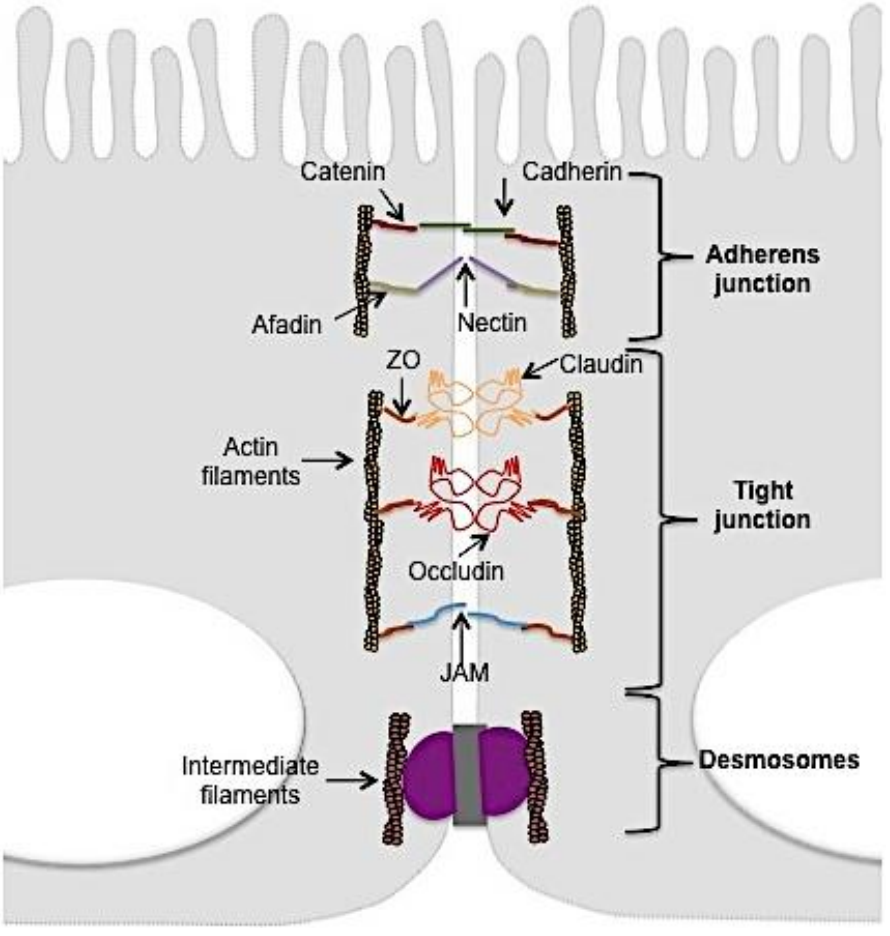

Figure 5. Cell junctions: mechanisms of transmembrane transport.

The major AJs include interactions among transmembrane glycoproteins of the cadherin superfamily, such as epithelial (E)-cadherins, and the catenin family, mainly p120-catenin, β-catenin and α-catenin [42]. The nectin-afadin complex is the other important AJ complex. The nectin family of immunoglobulin (Ig)G-like adhesion receptors establish interactions with nectins or nectin-like receptors [46, 47] and also interact with the cytoskeleton via afadin [48]. The cell type-specific expression of cadherins and nectins determine the strength and adhesive specificity of the AJs [44].

Tight junctions

TJs are dynamic multi-protein complexes that constitute a continuous belt-like ring around epithelial cells at the border between the apical and lateral

membrane that form a diffusion barrier allowing the regulated movement of ions and solutes through the paracellular pathway [49]. Four- and single-span transmembrane proteins are the two main classes of transmembrane protein comprising TJs [50, 51]. Claudins, occludin and tricellulin belong to the four-span transmembrane family. In contrast, members of the junctional adhesion molecule (JAM) family are the principal single-span transmembrane proteins. Similar to AJs, the intracellular domains of TJ proteins interact with scaffolding proteins, adaptor proteins and signalling complexes to control cytoskeletal attachment, cell polarity, cell signalling and vesicle trafficking [52].

Two canonical functions have been attributed to TJs. The first function (gate function by similarity) refers to their capacity to control the passage of substances through the paracellular pathway. In contrast, the second function (fence function by similarity) refers to their ability to maintain the polarity of the plasma membrane, allowing the vectorial transit of molecules across the epithelium [53]. Generally, the gate function is evaluated by measuring the transepithelial electrical resistance (TEER) of the intestinal tissue using the Ussing chamber technique or quantifying the transit of the following molecules, mainly transported by the paracellular route: fluorescein isothiocyanate (FITC)-dextran, H-mannitol or ovalbumin (OVA) [54–56] **(Figure 6)**. Conversely, the fence function is usually evaluated by inserting a fluorescent molecule, such as fluorescent sphingomyelin, into the apical membrane and determining whether the fluorescent label reaches the basolateral membrane in monolayers cultured in transwell filters or by chemically biotinylating the external domains of membrane proteins present at one of the cell surfaces [57, 58].

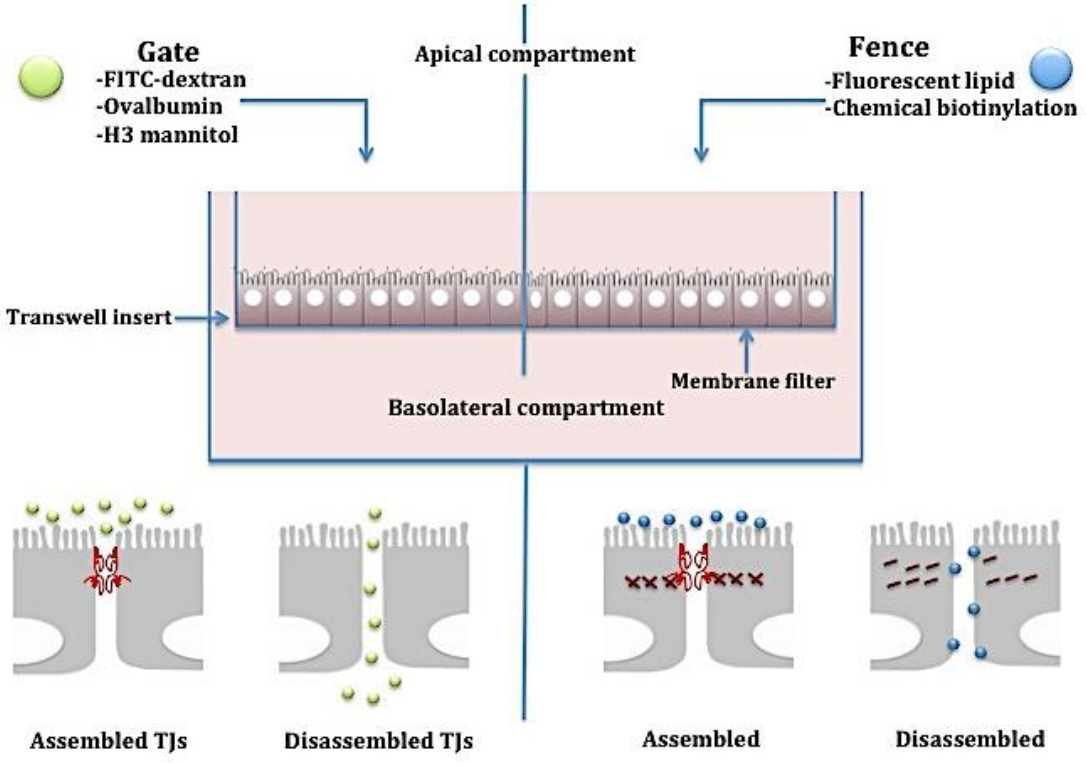

Figure 6. Canonical functions of tight junctions (TJs): paracellular gate and membrane fence. Adapted from [59]

Tight junction: occludin

Occludin was the first transmembrane component of TJs to be identified [60]. It is a 60-KDa integral membrane protein that contains four membrane domains, three cytoplasmic domains and two extracellular loops [61]. In addition to regulating paracellular permeability, occludin may also be involved in cellular adhesion [62, 63]. Considering that the TJ strands and barrier function were still present in occludin-deficient cells and epithelial tissues [64, 65] TJ formation and paracellular permeability barrier function do not exclusively depend on this protein.

Tight junction: tricellulin

Tricellulin is another transmembrane protein that constitutes the paracellular barrier and participates in epithelial barrier organization. Tricellulin is a 64-

KDa protein that is preferentially located at tricellular junctions and is structurally quite homologous to occludin [66].

Tight junction: claudin family

The main determinants of barrier properties of the TJs are the claudin family. Currently, 24 distinct claudin family members have been identified in humans, and their respective orthologues in other species have also been identified [35, 67, 68]. Claudins are 20-27 KDa transmembrane proteins with four hydrophobic transmembrane domains, two extracellular loops and N- and C-terminal cytoplasmic domains [69]. Claudins are divided into two main categories: pore-sealing and pore-forming claudins. Claudin-1, -3, -4, -5, -8, -11, -14 and -19 are known as "pore-sealing" claudins that reduce permeability [70–77]. In contrast, claudin-2 and -10 are classified as "pore-forming" claudins because they form paracellular anion/cation pores and water channels, thus decreasing epithelial tightness and increasing solute permeability [77–81]. In addition, some claudins have an ambiguous function: claudin-7, -12, -15 and -16 [75, 77, 82–86]. Claudin family members are differentially expressed among the intestinal sections, contributing to the local diversity of permeability and paracellular transport functions [87]. In humans, pore-forming claudins, such as claudin-2 are predominantly expressed in the proximal parts of the intestine [88]. In contrast, pore-sealing claudins, such as claudin-1, 3, and 4, are predominantly expressed in the distal parts of the intestine, consistent with their barrier properties [87–89]. The species is another factor that causes differences in the expression pattern of claudins [87]. Differences in the intestinal expression of representative claudin proteins between mammals respect are shown in **Table 1**.

Table 1. Intestinal expression of claudins in mammals. Adapted from [87].

Intestinal region	Claudins expression in humans	Claudins expression in rats	Claudins expression in mice
Small intestine			
Duodenum	1, 2, 3, 4, 5, 7, 12, 15 [88, 90, 91]	1, 2, 3, 4, 5, 8 [87, 92–94]	2, 3, 7, 15 [95, 96]
Jejunum	Not determined [88]	2, 3, 5, 7, 12 [87, 92, 94]	2, 3, 7, 12, 15 [95–98]
Ileum	2, 7, 8, 12, 15 [88]	2, 3, 4, 5, 7, 12 [87, 92–94]	1, 2, 3, 4, 7, 8, 12, 15 [95, 99–102]
Large intestine	1, 3, 4, 7, 8, 12, 15 [88, 89, 103, 104]	1, 2, 3, 4, 5, 8 [87, 92–94]	1, 2, 3, 4, 5, 7, 8, 12, 13, 15 [95, 96, 105–109]

Tight junction: junctional adhesion molecules

The JAM family is the principal single-span transmembrane protein expressed in TJs. JAMs are ≈40-KDa integral membrane proteins that belong to the Ig superfamily [110]. JAMs are subdivided into three classical members (JAM-A, JAM-B and JAM-C or JAM-1, JAM-2 and JAM-3, respectively) [111–113] and the atypical JAMs, including JAM-4 [114], the coxsackie and adenovirus receptor [115] and endothelial cell-selective adhesion molecule [116]. Structurally, JAMs comprise an extracellular amino terminal segment, a transmembrane domain and an intracellular carboxy terminal segment [110].

Scaffold proteins: zonula occludens family

Scaffold proteins provide the structural basis for the assembly of TJs at the cytoplasmic surface of intercellular contacts among epithelial cells [117]. Among these proteins, we highlight the role of zonula occludens (ZO) proteins: ZO-1 (220 KDa) [118], -2 (160 KDa) [119] and -3 (130 KDa) [120]. These molecules comprise three PZD domains, one SH3 domain and one GUK domain [121–123], and belong to the membrane-associated guanylate kinase family [124]. The canonical role of ZO proteins is to link the transmembrane proteins of multiprotein complexes to the cytoskeleton at cell-cell contacts [125]. They are multidomain polypeptides that also interact with several cytoplasmic

proteins, such as adapters, signalling molecules and transcriptional regulators, suggesting novel signalling functions for ZO proteins [126, 127].

Desmosomes

In addition to AJs and TJs, desmosomes have been distinguished of the barrier system in the intestine at the ultrastructural level. In contrast to AJs and TJs that are restricted to the apical domain, desmosomes are located subjacent to the AJs and are more widely distributed along the lateral membranes [128]. Desmosomes are button-like points of intercellular contact inside the cell that serve as anchoring sites for intermediate filaments, which form a structural framework with great tensile strength [129]. The major components of desmosomes are derived from three protein families: cadherins, armadillo proteins and plakins [128]. Desmosomal structures are present in tissue types that experience mechanical stress, such as the intestinal mucosa, not only providing mechanical stability but also facilitating cell–cell communication by transmitting signals [130].

Altogether, the intestinal epithelium is an essential compartment of the overall barrier function and plays a key role in maintaining intestinal health. However, alterations in its function could result in the development of a number of diseases that have also been associated with disruptions in the inner immunological barrier of the intestine and therefore affect the immune function of the mucosa. Mucosal surfaces are lined by epithelial cells that separate the external environment from the internal milieu, representing the first line of defence against pathogens. These cells constitute a barrier that provide physical protection, as well as an interface through which cells of the host immune system detect foreign substances and initiate immune responses. Consequently, this feature places the mucosal epithelium at the centre of interactions between the immune system and luminal contents, including dietary Ags and microbial products, enabling responses to physiological and

immunological stimuli. The next sections focus on the role of the intestinal mucosa in immune responses.

2.3.2. Immune function of the intestinal mucosa

The intestine is an organ that experiences a continuous antigenic challenge in the form of food Ags, Ags of the commensal bacterial flora, and pathogens. This challenge leads to the need for a mature intestinal immune system. Generally, the immune system is considered a complex and diverse system distributed throughout the body that provides host defence against pathogens [131]. In the intestine, the immune system must develop tolerance, a process that must be well balanced to produce an appropriate response to threatening food Ags and pathogens, as well as tolerate the commensal bacteria that inhabit the intestine. The intestinal immune system employs a number of key cells and molecules to develop this tolerance.

2.3.2.1. Components of the mucosal immune system

The mucosal immune system (MIS) is a compartment located near the surface that protects against toxic elements entering the body through mucous membranes [132]. It is viewed as a single layer epithelium covered with mucus and anti-microbial proteins produced by all lymphoid cells in the epithelia and in the *lamina propria* located below the mucosal surfaces in the body [133]. Considering its anatomical and functional properties, the MIS is separated into inductive sites such as Peyer's patches (PPs), mesenteric lymph nodes (mLNs) and isolated lymphoid follicles (ILFs) and effector sites, including the *lamina propria* and intraepithelial lymphocyte (IEL) compartment [134]. The mucosal inductive sites are collectively called mucosa-associated lymphoid tissue (MALT), which includes the gut-associated lymphoid tissue (GALT) in the intestine [132].

Peyer's patches

Mucosa domelike structures, known as PPs, are found along the MIS and are considered to be important sites for coordinating immune responses to pathogens in the intestine and for maintaining tolerance to food and commensal bacteria [135]. PPs are distributed throughout the small intestine, mainly in the distal section of the ileum and, to a lesser extent in the large intestine [136]. Based on the morphology of PPs, three areas have been categorized: the follicular area, the interfollicular area and the follicle-associated epithelium or FAE **(Figure 7)** [137]. Both follicular and interfollicular areas contain lymphoid follicles with a reservoir of proliferating B-lymphocytes, follicular dendritic cells (DCs) and macrophages comprising the germinal centre [138]. The follicle is surrounded by the subepithelial dome or corona that includes B cells, T cells, macrophages and antigen-presenting cells (APCs), including DCs; the FAE is located more externally and forms the interface between the GALT and the luminal microenvironment [138].

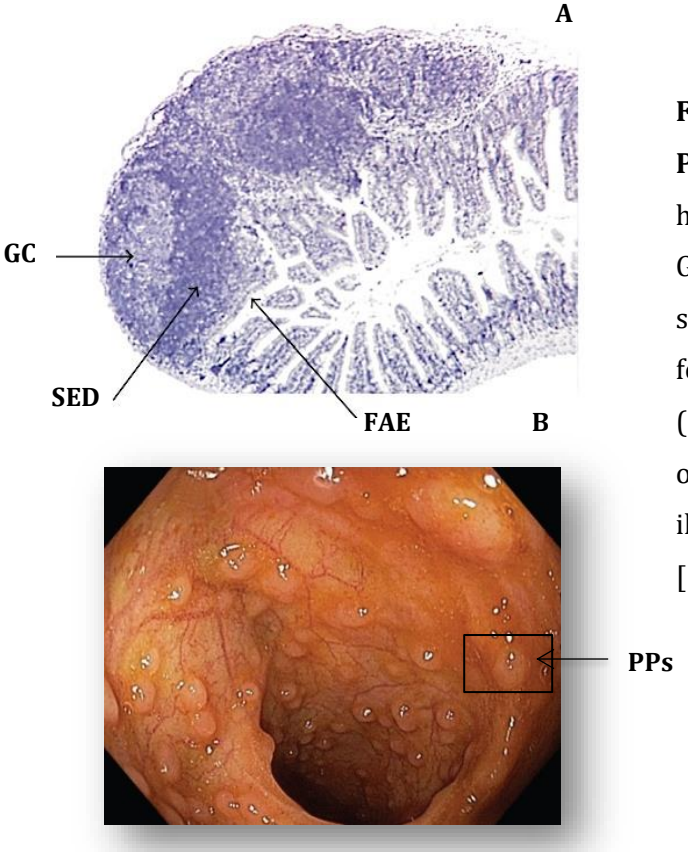

Figure 7. Representation of Peyer's Patches (PPs). A: histological features of a PP. Germinal centre (GC); subepithelial dome (SED); follicle-associated epithelium (FAE). B: PPs in the distal ileum of a 20-years-old man during ileocolonoscopy. Adapted from [138].

Isolated lymphoid follicles and microfold cells

ILFs consist of intestinal lymphoid structures containing B cell follicle and a few T cells observed in the small intestine and colon [139, 140]. As PPs, they act as a compensatory mechanism for humeral responses[141, 142]. Microfold cells (M cells) are unique subset of epithelial cells restricted to the region of the FAE covering both PPs and ILFs. These specialized cells lack a thick glycocalyx and do not secrete mucus. M cells vigorously participate in phagocytosis and transcytosis, by which they take up luminal particulate Ags, such as bacteria and deliver them to the immune cells in the GALT to initiate the intestinal immune response [143, 144].

Intraepithelial lymphocytes

In addition to the GALT, dispersed immune cells known as IELs reside throughout the small and large intestines. IELs consist of various T cell subsets that interact with epithelial cells to maintain normal homeostasis in the intestine; however, this regulatory mechanism is bi-directional, since epithelial cells also influence IEL T cell development and function [145, 146].

Mononuclear phagocytes: dendritic cells and macrophages

Other important intermediaries in the intestinal MIS include mononuclear phagocytes, comprising the above mentioned DCs and macrophages [147]. Both of these cell types are found throughout the *lamina propria* contiguous to the intestinal epithelial layer and in the GALT, including PPs and ILFs [148]. They perform disparate, yet complementary immunological functions in the intestine maintaining immune defence and homeostasis in this tissue. On one hand, DCs globally participate in the Ags presentation to T cells and polarize them, controlling the immune response [149]. Macrophages, on the other hand, contribute to translating danger signals to other immune cells, secreting cytokines to establish the homeostatic immune cell network and stimulating T cells [150].

Intestinal epithelial cells

Other central intermediaries of the defence mechanism of the mucosal surfaces are the intestinal epithelial cells. In addition to their classical absorptive and digestive functions, intestinal epithelial cells participate in transporting secretory Igs, produced by plasma cells in *lamina propria*, to the lumen, they allow the interaction with cells of the immune system and also express numerous molecules involved in antigen presentation [151].

Innate lymphoid cells

Innate lymphoid cells (ILCs) are another subgroup of immune cells of lymphoid origin that reside at the mucosal barriers [152]. Unlike adaptive lymphoid cells, such as T and B cells, ILCs lack antigen-specific receptors. Upon cytokine induction, ILCs release additional cytokines that mediate infections and tissue damage [153]. According to the cytokines production and transcriptional regulators they produce, three groups of ILCs have been distinguished: ILC-1, ILC-2 and ILC-3 [154]. Among their functions in the intestine, ILCs control pathogens and tolerance to commensal bacteria, contributing to intestinal homeostasis and the development of intestinal lymphoid tissue [155].

Mucus

Despite immune cells, the intestinal mucus has a central role in the MIS reducing the exposure of the immune system to Ags and protecting against self-digestion [156]. The mucus mainly comprises the mucin 2 (MUC2) protein produced by goblet cells [157, 158]. In addition, Paneth cells in the the intestinal crypts produce antibacterial peptides/proteins, highlighting the role of the anti-microbial peptide α-defensin in host protection [159, 160]. Fresh mucus is constantly secreted from globet cells in crypts and mixes with Paneth cell secretions containing MUC2, antibacterial peptides and lysozymes [161, 162].

Microbiota

In addition, the intestinal mucosa is a permanent home to bacteria that peacefully colonize the human intestine, named intestinal commensal microbiota [163]. The human GI microbiota comprises an ecosystem of approximately 300-500 bacterial species, including nearly 2 million genes, which together is known as the intestinal microbiome [164]. Although the microbiota is organized into different niches in the GI tract, the highest microbiota density is found in the human colon [165].

The intestinal microbiota is considered an "organ" with respect to its major role in the normal functioning of the body [166]. In mammals, the intestinal microbiota has central immunological functions. Commensal microbiota influence the development of the GALT and is crucial in pre-venting exogenous pathogen intrusion by directly interacting with pathogenic bacteria and stimulating the immune system [167]. More specifically, commensal bacteria control the GALT organization and the *lamina propria* immune network, influencing the development and maturation of cells of the MIS [163, 168] and subsequent IgA production [169].

2.3.2.2. Mucosal immune response

The innate immune system has the ability of distinguishing between pathogenic microbial components and harmless Ags by pattern recognition receptors (PRRs), including toll-like receptors (TLRs) [170]. In mammals TLRs are present on macrophages, neutrophils, DCs, intestinal epithelial cells and other cells belonging to innate immune system. TLRs recognize conserved characteristic molecules present on microorganisms and described as pathogen-associated molecular patterns derived from pathogens and tissue damage and inflammation-induced non-microbe danger signals, including damage-associated molecular patterns (DAMPs) [171, 172]. Microbial ligands expressed by pathogens but also by intestinal commensal are known as MAMPs

(microbe-associated molecular pattern). Lipopolysaccharides (LPS), peptidoglycans, flagellin and formylated peptides are among these MAMPs recognized by TLRs. In this sense, intestinal epithelial cells express an important LPS-binding molecule, cluster differentiation 14 (CD14), which collaborate, together with TLRs, in LPS signalling and cell activation [173]. Additionally, cells of innate immunity produce other critical factors such as myeloperoxidase (MPO), enzyme that plays a key role in the various functions of neutrophils in both innate and adaptive immunity [174]. Thus, innate immunity is tightly linked to adaptive immunity. Overall, the recognition of these MAMPs by TLRs leads to the activation of nuclear factor-kappa B (NF-κB) signalling pathway and consequently induces cytokine production, characterized by the induction of tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1) and IL-6, and up-regulates co-stimulatory molecules on APCs, leading to activation of adaptive cells [167]. In this sense, the migration of immune cells from mucosal inductive sites to effector tissues throughout the lymphatic system is the basis for the adaptive immune response in the intestine [132].

Lumenal Ags are easily sampled via M cells and transported to the underlying DCs. DCs mainly carry Ags to the inductive sites of the PPs and ILFs to induce mucosal T and B cell responses. In addition, DCs induce the expression of anti-inflammatory mediators, such as retinoic acid and transforming growth factor-β (TGF-β), subsequently increasing the expression of mucosal homing receptors ($\alpha_4\beta_7$ and CCR9) on activated lymphocytes. The homing of lymphocytes expressing specific receptors helps guide their eventual entry into major effector tissues, such as the *lamina propria*, via draining lymphatics into the mLNs and the bloodstream [142, 175, 176]. Ag-specific mucosal effector cells, such as IgA-producing plasma cells and B and T cells, are found in mucosal effector sites [177]. [178]. Adaptive mucosal immune responses result from $CD4^+$ helper T cells (Th), including both $CD4^+$ Th2 or $CD4^+$ Th1 cells, which

induces the development of IgA-producing plasma cells. Th cells are classified into Th1 cells, which produce IL-2 and interferon-γ (IFN-γ) and are involved in cellular immunity, Th2 cells, which produce IL-4, IL-5 and IL-1 and are involved in humoral immunity and Th17 cells, which produce the pro-inflammatory cytokine, IL-17, and plays important roles for the induction of inflammation [179, 180]. However, regulatory T (Treg) cells play central roles for immunoregulation, inhibiting proliferation and cytokine production in both $CD4^+$ and $CD8^+$T cells and Ig production by B cells [181] **(Figure 8)**.

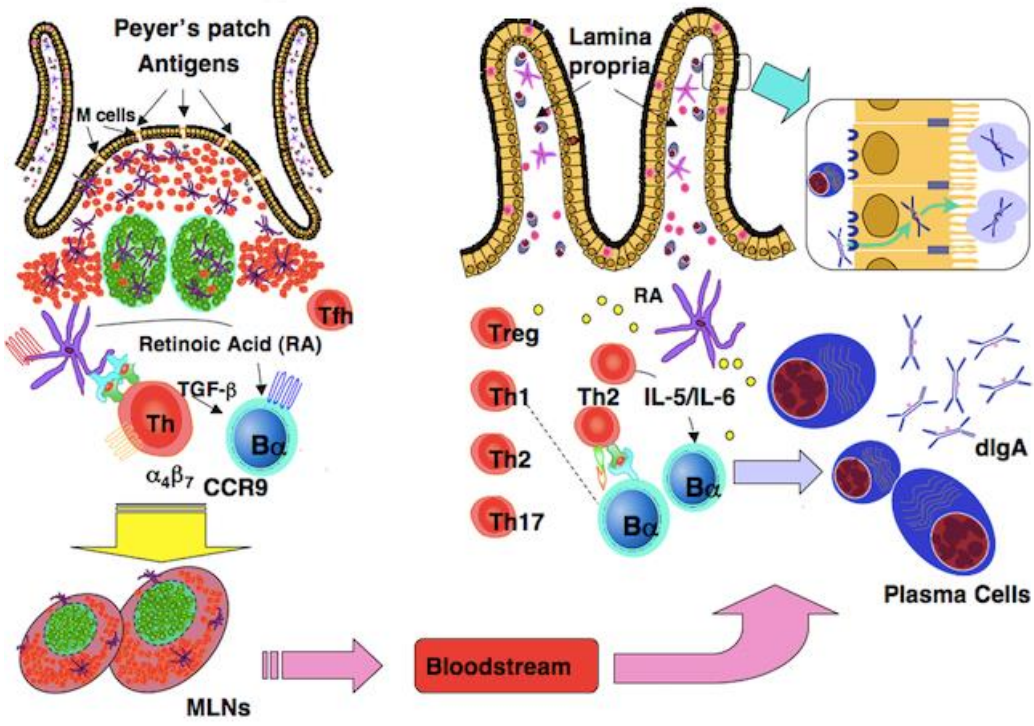

Figure 8. Regulation of the mucosal immune system (MIS) response in the intestine. Adapted from [132].

When the pathway is activated, epithelial cells produce the polymeric Ig receptor (pIgR) [182] that binds to pIgA in the *lamina propria*. The bound pIgA is internalized and transported apically across the epithelial cells, giving rise to secretory IgA antibodies with specificities for various Ags encountered in

mucosal inductive sites [146, 182]. Pathogens are ingested by epithelial DCs, which subsequently migrate to mLNs to induce T cell–independent, IgA-mediated B cell responses [183]. Principal intestinal mucosal immune components, function and location are summarized in the **Table 2**.

Table 2. Function and location of immune components in the intestinal mucosa.

Intestinal mucosal immune components	Function	Location
Lamina propria	Effector site of immune responses that hosts immune cells	Beneath the epithelium
Crypts	Houses Paneth cells and goblet cells	Invaginations of the epithelium around the villi
Mucus	Primary protection and surface lubrication	Covers the MIS
Globet cells	Secrete the mucus layer that protects the MIS	Intestinal crypts
Paneth cells	Produce antibacterial peptides	Intestinal crypts
Peyer's patches (PP)	Organized lymphoid follicles that act as an inductive site that coordinates the intestinal immune responses to pathogens	Component of the GALT located throughout the ileum of the small intestine
Isolated lymphoid follicles (ILFs)	Single follicles act as inductive sites that promote mucosal immune responses	Component of the GALT in the small and large intestines
Mesenteric lymph nodes (mLNs)	Inductive site for initiating intestinal immune responses to pathogens (adaptive immune response)	Between the layers of the intestine
Intraepithelial lymphocytes (IELs)	Effector cells that regulate mucosal innate and acquired immunity	Interspersed between epithelial cells in the epithelium of the small and large intestines
Microfold cells (M cells)	Specialized cells that take up Ags from the lumen and transfer them to the MALT initiating adaptive	Above PPs and ILFs

	immune response	
Intestinal dendritic cells (DCs)	Regulate T cell homing and present microbial peptides to T cells for priming and activation	*Lamina propia*, PPs, ILFs and mLNs
Innate lymphoid cells (ILCs)	Development of intestinal lymphoid organs and contribute to the immune response	*Lamina propria*
Intestinal macrophages	Mediators of the inflammatory response to pathogens and scavenge dead cells	*Lamina propia*, PPs and ILFs
Intestinal T helper (Th) cells	Mediators of immune homeostasis and protective responses against pathogens	*Lamina propia*, PPs and mLNs
Intestinal regulatory T (Treg) cells	Suppression of immune response	*Lamina propia*, PPs and mLNs
Intestinal B cells	Primary source of IgA antibodies	*Lamina propia*, PPs and role.
Intestinal epithelial cells	Recruit DCs and present Ags to T cells	Lining of the intestine
Toll-like receptors (TLRs)	Recognition of patogens	Cells belonging to innate immune system
Microbiota	GALT development and organization and IgA production	Within the MIS

Despite the panoply of antigenic substances in the lumen, a controlled equilibrium exists without inducing any overt pathology. However, perturbations of the immune homeostasis and the mechanisms that control it, including junctional multiprotein complexes or commensal microbiota, could result in intestinal dysfunction [167, 184]. In this sense, the MIS responds to peripheral substances, including food-derived molecular signals and pathogenic signals. Therefore, an understanding of the specific pathways that participate in sensing and managing peripheral signals is essential to determine the correct strategy to maintain intestinal homeostasis.

3. Intestinal dysfunction

A rapid expansion of our understanding of both the MIS and the intestinal barrier function has occurred recently, showing a close connection between these features and their implications for maintaining health [185, 186]. Additionally, the microbiota is viewed as part of this crosstalk because of the mutual influences of the host and the luminal microorganisms on the each other [31, 187, 188]. However, novel insights in this area of knowledge have revealed that genetic and environmental factors, including chemical reagents, the diet and the microbiota derivatives, can alter this balance and trigger intestinal dysfunction [3, 189–191].

Intestinal dysfunction is characterized by intestinal inflammation, and impaired intestinal barrier function [25]. Intestinal dysfunction imparts a significant and negative impact on human health. An understanding the underlying mechanisms involved is important in the development of effective prevention and treatment strategies. In some cases, intestinal diseases exert localized and temporal effects on the small or large intestine; however, uncontrolled intestinal alterations exert a systemic effect on the body [192, 193]. Thus, the aetiology of intestinal diseases is often confusing [194, 195], compromising treatment efficacy. In the last few years, this clinical condition has been linked with a variety of typical chronic intestinal diseases, such as inflammatory bowel diseases (IBDs) [196], as well as with metabolic pathologies with a non-specific basis in the intestine, including obesity [197, 198]. However, these terms are not entirely or clearly defined and established in the context of a metabolic disorder, and thus are a subject of debate. Consequently, the best approach to studying the complexity, pathogenesis and morphological changes in the human intestine caused by intestinal alterations in to induce them in animal models.

3.1. Chemical inducers of intestinal dysfunction

According to the literature, the widely used animal models of intestinal dysfunction consist of rodent models treated with chemical reagents [199]. Among these models, most include chemical-induced colitis models that morphologically and symptomatically reproduce human ulcerative colitis. Colitis models are sub-classified into: dextran sodium sulphate (DSS)-inducedcolitis [200–202], trinitrobenzene sulfonic acid (TNBS)-induced colitis [203–205], oxazolone-induced colitis [206–208] and acetic acid-induced colitis [209, 210].

Chemical models are extensively used, as they are easy to develop due to their wide availability, low cost and reproducibility [199]. Some hypotheses propose that these reagents may induce the hyper-polarization of the GI smooth muscle cells by modulating ion channels, subsequently resulting in reduced colonic contractility [199]. However, in general, the exact mechanisms underlying chemical-induced colitis models are poorly understood. Although some studies have suggested that DSS may also affect the distal small intestine [211, 212], these models are generally limited to recurrent episodes of inflammation and tissue degeneration located in the large intestine [213–216]. Moreover, these models do not recapitulate physiological conditions, but rather are chemical-induced models, making the identification of the mechanisms involved and a clear description of the aetiology of the clinical stadium difficult.

In the last few years, a few studies have been established a correlation between obesity and intestinal inflammation [8, 197, 217], but it was poorly explored until recently, possibly because chronic IBDs antecedents are related to intestinal epithelium damage, mal-absorption and weight loss. However, local tissue inflammation associated with obesity is less severe than in subjects with chronic IBD. Consequently, these findings have driven researchers to establish obesity-associated intestinal dysfunction models to elucidate the intimate

relationships between the intestine, diet and the host metabolism in a more physiological way.

3.2. Intestinal dysfunction associated with obesity

Obesity is characterized by low-grade chronic inflammation [218, 219]. To date, a majority of studies have focused on the central role of the adipose tissue in modulating inflammation in obesity. More specifically, the accumulation of activated pro-inflammatory immune cells within the visceral adipose tissue and reduction in the proportion of anti-inflammatory immune cells have been formulated as the causes of obesity-induced inflammation [220–222]. However, other organs also show low-grade chronic inflammatory changes that may also been related to obesity and other metabolic disorders, including the liver, muscle, pancreas, brain and the small and large intestines [223]. Among these tissues the intestine contains an extensive MIS that is continually exposed to both microbial and ingested Ags from the diet.

In the last few decades, the prevalence of obesity has increased, primarily due to the increasingly sedentary lifestyle and changes in the dietary habits in Western societies. Thus, obesity is becoming the major global public health in the developed world [224, 225]. The pandemic increase in obesity worldwide has prompted the search for animal models to study this condition and numerous comorbidities associated with obesity, such as intestinal dysfunction.

3.2.1. Animal models of obesity

The most commonly used animal models of obesity are small rodents (mice and rats), mainly including models of diet-induced obesity (DIO), and genetically obese animals to at lesser extent [226]. However, the choice of model depends upon the goal of the study.

The rat is an animal model that has been studied for many years because it resembles human physiology, metabolism and disease. With the recent sequencing of its genome, new biomedical interest in the development of new rat strains to tackle problems, such as obesity has resurged [227]. Principal genetic obese models include animals with a defect in the leptin signalling pathway in the hypothalamus, which develop a morbidly obese phenotype, such as obese fa/fa Zucker rats [228]. These animals have a point mutation that results in an alteration of just one amino acid that translates into decreased functional effectiveness of the leptin receptor (LepR); the receptor is generated but retained intracellularly, which translates to reduced numbers of LepRs on the cell surface. Consequently, these animals become noticeably obese between the third and the fifth week of life, and this for of obesity is associated with hyperphagia [229]. To date, few studies have used this model to study the correlation between obesity and intestinal dysfunction. Nevertheless, in the last few years, a new pathway in which the adipocyte-derived hormone leptin binds to its long form LepR to regulate intestinal epithelial barrier structure and function has been described [230–232], suggesting new clinical approaches using this animal model.

Some particular components of the Western diet are known to affect the intestine, microbiota, and subsequent health outcomes in the host, supporting the possibility that obesogenic diets deregulate intestinal homeostasis [233, 234]. Despite genetic models, DIO models are the most frequently used models to study obesity, including high-fat (HF) diets and cafeteria (CAF) diets, which are HF and high-carbohydrate diets (HC) [235, 236]. HF diets have been widely used by researchers to generate obese animal models [235]. However, a consensus regarding which HF diet is the most appropriate has not been achieved. Several different semipurified fat sources are usually used in different proportions, ranging between 20% and 60% of energy derived from fat, whereas 10% of energy is derived from fats in low-fat control diets. In addition,

the basic fat component also varies in these studies. For this reason, the phenotypes and biochemical and physiological parameters observed, might vary between different studies [235].

On the other hand, CAF diets are the hypercaloric diets that more accurately reflect the variety of palatable energy-rich foods present in the Western diet that are associated with obesity and other metabolic alterations [237]. In the CAF diet, animals are allowed free access to a variety of highly palatable, energy-dense, unhealthy human foods (high in fat and sugar) *ad libitum* plus a standard chow diet and water. CAF diets normally vary between 2 and 40 items; however, the usual components are cookies, chocolate, candy, cheese, milk, bacon and other meat sources, water and non-purified chow pellets, as well as mineral and vitamin supplements to prevent deficiencies [238]. CAF diets induce rapid weight gain, an increase in the fat pad mass and insulin resistance via hyperphagia [239–242]. Additionally, this diet activates hedonic feeding related to long-lasting neuronal alterations that regulate feeding behaviours and are associated with some patterns of obesity and related metabolic disorders in humans [243, 244]. Altogether, CAF diets induce obesity and related metabolic disorders more efficiently than HF diets [237, 245]. Despite the composition of the diet, another important factor to consider is the duration of the nutritional intervention. Although some CAF studies have been performed for a short period of time [246], CAF studies typically last a minimum of 3 weeks, although 5 months of diet intervention have also been reported [247–251].

Consequently, these models of obesity can be useful in deciphering and describing the pathways that are altered to produce obesity-associated intestinal dysfunction, pathways that will be explained in the following sections.

3.2.2. Dysbiosis and impaired barrier function in obesity

Increasing evidence links changes in the intestinal microbiota to the development of obesity [8]. In fact, significant changes in the composition of the intestinal microbiota have been observed in obese mice and obese patients [252, 253], through a process known as dysbiosis [254]. Lean individuals maintain a relative balance between the two main bacterial phyla, *Bacteroidetes* and *Firmicutes* [253]. In contrast, an increased ratio of *Firmicutes* to *Bacteroidetes* has been described in animal models of obesity [252, 254]. However, other studies show opposite results [255, 256]. Thus, these contradictory results suggest that this issue is not fully resolved. Overall, a decrease in bacterial richness may be considered an indicator of inflammation and metabolic disease.

Some mechanisms have been described that link bacteria to obesity. For example, intestinal bacteria suppress the synthesis of fasting-induced adipocyte factor/angiopoietin-like protein 4, the lipoprotein lipase suppressor in intestinal cells, thus increasing triglycerides storage in adipocytes and the liver [257, 258]. In addition, in an obese subject, the microbiota produces enzymes that degrade nutrients and harvesting energy from the diet more efficiently [254]. The first consequence of the alterations in the microbiota composition is barrier dysfunction and increased intestinal permeability [259, 260]. Intestinal bacteria seem to have a central role in intestinal barrier dysfunction. Changes in the microbiota induced by antibiotic treatment prevent the obesity-induced increase in intestinal permeability [260]. Additionally, interactions between the diet and microbiota induce effects on intestinal homeostasis and the integrity of the intestinal epithelial. In this sense, the intestinal microbiota ferment non-digestible carbohydrates, producing short chain fatty acids. These molecules bind to G protein-coupled receptors that inhibit inflammation and improve

barrier function [261–263]. Obesity models display a clear reduction in the expression of TJ proteins, such as ZO-1 and occludin [260, 264, 265]. Likewise, increased cytoplasmatic sequestration of occludin has been observed in an obese Sprague-Dawley rat model [8], and an abnormal distribution of occludin and ZO-1 has been detected in obese mice [266].

Some pathways that are over–activated in the obese model are involved in regulating intestinal homeostasis and barrier function. Much of this response is mediated by PRRs, including TLRs, which are required to maintain microbial and intestinal homeostasis [223]. TLRs start an immune cascade after recognizing the specific MAMPs and DAMPs [171, 172]. Saturated fatty acids, heat shock proteins, high-mobility group box 1, extracellular matrix degradation products, modified low-density lipoproteins and advanced glycation end-products are endogenous ligands that are recognized as DAMPs by TLRs, particularly TLR2 or TLR4, inducing a pro-inflammatory response [171, 172]. Among the TLRs, TLR4 is expressed at high levels in the intestine [267]. TLR4 activation is increased in intestinal epithelial cells in obesity-prone rats, and its activation is associated with a decrease in intestinal levels of alkaline phosphatase, a luminal enzyme that detoxifies LPS, abnormal TJ expression, and increased intestinal permeability [8].

Under normal conditions, MAMPs typically promote the secretion of anti-inflammatory agents, including some ILs and TGF-β, which promote intestinal tolerance and barrier function [32]. However, in the presence of an imbalance in intestinal homeostasis MAMPs induce the production of pro-inflammatory cytokines, including IL-1 and IL-6 by intestinal epithelial cells, macrophages and DCs [32]. Inflammatory cytokines have been reported to affect the expression levels and assembly of junctional complexes, exerting affect on TJs functions [268]. Indeed, pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-6 induced the opening of the intestinal TJs decreasing barrier function [269–

272].

Pro-inflammatory cytokine-induced TJ opening is mediated by myosin light chain kinase (MLCK) [273]. Cytokines increase MLCK transcription and thus induce its activation. Upon activation, MLCK catalyzes the phosphorylation of myosin light chain, inducing contraction of the peri-junctional actin-myosin filaments and the opening of the TJ barrier [273, 274]. In contrast, inhibition of MLCK activation prevents this effect [275].

3.2.3. Immune cell changes accompany obesity

Changes in innate immune cells and the levels of cytokines produced by these cells are observed during obesity after dysbiosis. The innate immune system not only maintains the immune tolerance to commensal bacteria but also acts as the first line of defence for pathogens. Some of the components that integrate the intestinal innate immune system are intestinal epithelial cells, ILCs and other rapidly responding immune cells, such as macrophages and neutrophils [223].

Regarding innate immune cells, ILC-3 is an important source of IL-22 in the intestine. IL-22 is a member of the IL-10 family and participates in host defence, tissue regeneration and maintaining intestinal epithelial integrity [276, 277]. During obesity, IL-22 production by ILCs is reduced [278]. These results are correlated with increased weight gain and insulin resistance in mice deficient in IL-22 receptor that are fed a HF diet. In contrast, exogenous IL-22 administration to genetically obese mice and mice fed a HF diet improves the metabolic parameters and preserves intestinal barrier function [278]. Eosinophils are another cell type affected by obesity and their numbers and proportions have been reported to decrease in animals fed a HF diet [279], results that were correlated with increased intestinal paracellular permeability in the ileum [279]. The composition of adaptive immune cells is altered in the

small intestine, mainly in the distal jejunum and ileum, and large intestine in the context of overnutrition and obesity [223]. After 12 weeks of HF diet, the proportions of IFN-γ-producing Th1 cells and $CD8^+$ T cells increased in the intestine of mice, whereas the Treg levels were decreased [280], and the same patron was observed in obese individuals [280].

3.2.4. Pro-inflammatory events associated with obesity

Altogether, this crosstalk stimulates the production of a cascade of pro-inflammatory cytokines and enzymes contributing to a pro-inflammatory state in the intestine.

Obese animals express higher levels of the gene encoding the pro-inflammatory cytokine TNF-α in both the small and large intestines [217, 264]. Some chemokines that attract pro-inflammatory immune cells, such as CCL5, and pro-inflammatory cytokines macrophage migration inhibitory factor are also up-regulated in the small intestine of obese animals [281]. In addition, the levels of the pro-inflammatory cytokine IL-1β are increased in the proximal colon [282]. In contrast, obesity is associated with decreased levels of IL-22 and IL-10 in the ileum, which are required to preserve the integrity of the epithelial barrier[283, 284].

In addition to cytokines, inducible enzymes are central intermediaries of intestinal inflammation. Cyclooxygenase (COX)-2 is a pro-inflammatory enzyme of the COX family that is related to intestinal inflammation [285]. COXs are bifunctional enzymes that contain both cyclooxygenase and peroxidase functions [286]. COXs exist as two distinct isoforms: COX-1 and COX-2. COX-1 is constitutively active in absence of stimuli and is expressed in most cells [287]. In contrast, COX-2 is an immediate-early response gene that is not normally expressed in most cells. However, this isoform is inducible expressed at sites of inflammation in response to inflammatory stimuli, including pro-inflammatory

cytokines (IL-1α/β, IFN-γ, and TNF-α) produced by inflammatory cells [288]. COXs are responsible for arachidonic acid metabolism by converting it into prostaglandins. These products influence a wide variety of biological processes, ranging from homeostasis to inflammation. COX-2 activation increases prostaglandin E2 levels at the site of inflammation and systemically [289, 290]. In obese subjects, COX-2 activity is induced in the intestinal epithelial fraction [223].

MPO is another enzyme involved in intestinal inflammation. MPO is a bactericidal enzyme that is abundantly expressed in polymorphonuclear neutrophils (PMNs) [291]. MPO has also been detected in monocytes and tissue macrophages [292]. MPO is a peroxidase that is a central component of the oxygen-dependent antimicrobial function of phagocytes by, reacting with hydrogen peroxide and a halide to synthesize hypochlorous acid or by reacting with tyrosine to form tyrosyl radicals [293]. In addition to its oxidative effects, MPO affects various processes involved in cell signalling and cell-cell interactions, and thus this enzyme is capable of modulating inflammatory responses [294]. In fact, intestinal inflammation is associated with increased MPO levels in the ileum of obese animals [8].

Inducible nitric oxide synthase (iNOS) is another enzyme involved in intestinal inflammation. Three isoforms of the NOS family have been identified, two of them are constitutively present in either endothelial (eNOS) or neuronal (nNOS) tissues, and the last isoform, iNOS, is expressed in response to stimulation with cytokines, microbes and bacterial products [295]. Nitric oxide (NO) synthesis by constitutive NOSs is being controlled at low levels lasts for a short period and is not cytotoxic to intestinal tissues [296]. In fact, eNOS-derived NO participates in regulating intestinal epithelial permeability [297] and modulates the major epithelial functions involved in host defence, such as mucus production [298]. In contrast, iNOS produces large amounts of NO [299]

and its production in epithelial cells is closely associated with the initiation and maintenance of intestinal inflammation [300].

Pro-inflammatory cytokines, adhesion molecules, acute phase proteins and inducible enzymes are nuclear factor-κB (NF-κB) target genes [301]. These genes contain NF-kB binding sites in their sequences, enabling their direct control by NF-kB [302]. NF-κB proteins form dimers that are inactive when they are associated with inhibitory proteins of the IκB family [303]. Inflammatory stimuli induce the activation of the NF-κB pathway by the IκB kinase (IKK) complex [304]. The IKK complex phosphorylates IκB proteins at serine residues, targeting them for polyubiquitination and proteasome-mediated degradation. Thus, NF-κB is released, accumulates in the nucleus and activates the transcription of its target genes [305, 306]. Studies have suggested that the activation of the NF-κB signalling pathway in intestinal tissues is the cause of local, low-grade intestinal inflammation associated with obesity [217, 307]

Finally, intestinal inflammation induces PMNs and macrophage infiltration [308]. These inflammatory cells participate in the maintenance of intestinal homeostasis and eliminate pathogens. However, under pathological conditions, the excessive recruitment and accumulation of activated immune cells in the intestine are associated with intestinal mucosal injury and oxidative stress [308, 309]. Cellular oxidative stress is another event that is closely linked to the uncontrolled activity of inflammatory cells and tissue inflammation. Ingested materials and pathogens induce inflammation by activating macrophages and PMNs, which in turn produces excessive amounts of reactive oxygen species (ROS) that lead to oxidative stress and damage in the intestine [310]. Moreover, excessive ROS production can damage cytoskeletal proteins [311] and subsequently induces intestinal barrier function disruption and increased permeability, contributing to intestinal inflammation [310].

3.2.5. Metabolic endotoxemia in obesity

Obesity is characterized by low-grade intestinal inflammation related to an impaired barrier function. Structural changes in the intestinal barrier trigger an increase in intestinal permeability, promoting the translocation of microbiota-derived factors across the intestinal barrier to the bloodstream [259, 312] and a two- to three-fold increase in its serum concentrations [259].

LPS, also known as lipoglycans and endotoxins, consist of lipid and polysaccharide fractions joined by a covalent bond. LPS is the major cell wall component of gram-negative bacteria [313]. LPS is a potent modulator of the inflammatory process in metabolic diseases [259]. LPS is initially transported by LPS-binding protein (LBP) from bacterial membranes and vesicles. Additionally, LBP transfers LPS to CD14 that splits LPS aggregates into monomeric fractions to present them to the TLR family [314]. Among the TLRs, TLR4 is considered the first barrier that recognizes bacteria present in the intestine and activates several signalling components, including NF-kB as the final effector transcription factor [268].

Different molecular mechanisms mediate intestinal LPS translocation. One possibility is paracellular transport through TJs [284]. An alternative possibility is that LPS enters the bloodstream by transcellular transport through M cells. These cells are permeable to bacteria and macromolecules and facilitate the sampling of intestinal Ags by the underlying lymphoid tissue [315]. The last mechanism is that LPS is absorbed and transported as chylomicrons via mLNs [316]. Under healthy physiological conditions, the intestinal epithelium acts as a barrier that prevents LPS translocation. In normal healthy individuals, plasma LPS concentrations are up to 0.2 ng/mL and is derived from the intestinal lumen [313, 317]. However, increased plasma levels have been observed obese animals and individuals, which is defined as metabolic endotoxemia [318, 319]. In this clinical condition, plasma LPS levels are chronically elevated compared

to normal individuals, but these levels are 10-50 times lower than subjects with sepsis [320]. Metabolic endotoxemia has been observed in genetic mouse models of obesity after the consumption of a normal chow diet [260], and is induced in lean mice and rats consuming obesogenic diets [259, 260].

Once in the circulation, LPS activates inflammatory pathways in the intestine and systemically contributing to a global state of low-grade pro-inflammatory and pro-oxidative stress associated with obesity and other metabolic disorders [321].

3.3. Intestinal dysfunction induced by an LPS injection

Endotoxin injections are considered a powerful model for studying acute inflammatory responses of the host [322, 323]. As mentioned in previous sections, plasma LPS concentrations in healthy individuals are approximately 0.2 ng/ml [313, 317]. Likewise, physiologically relevant plasma LPS levels range from 1 to 2 ng/ml, whereas plasma levels ranging 10 ng/ml are clinically relevant [3].

Based on the results from published *in vitro* studies, high pharmacological concentrations of LPS induce rapid the death and apoptosis of several cell types. However, these studies did not explain the biological activity of physiological concentrations of LPS [324, 325]. In contrast, human studies based on the intravenous administration of an endotoxin derived from *Escherichia coli* have been used to study experimental inflammation in humans, providing novel information about the interactions between inflammatory intermediaries activated cells [326–328].

Recently, studies focused on the intestine have observed that intraperitoneally (IP) injections with low doses of LPS selectively increases TJs permeability, without inducing epithelial cell death or intestinal mucosal damage [3]. The

effect of LPS on intestinal TJ permeability results from an increase in the enterocyte expression and membrane localization of TLR4 and TLR4-dependent expression and co-localization of CD14 [3]. This situation mimics a state similar to obese humans and it seems indicate that the same pathways are involved.

4. Dietary approaches that prevent intestinal dysfunction: flavonoids as bioactive compounds

Based on the direct contact and the dual interaction between the diet and the GI tract, screens for natural dietary compounds that might have beneficial health impacts, could result in the development of a useful strategy for ameliorating intestinal dysfunction.

Natural bioactive compounds from the flavonoid family have been described as anti-inflammatory agents in other tissues [329] and as antioxidant molecules in the intestine [330]. Moreover, when flavonoids reach the intestine, they directly interact with intestinal cells, which control several digestive and metabolic processes [331]. However, flavonoids are not completely absorbed in the GI tract and need to be metabolized by the colonic microbiota, thus modulating intestinal microbial ecosystem [332].

Consequently, flavonoids are postulated to have significant effects on the intestinal environment, which may lead an important influence on the physiology and biochemistry of the intestine.

4.1. Overview of dietary flavonoids

Phytochemicals or phytonutrients are secondary metabolites that are widely distributed in the plant kingdom. They are present in our diet following the consumption of plant-derived food sources and exert biological effects on mammalian systems by modulating enzymatic and chemical reactions [333]. Flavonoids are one of the largest and more prominent groups of phytochemicals in the family of polyphenols, which are characterized by the presence of more than one phenolic group [334].

Flavonoids are a very diverse family of polyphenols consisting of compounds with different chemical structures and characteristics [335]. Their basic structure is based on fifteen carbons, C6-C3-C6, with two aromatic rings (A and B-ring) connected by a three-carbon bridge (C-ring). Flavonoids are classified into flavones, flavonols, flavan-3-ols, flavanones, anthocyanidins and isoflavones according to the oxidation level of the C-ring [336]**.** However, they are subjected to further modifications, including glycosylation, malonylation, hydroxylation, hydrogenation, methylation, and sulfation, to generate numerous derivative forms [337]. Flavan-3-ols are the most abundant subgroup of flavonoids in the human diet **(Figure 9)**.

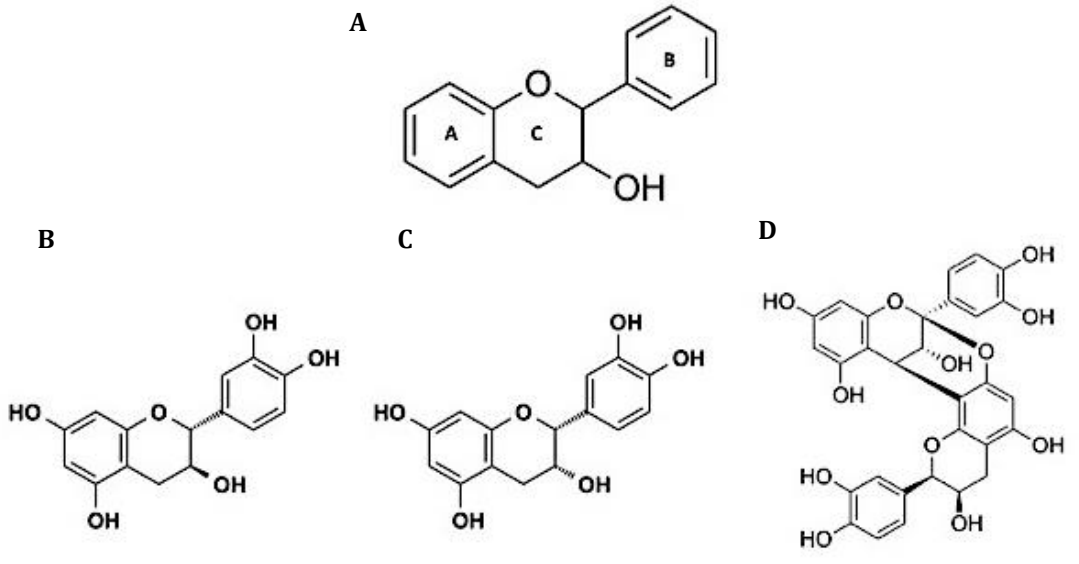

Figure 9. Basic structures of flavan-3-ols (A), catechin (B), epicatechin (C) and the proanthocyanidin dimer (D).

Flavan-3-ols include simple monomers, such as (+)-catechin and its isomer (-)-epicatechin, as well as oligomers and polymers, known as the so-called proanthocyanidins or condensed tannins [338]. In addition, proanthocyanidins that exclusively comprise epi or catechin monomers are known as procyanidins [339].

Interest in research on dietary flavonoids has increased, given their reported versatile health benefits [340, 341]. Several members of the flavonoid family have been described to function as anti-inflammatory agents that are also able to prevent increased intestinal permeability [342]; thus, their properties make them good candidates for affecting intestinal physiology. Likewise, the beneficial roles and significance of flavonoids are directly associated with their structural features and metabolism [343].

Taking all this information into account, in the next section we focus on reviewing the available evidence regarding flavonoid bioavailability, intestinal absorption and metabolism in the GI tract, as well as their protective effects on intestinal inflammation, barrier integrity and intestinal microbiota using *in vivo* and *in vitro* models. Finally, because this review was written in 2015, recent publications describing these effects of flavonoids were collected in the **Table 3** presented in **Annex 1**.

4.2. MANUSCRIPT 1

Literature review

Effects of flavonoids on intestinal inflammation, barrier integrity and changes in the gut microbiota during diet-induced obesity

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Effects of flavonoids on intestinal inflammation, barrier integrity and changes in gut microbiota during diet-induced obesity

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Abstract

Diet-induced obesity is associated with low-grade inflammation, which, in most cases, leads to the development of metabolic disorders, primarily insulin resistance and type 2 diabetes. Although prior studies have implicated the adipose tissue as being primarily responsible for obesity-associated inflammation, the latest discoveries have correlated impairments in intestinal immune homeostasis and the mucosal barrier with increased activation of the inflammatory pathways and the development of insulin resistance. Therefore, it is essential to define the mechanisms underlying the obesity-associated gut alterations to develop therapies to prevent and treat obesity and its associated diseases. Flavonoids appear to be promising candidates among the natural preventive treatments that have been identified to date. They have been shown to protect against several diseases, including cardiovascular diseases and various cancers. Furthermore, they have clear anti-inflammatory properties, which have primarily been evaluated in non-intestinal models. At present, a growing body of evidence suggests that flavonoids could exert a protective role against obesity-associated pathologies by modulating inflammatory-related cellular events in the intestine and/or the composition of the microbiota

populations. This paper will review the literature to date that has described the protective effects of flavonoids on intestinal inflammation, barrier integrity and gut microbiota in studies conducted using *in vivo* and *in vitro* models.

Keywords: flavonoids, intestinal inflammation, obesity, barrier integrity, microbiota

Introduction

It is now widely accepted that obesity is associated with low-grade chronic inflammation, which contributes to an increased risk of insulin resistance and type 2 diabetes mellitus, as well as other detrimental health consequences linked to obesity [1]. Most prior studies have focused on adipocytes as the source of inflammatory mediators in this pathology [2–5]. Recently, the gastrointestinal tract has been described as another potential source of inflammation that is associated with diet- and/or obesity-related pathologies [6]. The intestine is essential for the digestion and extraction of nutrients, such as lipids, carbohydrates and proteins, but its role in metabolic diseases has been poorly investigated over the years. Increased attention has been paid to the link between the gut microbial composition and obesity. The gut microbiota is a source of endotoxins, whose increase in plasma is related to obesity and insulin resistance through increased intestinal permeability in animal models; however, this relationship still needs to be confirmed in humans [7]. Furthermore, different studies suggest that normal non-pathogenic enteric bacteria play a key role in diet-induced adiposity because germ free mice were reported to have less body fat [8] and do not become obese or insulin resistant when subjected to a HF diet [9]. In addition, strong evidence supports a direct link between metabolic diseases encompassing obesity and intestinal dysbiosis,

namely, alterations of the gut microbial composition [10]. These findings have driven research interest to converge on making clearer the relationships between the gut microbiota, diet, host metabolism and the immune system [11].

Defining the sources, causes and mechanisms underlying the development of inflammation during progressive increases in body weight and adiposity is a powerful tool for developing strategies and therapies that prevent or limit the adverse effects of obesity on health. A growing body of evidence suggests that some bioactive compounds present in food, particularly flavonoids, could play a role in obesity through their effects on inflammatory mediators and pathways [12, 13], barrier integrity [14, 15] and/or gut microbiota composition [16–19]. Dietary flavonoids are not completely absorbed from the gastrointestinal tract and are metabolized by the gut microbiota, which reaffirms that they and their metabolites may play a key role in the maintenance of intestinal health.

In view of the direct contact and the dual interaction existing between these natural bioactive compounds and the gut microbial ecosystem coupled with their involvement in modulating obesity and inflammation, it can be hypothesized that the effects of flavonoids on obesity-associated pathologies can be partially explained through the flavonoid-mediated beneficial effects on intestinal alterations. It is worth mentioning that most of the studies performed to date on the effects of flavonoids, both *in vitro* and *in vivo*, address the issue using models of cytokine, endotoxin or chemically induced intestinal inflammation to address severe, acute or chronic inflammation, but few studies address the specific effect of flavonoids on obesity-associated intestinal alterations. However, although severe intestinal inflammation, including inflammatory bowel disease and obesity-associated intestinal inflammation, show different degrees of severity, both pathologies share common pathways and mechanisms. Taking into account all these factors, the present review will

describe the *in vivo* and *in vitro* evidence on the effects of flavonoids in modulating the intestinal inflammatory response, barrier integrity and changes in gut microbiota.

Overview of dietary flavonoids: classification, metabolism, absorption and bioavailability

The term flavonoid refers to certain plant-derived bioactive substances, which exert biological responses in mammalian systems. Flavonoids are classified into flavones, flavonols, flavan-3-ols, flavanones, anthocyanidins and isoflavones [20], depending on the level of oxidation of the C-ring. These bioactive compounds are particularly abundant in certain vegetables, fruits, fruit juices, green and black tea, red wine, chocolate and coffee [21].

The biological properties of flavonoids are closely linked to their bioavailability, intestinal absorption and metabolism in the gastrointestinal tract, which, in turn, depend on their chemical structure and the degree of polymerization [22]. Flavonoid metabolism occurs via a common pathway that results in an aglycone form, which can be absorbed from the small intestine. However, most flavonoids are present in food, as esters, glycosides, or polymers that cannot be absorbed in their original form [21]. To be absorbed, these molecules must be previously hydrolyzed by intestinal enzymes, such as lactase phloridzin hydrolase or cytosolic β-glucosidase, or by the colonic microflora. Before they pass into blood stream, the aglycones are subjected to some degree of phase II metabolism to obtain methylated, sulfated and/or glucuronidated metabolites [23]. Then, the metabolites reach the liver via the portal bloodstream, where they can also undergo further phase II metabolism. The resulting metabolites can enter into the systemic circulation or can return to the small intestine by means of enterohepatic recirculation [22]. Flavonoids that are resistant to the action of hydrolytic enzymes are not absorbed in the small intestine and reach the colon. The colonic microbiota hydrolyzes glycosides into aglycones and

metabolizes them into different aromatic acids. Finally, the flavonoids and their metabolic derivatives are mainly excreted through both biliary and urinary pathways [21]. Despite all of this literature, the bioavailability of these compounds remains a controversial point. Most of them are detected in several tissues inside the organism [21]. However, the enormous diversity of chemical structures that can arise after their metabolization makes it difficult, most of the time, to identify the compound(s) that are responsible for the described effect. In contrast, it is very clear that flavonoids reach the gastrointestinal tract, where they can directly interact with the intestinal cells, which control several digestive and metabolic processes. Consequently, flavonoids and their aromatic bacterial metabolites are postulated to have significant effects on the intestinal environment, which may lead an important influence on the physiology and biochemistry of the gut, mainly in situations of metabolic disruption such as obesity.

Intestinal alterations in obesity

Obesity-associated intestinal inflammation: the link between HF diet, changes in the gut microbiota and metabolic endotoxemia

The gut microbiota represents an ensemble of microorganisms that resides in the intestine, where it plays important roles in ensuring proper digestive functioning, in the immune system and in performing a barrier effect. With respect to the major role that gut microbiota plays in the normal functioning of the body and the different functions it accomplishes, experts currently consider it an “organ”. In addition, certain bacteria tend to adhere to the surface of the intestinal mucosa, while others inhabit the lumen. Whereas the bacteria of the mucosal surface interact with the host immune system, the micro-organisms residing in the lumen may be more relevant to metabolic interactions with food or other digestion derivatives [24]. In fact, compelling evidence supports the

role of the intestinal microbiota in the regulation of adiposity and body weight, and it has received increased attention from researchers worldwide [8, 25–28].

The highest microbiota density in the human body is found in the colon. This compartment is primarily composed of anaerobic bacteria, such as *Bacteroides, Porphyromonas, Bifidobacterium, Lactobacillus and Clostridium*, that belong to the most abundant phyla: Bacteroidetes, Actinobacteria, and Firmicutes [29–31]. The proportion of each phyla varies between individuals and depends on age, stress, geographical location, as well as on diet [32–37]. Data in human subjects and rodents revealed that 90% of the normal gut microbiota consists of the Bacteroidetes and Firmicutes phyla, while obesity is linked to changes in their proportions [10, 38–40], which can lead to dysbiosis. In this sense, transfer of the colonic microbiota from *ob*/*ob* mice to germ-free animals led to increased fat gain, equivalent to an extra 2% energy retention of the calories consumed, compared with transfer from control mice. These changes were associated with a dysbiosis in obese mice characterised by an enhanced representation of Firmicutes and a reduced representation of Bacteroidetes [10]. In obese patients the same results were obtained: the relative proportion of Bacteroidetes was decreased in comparison with lean people. Interestingly, this proportion increased with weight loss on two types of low-calorie diet [40]. However, this modification of the Firmicutes:Bacteroidetes ratio observed in obese individuals was not observed in other studies [41], requiring further studies in this area. In addition, it has been suggested that changes in the composition of the gut microbiota and epithelial functions may play a role in obesity-associated inflammation [42]. However, due to the high microorganism diversity found between subjects, it has been difficult to obtain clear conclusions about the predominant phyla present in metabolic diseases [43].

Components that originated from gut microbiota, such as lipopolysaccharides (LPS), lipoteichoic acid, peptidoglycan, flagellin and bacterial DNA, can cause

immune system activation. Among them, LPS is thought to be a major inducer of the inflammatory response [44, 45]. LPS are large glycolipids that consist of lipid and polysaccharide fractions joined by a covalent bond. They are found in the outer membrane of Gram-negative bacteria, act as endotoxins, and can elicit strong immune responses. The ability of LPS to promote low-grade inflammation and metabolic disturbances may differ, primarily, because of the chemistry of its components, due to the existing variations between strains [46]. Under normal conditions, the presence of this endotoxin in the intestinal lumen does not cause negative health effects. However, some factors can favour the transfer of LPS into the circulatory system and cause metabolic endotoxemia [47]. It is suggested that the consumption of a HF diet induces changes in the gut microbiota, which leads to excessive energy harvesting and storage, and increases intestinal permeability, leading to metabolic endotoxemia [48].

Animal research has indicated that germ-free mice fed a high-fat diet do not gain weight, exhibit adiposity, or display other metabolic effects, such as insulin resistance. When the microbiota was transplanted from lean mice or genetically or diet-induced obese mice into germ-free mice, the recapitulation of the original phenotype was observed, showing an increase in body fat [9]. In genetically obese mice and obese patients, there is a significant change in the composition of the gut microbiota compared with lean controls [10, 40], and in rodents, these modifications can be induced by ingestion of a high-fat diet [49]. It has been hypothesized that the body weight gain is associated with an increase in the capacity of the microbiota to extract nutrients from the diet [50]. However, other mechanisms, such as changes in gut function, have not been fully explored [51]**.** It has been suggested that the type of diet consumed, particularly HF diets, can contribute to metabolic endotoxemia [52].

In this respect, it has been observed that a high-fat meal promotes the translocation of intestinal endotoxins into the circulation in human subjects (10, 11) and in mice (12, 13). Plasma levels of LPS were also shown to increase in response to a 4-w high-fat diet, by genetically induced hyperphagia (13), or in the blood of mice orally gavaged with LPS (14). Moreover, a link was revealed between HF diet, inflammation, and the occurrence of pro-inflammatory products from gut microbiota Gram-negative bacteria in plasma [7, 53].

Therefore, an important question is how dietary fat promotes intestinal LPS absorption. One possibility is that dietary fat promotes paracellular leakage of LPS across the intestinal epithelium. This possibility is supported by observations that intestinal-epithelial tight-junction (TJ) integrity is compromised in obese mice [26] and by studies demonstrating that experimental exposure of the intestinal lumen to some fatty acids can cause small-intestinal epithelial damage [54]. An alternative possibility could be that LPS enters the bloodstream by transcellular transport through intestinal epithelial cells. This process could occur through the so-called intestinal-epithelial microfold cells (M-cells), which are permeable to bacteria and macromolecules and facilitate sampling of gut antigens by the underlying lymphoid tissue [55].

Once in circulation, LPS initiates the activation of Toll-like receptor (TLR) 2 and 4, and LPS receptor CD14, leading to increased activation of inflammatory pathways through cytokine release [56–58], all generally contributing to a state of systemic inflammation associated with obesity and other metabolic disorders **(Figure 1)**.

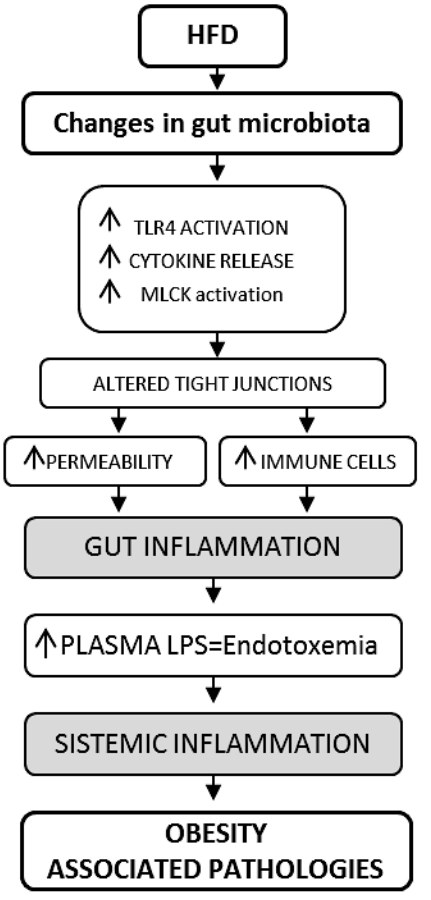

Figure 1. Hypothesis for gut inflammation after a high-fat diet challenge. Changes in gut microbiota after a high-fat diet (HFD) induce an increase in intestinal permeability and activation of immune cells. Consequently, endotoxemia increases and triggers systemic inflammation and metabolic disorders. MLCK, myosin L chain kinse; LPS, lipopolysaccharide; TLR4, toll like receptor 4.

Altered networks in intestinal inflammation: barrier integrity and inflammatory response

The paracellular and transcellular pathways are the two major pathways mediating transmembrane transfer of intestinal bacterial substances. Both mechanisms may be involved in intestinal mucosal barrier damage and bacterial translocation. The paracellular pathway is integrated by TJs,

consisting of zonulin/zonula occludens (ZO)-1, occludin, claudins, and actin-myosin cytoskeletal proteins. Previous studies have shown that inflammatory cytokines and bacterial antigens can affect the expression level and assembly of these elements, thereby exerting an influence on TJ functions [59]. Immune cells, including neutrophils, dendritic cells, and monocytes, have also been directly implicated in inducing disturbances in TJ barrier function. It has been postulated that pro-inflammatory cytokine-induced opening of the intestinal TJ barrier is an important mechanism contributing to the TJ barrier defects present in various inflammatory conditions of the gut [60]. Previous studies [61–64] have shown that myosin light chain kinase (MLCK) plays a central role in the regulation of intestinal TJ permeability. The activation of MLCK catalyses the phosphorylation of myosin light chain (MLC), inducing contraction of the peri-junctional actin-myosin filaments and the opening of the TJ barrier. In contrast, inhibition of MLCK activation prevents this effect [63]. It has been suggested that the cytokine-mediated barrier dysfunction could be mediated by an increase in Nuclear Factor (NF)-kB, which, in turn, activates MLCK gene and protein expression [65].

Once intestinal bacteria and endotoxins enter the portal vein and/or lymphatic system, they can reach other tissues and organs, leading to a cascade response modulated by inflammatory mediators. This situation can induce a systemic inflammatory response, which further damages the function of the intestinal barrier [59]. The endotoxin-signalling pathway includes the binding of LPS to LPS-binding protein (LBP) and its subsequent transfer to the CD14 receptor. LBP-bound LPS initiates inflammation via TLRs associated with membrane-anchored CD14 [54]. TLRs are a family of pattern-recognition receptors that play a key role in the innate immune system. Among all, the TLR4 is expressed at high levels in the intestinal tract, and given that LPS is its specific ligand, TLR4 could be considered the first barrier for recognition of bacterial presence in the gastrointestinal tract. NF-κB is the final effector transcription factor of

the TLR4 signalling pathway pivotal role in the translation and transcription of inflammatory mediators [59].

In mammals, the NF-κB family comprises five proteins, including p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB 1), and p100/p52 (NF-κB 2), which associate with each other to form transcriptionally distinct homo- and heterodimeric complexes; the p65:p50 heterodimer is the most abundant and the most relevant for inflammation [66]. In resting cells, the p65:p50 NF-κB heterodimer is sequestered in the cytoplasm by binding to its inhibitory protein, IkappaB (IκB). In response to an inflammatory stimulus, such as LPS, the classical NF-κB activation pathway leads to the activation of the IκB kinase (IkkB), a member of the IKK complex, triggering IκB -a phosphorylation (pIkB-a). Then, pIkB-a is recognized by the ubiquitin ligase machinery, resulting in its polyubiquitination and subsequent proteasomal degradation. After pIκB-a degradation, the p65:p50 heterodimers are able to translocate to the nucleus, where they bind to the κB motif found in the promoter or enhancer regions of numerous pro-inflammatory genes to induce their expression [67].

NF-κB target genes include cytokines (for example, tumor necrosis factor (TNF)-α and interleukins, IL), adhesion molecules, acute phase proteins and inducible enzymes (inducible nitric oxide synthase (iNOS) and cyclo-oxygenase- 2 (COX-2)) among others [68]. All of these genes contain verified NF-κB binding sites in their sequences, providing strong experimental evidence for their direct control by NF-κB [69]. Among all of these genes, the expression of iNOS and COX-2 has been widely studied in relation to intestinal inflammation. In this regard, sustained high nitric oxide (NO) production by iNOS plays a role in the pathology of chronic inflammatory bowel disease [70, 71]. During the last decade, it has become increasingly clear that NO overproduction by iNOS is deleterious to intestinal function [72], thus contributing significantly to gastrointestinal immunopathology. COXs are

enzymes that are responsible for the metabolism of arachidonic acid, converting it into prostaglandins (PG). These products influence a wide variety of biological processes, ranging from homeostasis to inflammation [73]. There are two COX isoforms, the constitutive COX-1 isoform, and the inducible COX-2 isoform [73, 74]. As a result of COX-2 induction, prostaglandin E_2 ($PGE_{2)}$ levels increase at the site of inflammation and can also be detected systemically.

Taken together, these data suggest that HF diet-induced changes in the intestinal microbiota could be responsible for metabolic endotoxemia and for the onset of the corresponding diseases. The causative link between changes in intestinal bacteria populations, endotoxemia, and metabolic disease needs further assessment [56], but the mechanisms likely include altered epithelial permeability, translocation of bacterial products and up-regulation of pro-inflammatory cytokines and hormones produced by gut endocrine cells, mechanisms which might be modulated by flavonoids.

Flavonoid modulation of intestinal inflammation, barrier integrity and gut microbiota

Flavonoid effects on inflammatory pathways

NF-κB plays a key role in the intestinal inflammatory response [75]; therefore, the compounds that could modulate this inflammatory pathway are an interesting field of investigation. Flavonoid-mediated modulation of the inflammatory response has been extensively studied in several *in vivo* and *in vitro* models [76–80]; however, there are fewer studies regarding its effects on intestinal inflammation **(Table 1)**.

The initial step in the activation of the NF-κB pathway by endotoxins is LPS binding to its receptor TLR4. Dou *et al.* [81] studied the effect of naringenin on flavonoid modulation of TLR4 expression in colonic inflammation using female C57BL/6 mice. Naringenin is a flavanone present in citrus fruits that plays an

important role as anti-inflammatory and antioxidant agent [14, 82]. In this report, colonic inflammation was produced using dextran sulphate sodium (DSS), one of the most widely utilized chemical compounds for inducing an intestinal inflammatory model [83–85]. After a 6 d DSS treatment, both the mRNA and protein expression of TLR4 were significantly increased, however naringenin treatment inhibited its expression. In presence of intestinal inflammation, a situation of dysbiosis is observed accompanied by a deregulation of pattern-recognition receptors that recognize pathogen-associated molecular patterns. One of this pattern-recognition receptor is TLR4, a key receptor for commensal recognition in gut innate immunity and the initial modulator in the activation of the NF-κB pathway. Given that many therapeutic targets that abrogate intestinal inflammation might transect with the TLR4 signalling pathway [86, 87], the results observed by Dou *et al*. [81] may give insight into further evaluation of naringenin as a food supplement in the treatment of intestinal inflammation by suppressing TLR4/NF-κB signalling pathway.

Luteolin is another flavonoid that has been related to NF-κB pathway inhibition. It is a flavone that is abundant in carrots, peppers, celery, olive oil, peppermint, thyme, rosemary, and oregano. Luteolin has been shown to produce various beneficial health effects, including antioxidant, anti-inflammatory, antimicrobial and anti-cancer activities [88, 89]. Once the membrane receptor is activated by *e.g.* LPS, the classical pathway of NF-κB activation leads to the phosphorylation of IKK. Kim *et al.* [90] observed that IKK activity was suppressed by pre-treating IEC-18 cells (a rat non-transformed small intestinal cell line) with luteolin followed by LPS stimulation. Luteolin effect resulted in an inhibition of NF-κB signalling and the consequent pro-inflammatory gene expression in these intestinal epithelial cells. Ruiz *et al*. [91] found that treatment with functionally diverse flavonoids, such as 3'-hydroxy-flavone and also luteolin, followed by TNF-α stimulation, inhibited NF-κB signalling by targeting different

points of the pathway. They observed that 3'-hydroxy-flavone was able to inhibit IKK activity and that luteolin inhibited NF-κB RelA transcriptional activity in Mode-K cells, a murine intestinal epithelial cell line.

Some authors have demonstrated that flavonoids are able to inhibit the NF-κB translocation to the nucleus, preventing pro-inflammatory gene transcription. This effect can be explained by the protective role that some flavonoids exert over IκB degradation. Nunes *et al.* [92] found that a treatment with a red wine extract rich in procyanidins and anthocyanidins significantly inhibited IκB degradation. These results were observed in HT-29 cells (human epithelial colorectal adenocarcinoma cells) stimulated with TNF-α, IL-1β and interferon (INF)-Ɣ. Some *in vitro* and *in vivo* studies have proven the effect of flavonoids on IκB degradation. An *in vitro* study showed that *Opuntia ficus-indica* juice, also known as cactus pear juice, acted as an antioxidant and anti-inflammatory agent in Caco-2 cells [93]. The extract constituents were flavonoids, such as isorhamnetin and some of its derivatives. Pre-treatment with *Opuntia* extract followed by stimulation with TNF-α, IL-1β and LPS slightly prevented IκB depletion. Moreover, the co-incubation of the extract with these inflammatory inducers led to a more significant effect, showing higher levels of IκB. Other authors [81] also showed similar effects of flavonoids on NF-κB translocation. Naringenin significantly blocked the NF-κB signalling pathway in DSS-induced colitis by suppressing IκBα phosphorylation/degradation, blocking NF-κB p65 nuclear translocation and inhibiting NF-κB-mediated transcriptional activity.

Upon activation, NF-κB regulates the transcriptional activation of many genes involved in the immune and inflammatory responses, such as pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) and enzymes [68]. The beneficial effect of flavonoids on intestinal inflammation has directly been related to the suppression of pro-inflammatory enzyme expression, such as COX-2 and iNOS. Nunes *et al*. observed that pre-treatment with a red wine extract rich in

catechins, oligomeric procyanidins and anthocyanidins inhibited COX-2 and iNOS cytokine-induced expression and it also suppressed interleukin (IL)-8 overproduction in HT-29 cells [92]. In another study, also in HT-29 cells, pre-treatment with pomegranate juice, which is rich in anthocyanidins and catechins, reduced TNF-α-induced COX-2 expression [94]. This finding may be related to the inhibition of phosphatidylinositide 3-kinase (PI3K) and protein kinase B, preventing the translocation of NF-κB to the nucleus, inhibiting the transcription of genes encoding these inflammatory enzymes. Another hypothesis might be that flavonoids are acting at the same level of NF-κB pathway but modulating the mitogen activated protein kinase (MAPK) activity. Either way, the selective inhibition of COX-2 by flavonoids could be an interesting strategy to reduce inflammation without altering the protective role of PG synthesized by COX-1.

Other authors found that the pre-treatment of Caco-2 cells with a Sardinian red wine extract, rich in flavanols, flavolons and anthocyanidins, prevents IL-6 and IL-8 expression and synthesis after being challenged with an oxysterol mixture [95]. In addition, During *et al*. [96] studied the effects of chrysin, a flavone found in some plants, such as passionflowers or chamomile. They concluded that o-methylated chrysin was able to modulate intestinal inflammation in Caco-2 cells. The cells were pre-treated with both the o-methylated and the non-methylated forms of chrysin and then, stimulated with IL-1β. The results indicated that the o-methylated form was able to reduce IL-6 and IL-8 secretion and COX-2 activity more effectively than the non-methylated form, indicating a structure-related effect. These results are in agreement with other studies that have demonstrated that the o-methylation of flavones improves their intestinal absorption and metabolic stability [97, 98]. Due to their increased lifespan in our body o-methylated flavones are more able to induce potential health effects as compared to their parent unmethylated analogues, according to the observations of During *et al.* [96].

It has also been reported that naringenin is able to down-regulate the expression of adhesion molecules (ICAM-1), chemokines (monocyte chemotactic protei-1; MCP-1), iNOS, COX-2, TNF-α and IL-6 [81] in a model of DSS-induced colitis using female C57BL/6 mice. Furthermore, in a rat model of spontaneous inflammatory bowel disease, Castagnini *et al.* [99] found that Marie Ménard lyophilized apples, which are rich in flavonols and flavan-3-ols, reduced myeloperoxidase (MPO) activity and COX-2 and iNOS gene expression. MPO is a key component of the oxygen-dependent microbial activity of phagocytes but it has been also linked to tissue damage in acute or chronic inflammation. Beyond its oxidative effects, MPO affects various processes involved in cell signalling and cell-cell interactions and are, as such, capable of modulating inflammatory responses. MPO is considered a marker of disease activity in patients with intestinal inflammation further highlighting the modulatory effect of flavonoids on this enzyme.

Very recently, Mascaraque *et al.* [100] tested the intestinal anti-inflammatory activity of apigenin K, a soluble form of apigenin, in two models of rat colitis, namely, the trinitrobenzenesulfonic acid model and the DSS model. Apigenin K pre-treatment ameliorated the morphological signs and biochemical markers in both models. Specifically, Apigenin K pre-treatment tended to normalize the expression of a number of colonic inflammatory markers (*e.g.*, TNF-α, transforming growth factor-β, IL-6, intercellular adhesion molecule 1 or chemokine ligand 2) and to reduce colonic MPO and alkaline phosphatase activities.

Table 1. Summary of flavonoid effects on intestinal inflammatory response and barrier function *in vivo* and *in vitro*.

Flavonoid	Concentration	Cell type or animal model	Induction of inflammation	Effect	Reference
In vivo					
Naringenin (flavanone)	50mg/kg, 10 d	Female C57BL/6 mice (colon)	4% (w/v) DSS in drinking water, 6 d	Suppression of TLR4/NF-kB Suppression of IkBα phosphorylation/ degradation Down-regulation of iNOS, ICAM-1, MCP-1, COX-2 TNF-α and IL-6 expression	Dou *et al.,* 2013 [81]
	Diet containing a 0.3 % (w/w), 9 d	Male BALB/c mice (colon)	2% (w/v) DSS in distilled water, 9 d	Prevention of JAM-A, occludin and claudin-3 decrease and claudin-1 increase	Azuma *et al.,* 2013 [14]
Marie Ménard lyophilized apples (flavonols and flavan-3-ols)	7.6% of total diet, 12 w	HLA-B27 transgenic rats (colon mucosa)	Genetic IBD	Reduction of MPO and COX-2 activity and iNOS gene expression	Castagnini *et al.,* 2009 [99]
Quercetin (flavonol)	200 μM, 24 h	Male Wistar rats (distal colon)	TNF-α 10^4 U/ml INF-Ɣ 100 or 1000 U/ml, 24 h ex vivo	Down-regulation of Claudin-2 expression	Amasheh *et al.,* 2012 [15]
Puerarin (isoflavone)	180mg/kg and 90 mg/kg, 5 w	Male Sprague-Dawley rats (small Intestine)	Lieber-DeCarli diet [101](1000 KCal liquid diet with 36% etanol), 8 w	Up-regulation of ZO-1 expression	Peng *et al.,* 2013[103]
Apigenin K	3 mg/kg, 7-9 d	Female Wistar rats (colon)	TNBS 40mg/ml or 4% (w/v) DSS, single dose	Normalization of inflammatory markers expression and reduction of MPO and alkaline phosphatase activities.	Mascaraque *et al.,* 2015 [100]

Hydrocaffeic acid	50 mg/kg/d, 18 d	Male Fischer 344 rats (colon)	4% (w/v) DSS in drinking water, 4 d	Down-regulation of TNF-a, IL-1β, and IL-8 expression	Larrosa *et al.,* 2009 [103]
In vitro					
Luteolin (flavone)	50 μM, 1 h	IEC-18 cells	LPS (1μg/ml) 1 h	Inhibition of LPS-induced IKK activity	Kim *et al.,* 2005 [90]
	100 μM, 24 h	Mode K cells	TNF-α (5 μg/ml) and IL-1β (5 μg/ml), 24 h	Inhibition of NF-κB transcriptional activity	Ruiz *et al.,* 2006 [91]
3'-hidroxy-flavone	100 μM, 24 h	Mode K cells	TNF-α (5 μg/ml) and IL-1β (5 μg/ml), 24 h	Inhibition of IKK activity	Ruiz *et al.*, 2006 [91]
Red wine extract (procyanidins, catechins and anthocyanidin)	100, 200, 400 and 600 μg/mL, 30 min	HT-29 cells	TNF-α (20 ng/ml), IL-1β (10 ng/ml) and INF-ϒ (50 ng/ml), 24 h	Inhibition of the degradation of IkB protein Inhibition of COX-2 and iNOS and suppression of IL-8 overproduction	Nunes *et al.*, 2013 [92]
Opuntia ficus-indica extract (isorhamnetins and derivates)	*50 mg gallic equivalents/L, 4 or 48 h*	Caco-2 cells	TNF-α (50 ng/ml), IL-1β (25 ng/ml) and LPS (10 μg/ml), 48 h	Inhibition of the depletion of IkB protein	Matias *et al.*, 2014 [93]
Pomegranate juice (anthocyanidis catechins)	50 mg/L, 1 h	HT-29 cells	TNF-α (20 μg/L), 24 h	Reduction of TNF-α COX-2 expression	Adams *et al.*, 2006 [94]
Sardinian red wine extract (flavanols, flavonols and anthocyanidin)	25 μg/mL Sardinian wine, 1 h	Caco-2 cells	Oxy-mixture (30 and 60 μM), 4 or 24 h	Prevention of IL-6 and IL-8 expression and synthesis	Biasi *et al.*, 2013 [95]

Chrysin o-methylated (flavone)	50 µM, 1 h	Caco-2 cells	IL-1β (25 µ/mL), 24 h	Reduction of IL-6 and IL-8 secretion and COX-2 activity	During *et al.*, 2013 [96]
Quercetin (flavonol)	200 µM, 24 h	HT-29 cells	100 U/ml TNF-α, 24 h	Inhibition of TNF-α-decreased TEER Down-regulation of Claudin-2 expression	Amasheh *et al.*, 2012 [15]
	218 µM, 48 h	Caco-2 cells	500 µM indomethacin, 48 h	Protection against intestinal permeability alterations Protection of ZO-1 delocalization and prevention of the decrease of ZO-1 and occluding expression	Carrasco-Pozo *et al.*, 2013 [104]
EGCG (flavanol)	218 µM, 90 min	Caco-2 cells	500 µM indomethacin, 90 min	Protection against intestinal permeability alterations	Carrasco-Pozo *et al.*, 2013 [104]
	100 µM, 48 h	T84 cells	INF-Ɣ (20 ng/ml), 48 h		Watson *et al.*, 2004 [105]
Genistein (isoflavone)	300 µM, 3 h	Caco-2 cells	Mixture of xanthine oxidase (20 mU/mL) and xanthine (0.25 mM), 3 h	Prevention of ZO-1 tyrosine phosphorylation	Rao *et al.*, 2002 [106]

Abbreviations: COX-2, cyclooxygenase-2; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule-1; IFNɣ, interferon-ɣ; IKK, IκB kinase; IκB, inhibitory protein kappaB; IL, interleukin; iNOS, inducible nitric oxide synthase; JAM-A, junction adhesion molecule ; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; MPO, myeloperoxidase; NF-κB, nuclear factor-kB; PGE2, prostaglandin E2; STAT, signal transducer and activator of transcription protein; TEER, transepithelial electrical resistance; TLR4, toll-like receptor 4; TNBS, trinitrobenzenesulfonic acid; TNF-α, tumor necrosis factor-α; ZO-1, zonula occludens-1.

It should be noted that flavonoids are metabolized by intestinal cells and gut bacteria, and it is possible that some of the anti-inflammatory properties of flavonoids in the gut might be mediated by their metabolites in addition or in place of the original compound present in food, however, it has been studied much less. In this sense, Larrosa et al. [103] concluded that some polyphenol-derived metabolites from the colon microbiota inhibit DSS-induced colitis lipid peroxidation and DNA damage in the colon mucosa and down-regulate the fundamental cytokines involved in the inflammatory process (TNF-a, IL-1b, and IL-8).

In summary, the literature suggests that flavonoids reduce intestinal inflammatory processes driven by NF-κB activation by inhibiting cytokine expression and synthesis and down-regulating TLR-4/ NF-κB pathway in intestinal models **(Figure 2)**. If these observations are confirmed in clinical trials, flavonoid-rich foods or flavonoid supplements may have potential therapeutic and/or preventive applications in the management of intestinal inflammation.

Flavonoid effects on intestinal mucosal barrier integrity

Because the integrity of the intestinal barrier has been compromised in several intestinal pathologies [51, 107, 108], the potential protective effects of naturally occurring bioactive compounds have been evaluated in some *in vitro* and *in vivo* models **(Table 1)**.

Quercetin has been proposed to exert beneficial effects over the intestinal barrier function [109]. It is the most common flavonoid in nature and can be found in fruits and vegetables, including onions, kale and apples [110]. Amasheh *et al*. tested the effect of quercetin on cytokine-induced intestinal barrier damage both in HT-29 cells and in the distal colon from male Wistar rats *ex vivo* [15]. *In vitro*, quercetin was added on both sides of the culture insert and TNF-α was added only to the basolateral side, which produced a

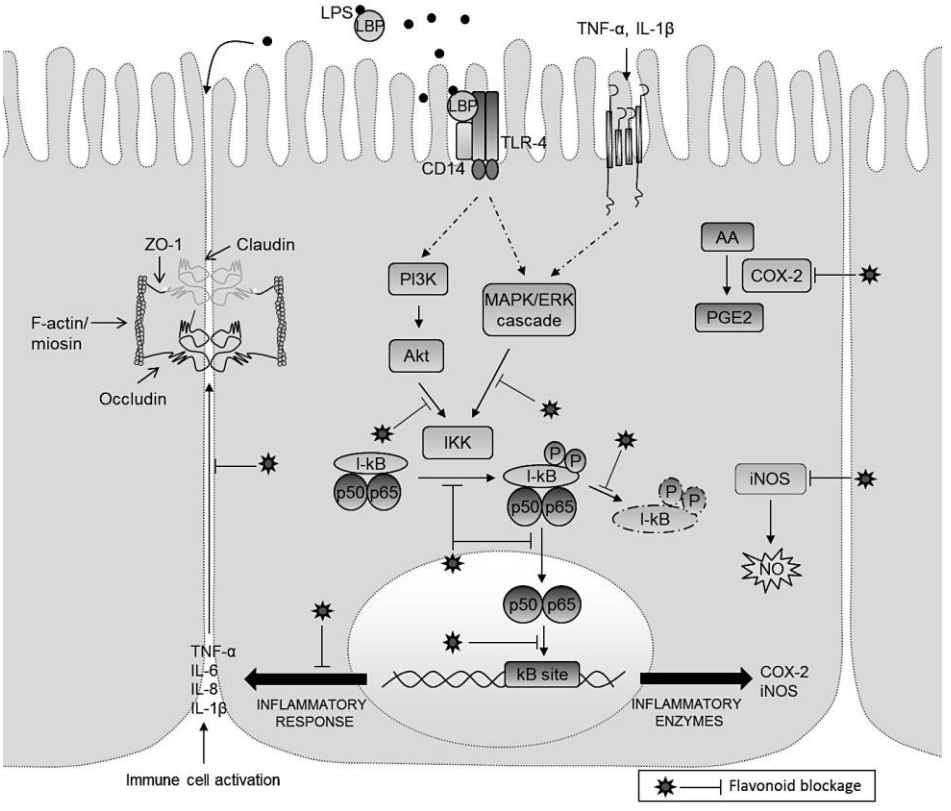

Figure 2. Schematic view of the anti-inflammatory mechanisms of flavonoids on intestinal inflammation. The mechanisms underlying the anti-inflammatory effects of flavonoids involve, among others, the production and secretion of inflammatory mediators, protection of TJ cytokine-induced damage and the modulation of the MAPK and NF-kB pathways. AA, arachidonic acid; DSS, dextran sulfate sodium; MyD88, myeloid differentiation primary response gene 88; NIK, NF-kB-inducing kinase

decrease in transepithelial electrical resistance (TEER). Interestingly, quercetin treatment partially inhibited this effect. In this study, the expression of claudin-2 was also evaluated. Claudin-2 forms cation-selective channels, and consequently, its up-regulation could contribute to the altered barrier function by allowing the massive transit of cations and water to the lumen [111]. In this context, the authors found that quercetin exerts a protective effect on the intestinal barrier by down-regulating claudin-2. The analysis of intestinal permeability in rat colon *ex vivo* revealed that the application of TNF-α and INF-

ϒ reduced the total resistance of the intestinal barrier, which was partially inhibited by quercetin.

Carrasco-Pozo *et al*. tested the effect of quercetin and epigallocatechin gallate (EGCG) against the indomethacin-induced disruption of epithelial barrier integrity in Caco-2 cells [104]. Indomethacin is a non-steroidal anti-inflammatory drug that causes mitochondrial dysfunction, oxidative stress and apoptosis in chronic administration [112, 113]. The results showed that quercetin and EGCG completely protected against the indomethacin-induced decrease in TEER. The same results were obtained when the permeability was assessed by measuring fluorescein isothiocyanate-labeled dextran (FD-4) transport across the Caco-2 cell monolayer [104]. Finally, they evaluated the protective effect of quercetin on ZO-1 and occludin in Caco-2 cells treated with indomethacin and rotenone (an environmental toxin). Immunofluorescence analysis revealed that either indomethacin or rotenone, both inhibitors of mitochondrial complex I, caused TJ disruption through ZO-1 delocalization. Treatment with quercetin protected ZO-1 delocalization and also prevented the decrease in ZO-1 and occludin expression. The authors hypothesized that quercetin's effects may be due to its mitochondrial-protecting property. However, it could also be the result of a modulatory effect of quercetin on the activity of various intracellular signalling molecules that regulate the integrity of TJ. In fact, Quercetin has been reported to inhibit isoform-mixed protein kinase C (PKC) [114]-and PI3K [115]. The PKC family have been shown to be involved in the barrier function in an isoform-specific manner [116, 117]. Atypical PKCς and -λ are necessary for the maintenance of TJ [118], whereas a novel PKCδ is activated by hydrogen peroxide and induces TJ disruption [119]. PI3K, also, negatively modulates the intestinal barrier function. Activation of PI3K by oxidative stress dissociates occludin and ZO-1 from the actin cytoskeleton and disrupts barrier function in epithelial cells [120].

Nevertheless, the mechanisms underlying these flavonoid-mediated biological effects have not been fully clarified yet.

The effect of naringenin was evaluated in a murine model of chronic intestinal inflammation [14]. To induce intestinal damage, male BALB/c mice were fed with 2% (w/v) DSS. The colonic permeability was studied by measuring FD-4 paracellular transport. The authors found that the animals fed with DSS exhibited higher permeability than the control group. In contrast, the DSS + naringenin group did not differ from the control group. Furthermore, the expression of the occludin, junctional adhesion molecule (JAM)-A, claudin-3 and claudin-1 proteins was decreased in the DSS group. However, the level of these proteins was equivalent to the control group after treatment with naringenin. Taken together, all of these findings suggested that naringenin was able to protect TJ by suppressing DSS-induced damage in the intestinal epithelial cells.

Puerarin [7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one- 8-(b-D-glucopyranoside)], an isoflavone extracted from a Chinese medicinal herb, can modulate TJ expression in the altered intestinal barrier *in vivo* [102]. Male Sprague-Dawley rats were fed an ethanol liquid diet producing intestinal barrier dysfunction. In this study, ZO-1 protein expression was significantly down-regulated by ethanol intake, whereas the groups treated with puerarin exhibited an up-regulation of this protein. The authors concluded that the expression of ZO-1 in the rats that received ethanol was indicative of injury to the intestinal barrier function and that puerarin mitigated such intestinal alterations. The negative effect that ethanol exercises at intestine level is not only due to the alteration of gastrointestinal epithelial barrier function, and the increment of intestinal permeability, but also includes diminished phagocytosis mediated by Kupffer cells [121] and bacterial overgrowth, among others [122]. Then puerarin may be acting against ethanol-induced injury at different levels to modulate intestinal health, but this hypothesis needs further assessment.

The molecular mechanisms of genistein, quercetin, myricetin and EGCG in protecting the intestinal barrier have been extensively reviewed by Suzuki *et al*. [123]. These molecules exerted protective and promoting effects on intestinal TJ barrier function. In particular, genistein and quercetin interact with intracellular signalling molecules, such as tyrosine kinases and PKCδ, resulting in the regulation of TJ protein expression and assembly. More specifically, it has been demonstrated that oxidative stress-induced TJ dysfunction is related to the tyrosine phosphorylation of occludin, ZO-1 and E-cadherin in Caco-2 cells [106]. It has been hypothesized that genistein acts against the oxidative stress in the intestinal barrier by suppressing c-Src kinase (a tyrosine kinase) activation, which inactivates tyrosine phosphorylation of the TJ. Furthermore, EGCG's effects on IFNγ-induced intestinal barrier dysfunction were evaluated in T84 human colonic cells [105]. The results showed that EGCG restored the decreased TEER values caused by IFNγ. The authors suggested that the ability of EGCG to limit the IFNγ-induced increases in epithelial permeability is likely a component of the anti-inflammatory nature of this polyphenol.

Flavonoid-microbiota interaction: modulation of the gut microbiota composition

In mammals, the microbiota is involved in the maintenance and development of the immune system, in the regulation of several metabolic pathways, and in general body homeostasis [124, 125]. It has been suggested that both dietary flavonoids, which are the substrates of intestinal bacteria, and the metabolites produced during flavonoid degradation in the colon may modulate and induce oscillations in the composition of the microbiota populations by means of prebiotic and antimicrobial effects against gut pathogenic microorganisms [126–128]. However, the mechanisms involved are still poorly understood. In the following section, we summarize the effects of flavonoids and their

metabolites from colonic metabolism on the gut microbiota composition **(Table 2)**.

Interesting results were obtained in human studies from Tzounis *et al.* [127] who evaluated the prebiotic effect of cocoa flavanols in a randomized, double-blind, crossover intervention study that included twenty-two human volunteers. The administration of 494 mg of cocoa flavanols for 4 w significantly increased the number of *Lactobacillus* and *Bifidobacterium* populations but significantly decreased the *Clostridia* counts. These microbial changes were correlated with reductions in plasma C-reactive protein (CRP) concentrations, which is considered to be a blood marker of inflammation and a hallmark of the acute phase response [127]. These changes in the dominant bacterial communities were similar to those found by Clavel *et al.* [129] in a randomized, double-blind, placebo-controlled study undertaken by 39 postmenopausal women. After one month of supplementation with 100 mg/d of isoflavones, the percentages of the *Lactobacillus-Enterococcus group*, the *Faecalibacterium prausnitzii* subgroup and the genus *Bifidobacterium* were significantly increased [129]. Queipo-Ortuó *et al.* [128] performed a randomized, crossover, controlled intervention study, in which 10 adult men participated. The results showed that a daily consumption of 272 ml of red wine, which is mainly rich in flavanols, anthocyanins, flavonols, and other flavonoids, decreased the plasma levels of triglycerides and high-density lipoprotein cholesterol, and these significant reductions may be partly due to the flavonoid-induced increase in the number of Bacteroidetes phyla [128]. Other authors noted a significant reduction in the plasma concentration of CRP after red wine treatment, which was related to an increase in the percentage of *Bifidobacterium* [130].

Table 2. Summary of flavonoid effects and their metabolites on the modulation of gut microbiota composition.

Flavonoid	Gender/Specie	Dose	Effect on the microbial populations	Reference
Human studies				
Cocoa flavonols	22 human volunteers	494 mg, 4 w	Increase the number of *Lactobacillus* and *Bifidobacterium*	Tzounis *et al.*, 2011 [127]
Isoflavones	39 postmenopausal women	100 mg/d	Increase the number of *Lactobacillus-Enterococcus* group*, Faecalibacterium prausnitzii* subgroup and the genus *Bififobacterium*	Clavel *et al.*, 2005 [129]
Red wine (flavanols, anthocyanins, flavonols, etc.)	10 adult men	272 mL	Increase the number of Bacteroidetes phyla	Queipo-Ortuño *et al.*, 2012 [128]
Animal studies				
Quercetin	*Wistar* rats	30 mg/kg body weight per d during the experiment	Decrease Firmicutes populations, *Erysipelotrichi* class and *Bacillus* genus Down-regulation of *Erysipelotrichaceae*, *Bacillus* and *Eubacterium cylindroides* species	Etxeberria *et al.*, 2015 [16]
Cranberry extract (proanthocyanidins and flavonols)	Mice	200 mg/kg 8 w	Increase the proportion of *Akkermansia*	Anhê *et al.*, 2015[132]
Green tea leaves (flavanols)	Mice	4% w/w	Increase the proportion of *Akkermansia*	Axling *et al.*, 2012 [132]
In vitro				
Tea extract (catechin, epicatechin and	-	0,1% w/v of aromatics	Repression of the growth of *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides spp*	Lee *et al.*, 2006 [18]

their aromatic metabolites)				Parkar *et al.*, 2008 [19]
Catechin, epicatechin, naringenin, diadzein, genistein and quercetin	*Caco-2 cells*	Dose likely to be present in the gastrointestinal tract	Affection on the viability of *Lactobacillus rhamnosus, Escherichia coli, Staphylococcus aureus* and *Salmonella typhymurium*	
Flavonoid metabolites				
Phenolic acids of the gut (benzoic, phenylacetic and phenylpropionic acids)	-	1000 µg/mL	Inhibitory effect in the growth of *Escherichia Coli, Lactobacillus paraplantarum, Lactobacillus plantarum, Lactobacillus fermentum, Lactobacillus brevis, Lactibacillus corynifirmis*, *Staphylococcus aureus* and *Candida albicans*	Cueva *et al.*, 2010[133]

Regarding animals studies, Etxeberria *et al.* [16] assessed in Wistar rats, the potential of quercetin to reverse alterations of the gut microbial composition associated with diet-induced obesity. All of the animals were fed a high-fat sucrose diet, containing 17% of the energy as sucrose, for 6 w, and the treated group was also supplemented with quercetin at 30 mg/kg body weight per d during the experiment. According to the results, quercetin generated a significant impact on different taxonomic grades of the gut microbiota composition. At the phylum level, quercetin administration attenuated the Firmicutes:Bacteroidetes ratio, decreasing Firmicutes populations by 34.2%. Quercetin also significantly reduced *Erysipelotrichi* (-83.9%) and *Bacillus* (-74.3%) abundance at the class and genus level, respectively. Furthermore, the treated group showed a statistically significant down-regulation detected in the mean relative abundance of some bacterial species previously associated with diet-induced obesity (*Erysipelotrichaceae, Bacillus,* and *Eubacterium cylindroides*). Overall, quercetin administration effectively reduced the high-fat sucrose diet-induced gut microbiota dysbiosis. Meanwhile, Anhê *et al*. [131] evaluated the impact of a cranberry extract rich in proanthocyanidins and flavonols in the modulation of the gut microbiota on mice fed high-fat sucrose diet. The daily supplementation with 200 mg/kg of cranberry extract for eight w noticeably increased the proportion of the mucin-degrading bacterium *Akkermansia* [131]. Similarly, in another study high-fat-fed mice supplemented with 4% w/w powered green tea leaves high in flavanols has also been recently associated with an increase in the proportion of *Akkermansia* after 22 w [132]. According to the literature, *Akkermansia* administration as a probiotic was reported to reduce systemic LPS levels in high-fat-fed mice, which is possibly associated to the ability of *Akkermansia* to preserve the mucus layer thickness, therefore reducing gut permeability and LPS leakage [134]. Therefore, the results discussed previously might suggest another possible modulatory pathway of intestinal barrier integrity by flavonoids, resulting from their

demonstrated effect on *Akkermansia* abundance, in a dysbiosis situation, through the preservation of the mucus layer thickness, although further studies are required in order to confirm this issue.

In vitro studies have focused on evaluating the effect of flavonoids on the growth pattern of intestinal bacteria as an approach to understanding the role of these phytochemicals in the gut microbiota. Lee *et al*. [18] assessed the influence of the phenolic components of a tea extract, rich in catechin and epicatechin, and their aromatic metabolites upon the growth of common pathogenic, commensal and probiotic intestinal bacteria as representative intestinal microflora. Tea phenolics and their derivatives significantly repressed the growth of specific pathogenic bacteria, such as *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides spp* [18]. Furthermore, Parkar *et al*. [19] also tested the effect of the most representative dietary flavonoids on growth of probiotic (*Lactobacillus rhamnosus*), a commensal (*Escherichia coli*) and two pathogenic bacteria (*Staphylococcus aureus*, *Salmonella typhimurium*), together with their effects on adhesion of pathogenic and probiotic bacteria to cultured Caco-2 cells. The incubation with catechin, epicatechin, naringenin, diadzein, genistein or quercetin affected the viability of representative gut flora *in vitro*, at doses likely to be present in the gastrointestinal tract. In addition, naringenin showed an effective inhibition of *S. typhimurium* adherence to Caco-2 enterocytes [19].

In accordance with the literature, the proportion of Bacteroidetes to Firmicutes is altered in obese individuals, which produces signals that control gene expression in epithelial intestinal cells [10, 38, 135]. In addition, the metabolism of flavonoids by gut microbiota includes the cleavage of glycosidic linkages, which generates different products, such as glycans, that are necessary for the survival of the intestinal microbiota. The Firmicutes family possess fewer glycan-degrading enzymes than Bacteroidetes and is more

repressed by antimicrobial effects of flavonoid compounds than the Bacteroidete*s* family. Instead, the Bacteroidete*s* family prevail following dietary flavonoid intake, and the flavonoids are fermented to phenolic compounds due to the presence of more glycan-degrading enzymes [136]. Therefore, taken into account the previous evidences, the prebiotic power of dietary flavonoids could be a possible mechanism by which these phytochemicals substances exert their beneficial effects.

Oligomeric and polymeric forms of flavonoids are metabolized by the intestinal microbiota into various phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives [137]. It has been reported that these metabolites may modulate the growth of bacteria in the gut microbial milieu. As an approach towards the evaluation of their effect in the gut, Cueva *et al*. assessed the antimicrobial activity of different phenolic acids against different commensal, probiotic and pathogenic bacteria [133]. Some phenolic acids demonstrated an inhibitory effect in the growth of *E. coli* ATCC 25922, a non-pathogenic strain, at a concentration of 1000 μg/mL, as well as, in the growth of lactobacilli (*Lactobacillus paraplantarum* LCH7, *Lactobacillus plantarum* LCH17, *Lactobacillus fermentum* LPH1, *L. fermentum* CECT 5716, *Lactobacillus brevis* LCH23, and *Lactobacillus coryniformis* CECT 5711) and pathogens (*S. aureus* EP167 and *C. albicans* MY1055) [133]. Recently, it has been reported that these metabolites may also exert several biological activities, such as the inhibition of platelet aggregation and activation function [138], inhibition of COX-2 in HT-29 colon cancer cells [139], reduction in the synthesis of prostanoids in colon cells [140], anti-proliferative activity in prostate and cancer cells [141] and, finally, influence cell proliferation, apoptosis and signalling pathways in human colon carcinoma cells [142].

To sum up, although there are few studies regarding the effects of flavonoid consumption on the gut microbiota composition, results in this field seem to

indicate that the effects of flavonoids in human health depend, to a large degree, on their transformation by the gut microbiota. In turn, flavonoids and their metabolites contribute to the maintenance of gut health, inducing the growth of beneficial bacteria and inhibiting the growth of pathogen species. However, the mechanisms involved in this two-way relationship remain to be clearly elucidated.

Conclusions

Flavonoids are a large and diverse group of natural compounds of which only a few have been evaluated regarding their effect on intestinal alterations. The strongest conclusion that can be drawn from the revision of the current literature is that some flavonoids are able to reduce the intestinal inflammatory processes targeting the TLR4/ NF-κB pathway. Although there are few studies regarding the flavonoid effects on intestinal permeability, most of them point out that flavonoids are able to protect barrier integrity by primarily acting on TJ stability. Finally, the review of the literature on the effects of flavonoid consumption on the gut microbiota populations suggests that flavonoids may modulate the microbiota composition by means of prebiotic and antimicrobial properties. However, the mechanisms involved are still poorly understood.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Authorship

Both K. G.-C. and I. G. initiated the literature search, were in charge of drafting the manuscript and designed the figures. Both K. G.-C. and I. G. contributed equally to all parts of the paper. M.P., A.A. and M.B. revised the first drafts. X.T. was responsible for final editing and was responsible for final content. All authors critically reviewed the manuscript and approved the final version.

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4.3. Annex 1

Table 3. Publications from 2015 to 2017 evaluating the effects of flavonoids on intestinal inflammation, barrier function and microbiota *in vivo* and *in vitro.*

Flavonoid	Concentration	Cell type or animal model	Induction of disruption	Effect	Reference
In vivo					
Anthocyanin-rich fraction from blueberries (anthocyanidins)	*10 mg/kg, 8 d*	Male Wistar rats	10 mg of TNBS, single rectal administration	Inhibition of MPO activity and COX-2 and iNOS expression and increased glutathione peroxidase activity i in the colon	Pereira *et al*., 2017 [344]
Apple polymeric procyanidins (flavan-3-ols)	*Diet containing a 0.5%, 20 w*	Male C57BL/6J mice	HF diet, 20 w	Attenuation of plasma LPS levels, up-regulation of TJ proteins (ZO-1 and occludin) and down-regulation of TNF-α expression in the ileum. Increased levels of *Akkermansia* and a decrease in the *Firmicutes* to *Bacteroidetes* ratio	Masumoto *et al*., 2016 [345]
(-)-Epigallocatechin-3-gallate (flavan-3-ols)	*3.2 mg/g, 3 d*	Male CF-1 mice	1.5 % w/v DSS, 7 d	Reductions in IL-1, IL-6, TNF-α and MCP-1 levels in the colon and *in vivo* intestinal permeability (lactulose/rhamose ratio)	Bitzer *et al*., 2016 [346]
Grape pomace extract (anthocyanidins,	*Diet containing 1% of the extract,*	Male C57BL/6J mice	HF diet, 13 w	Attenuation of plasma LPS levels, reduction in the expression of intestinal inflammatory markers (TNF-α, IL-6 and iNOS) and up-regulation of TJs protein	Roopchand *et al*., 2015 [347]

flavan-3-ols and flavonols)	*13 w*			expression. Increased levels of *Akkermansia muciniphila* and a decrease in the *Firmicutes* to *Bacteroidetes* ratio	
Cranberry extract (flavonols, flavan-3-ols and anthocyanidins)	200 mg/kg, 8 w	C57BL/6J mice	HF/HC diet, 8 w	Attenuation of plasma LPS levels and intestinal oxidative stress and an increase in *Akkermansia* levels	Anhê *et al.*, 2015 [348]
Quercetin (flavonol)	30 mg/kg BW/day, 6w	Wistar rats	HF/HC diet, 6 w	Increased expression of the IL-18 gene in the colon. Reduction in the *Firmicutes* to *Bacteroidetes* ratio and inhibition of *Erysipelotrichaceae*, *Bacillus, Eubacterium cylindroides*	Etxeberria *et al.*, 2015 [349]
In vitro					
Cyanidin-3-O-glucoside (anthocyanidins)	20 and 40 μM, 24 h	Caco-2 cells	TNF-α (50 ng/ml), 3h 6 h	Inhibition of NF-kB transcriptional activity Inhibition of IL-6 expression	Ferrari *et al.*, 2016 [350]
(-)-Epigallocatechin-3-gallate (flavan-3-ols)	10 and 50 μM, 48 h	Caco-2 cells	DSS (2%), 48 h	Inhibition of intestinal permeability (FITC-dextran) *in vitro*	Bitzer *et al.*, 2016 [346]
Apigenin (flavone)	25 μM, 1h	Nontransformed porcine intestinal epithelial cell line IPEC-J2	LPS 10 μg/mL, 1h	Inhibition of the expression of the IL-6, IL-8 and COX-2 genes	Farkas *et al.*, 2015 [351]

(-)-Epicatechin (flavan-3-ols)	*0.05-5 μM, 6h*	Caco-2 cells	TNF-α (20 ng/ml),3h	Inhibition of NF-kB activation	Contreras *et al.*, 2015 [352]
			24h	Prevention of increase in the levels of the MLCK and ZO-1 proteins	
			48h	Prevention of the cellular redistribution of ZO-1 and increased permeability	

II. HYPOTHESIS AND OBJECTIVES

1. Hypothesis and objectives

Flavonoids are plant-derived bioactive compounds that are present in the human diet, and exert a biological response in our body. Amongst flavonoids, proanthocyanidins have demonstrated numerous beneficial properties in health [353]. In this sense, the consumption of a grape seed proanthocyanidin extract (GSPE) has clear anti-inflammatory properties, which have primarily been evaluated in non-intestinal tissues [354, 355]. In addition the same extract has been shown to protect against metabolic diseases, including obesity [356], resulting interesting from a therapeutic nutritional perspective. Another important point is that the behaviour of these compounds is linked to their bioavailability, intestinal absorption and metabolism in the GI tract [332]. Thus, proanthocyanidins appear to be promising candidates to protect intestinal health.

According to our review, evidences on the protective role of flavonoids against intestinal inflammation and permeability can be found in the biomedical literature [357]. However, most of the studies that evaluated proanthocyanidins and intestinal alterations were limited to *in vitro* models [346, 350–352, 358–360], and the *in vivo* studies were performed in chemical animal models of IBD [344, 346, 361, 362].

On the other hand, as we mention in the **Annex 1**, in the last three years some studies have evaluated the role of these substances in animal models of intestinal dysfunction induced by obesity and diet, but to date all of these studies have been developed in HF-fed animals and not in other DIO models, such as CAF diet, which mimetic more accurately obesity and other metabolic alterations developed in the Westerns societies.

Based on these recent findings and the potential of proanthocyanidins, we hypothesised that ***the consumption of a grape seed proanthocyanidin extract***

(GSPE) influences on intestinal health, protecting against intestinal inflammation and impaired barrier function in vivo. Therefore, the main objective of this thesis was to ***elucidate whether the administration of GSPE can modulate the intestinal inflammatory response and permeability in two different rat models of intestinal dysfunction.***

Different specific objectives were established to reach this main aim:

1. Since intestinal dysfunction features have been observed in an obesity context, and considering that some components of the Western diet have a negative impact on intestinal homeostasis, firstly we wanted to ***examine the impact of the CAF diet on intestinal health status over time, and to compare it with a genetic model of obesity, in order to define a chronic robust model of intestinal dysfunction associated with obesity in rats.***

To achieve this objective, obesity was induced in healthy female Wistar rats after a chronic CAF diet administration for 12, 14.5 or 17 weeks. We focused on examining the effect of the diet intervention on morphometric variables, inflammatory response, TJs and oxidative state in the intestine. Additionally, these effects were compared with genetic obese fa/fa Zucker rats fed a standard diet for 10 weeks, with the aim of distinguishing between effects derived from the genetic background or the diet.

2. Once we established the weeks of chronic overfeeding required to obtaining a solid model of intestinal dysfunction, we secondly wanted to ***evaluate whether a corrective treatment with dietary doses of GSPE had a beneficial effect on obesity-associated intestinal alterations in response to the CAF diet.***

In order to obtain an advanced stage of obesity, healthy female Wistar rats were fed a CAF diet for 18 weeks, and the last three weeks they were also supplemented with GSPE at three dietary doses as a corrective treatment with

proanthocyanidins. Finally, we checked the effect of the extract on inflammatory response, oxidative stress markers and TJs in the intestine.

3. The protective effect that the dietary doses of GSPE administered in a corrective way have shown protecting TJs alterations suggests a subtle modulation of barrier function by these bioactive compounds at these doses, and it leads to the possibility of trying new doses and treatments. To study it in more depth, we decided to ***analyse the effect of a pharmacological dose of GSPE in the modulation of barrier disruption induced by CAF diet, and to compare the effectiveness of this dose administered at different time points of the experiment.***

For this purpose, female healthy Wistar rats were fed with the CAF diet during 17 weeks, and evaluated the effect of a preventive and an intermittent treatment with a pharmacological dose of GSPE on the modulation of intestinal barrier function, measuring *in vivo* and *ex vivo* markers of intestinal permeability and metabolic endotoxemia.

4. After confirming the effect of proanthocyanidins on intestinal health as anti-inflammatory agents and modulators of barrier function in the DIO phenotype, we decided to move to other scenery of intestinal dysfunction. Taking into account that the exposition of low doses of LPS causes intestinal alterations similar to those occurring in obesity, our final objective was *to* ***evaluate the preventive effects of GSPE in a rat model of acute intestinal inflammation and impaired intestinal permeability induced by LPS injection.***

For this purpose, we analysed intestinal inflammatory and permeability markers in male Wistar rats supplemented with GSPE before the IP injection of LPS to induce intestinal dysfunction.

III. RESULTS

1. MANUSCRIPT 2

Results

A cafeteria diet triggers intestinal inflammation and oxidative stress in obese rats

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A cafeteria diet triggers intestinal inflammation and oxidative stress in obese rats

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Abstract

Background: The gastrointestinal alterations associated with the consumption of an obesogenic diet, such as inflammation, permeability impairment and oxidative stress, have been poorly explored in both diet-induced obesity (DIO) and genetic obesity.

Objective: To examine the impact of an obesogenic diet on the gut health status of DIO rats in comparison to the Zucker (fa/fa) rat leptin receptor–deficient model of genetic obesity over time.

Design and Methods: Female Wistar rats (n=48) were administered a standard or a cafeteria (CAF) diet for 12, 14.5 or 17 weeks and were compared with *fa/fa* Zucker rats fed a standard diet for 10 weeks. Morphometric variables, plasma biochemical parameters, myeloperoxidase (MPO) activity and reactive oxygen species (ROS) levels in the ileum were assessed, as was the expression of pro-inflammatory genes (TNF-α, and inducible nitric oxide synthase (iNOS)) and intestinal permeability genes (zonula occludens-1 (ZO-1), claudin-1 and occludin).

Results: Both the nutritional and the genetic obesity model showed increased body weight and metabolic alterations at the final time point. An increase in

intestinal ROS production and MPO activity was observed in the gastrointestinal tract of rats fed a CAF diet but not in the genetic obesity model. TNF-α was overexpressed in the ileum of both CAF diet and *fa/fa* groups, and ileal inflammation was associated with the degree of obesity and metabolic alterations. Interestingly, the 17-week CAF group and the *fa/fa* rats exhibited alterations in the expression of permeability genes.

Conclusion: Relevantly, in the hyperlipidic refined sugar diet model of obesity, the response to chronic energy overload led to time-dependent increases in gut inflammation and oxidative stress and changes in intestinal permeability genes.

Keywords: Gut, inflammation, obesity, cafeteria diet, oxidative stress.

Introduction

The regular consumption of a high-fat/high-refined carbohydrate diet such as a typical cafeteria (CAF) diet contributes to hyperphagia and obesity as well as other detrimental health consequences linked to obesity, such as insulin resistance and low grade inflammation [1, 2]. The hyperphagic and obesogenic effect of a CAF diet is produced via induction of a number of different mechanisms [3]. High-fat meals can stimulate innate immune cells and lead to a transient postprandial inflammatory response, altering our immune system and subsequently our inflammatory status [4]. Previous studies have pointed to the adipose tissue together with the liver as the main sources and focus of inflammation in obesity [5–8]. In addition, the gastrointestinal (GI) tract is another potential target for treating diet-associated inflammation. Among its various functions, the GI tract has the responsibility of informing and protecting the body from diverse chemical structures and metabolizing the nutrients required for energy and the plastic functions of human beings [9]. In addition,

the intestinal epithelium has a conditioning effect on gut homeostasis, regulating the mucosal immune response and the metabolic activity of the intestine and the entire organism [10]. The impact of various dietary constituents including fats and carbohydrates on the GI tract and the microbiota, and subsequent health outcomes in the host, is another exciting and novel area of inquiry. The currently existing knowledge regarding the cellular effects that nutrients exert through the gut supports the possibility of deregulation of gut homeostasis by obesogenic diets [11]. High-fat/high-refined carbohydrate diets have been linked to a pro-inflammatory profile, although this has been poorly studied in the GI tract. Intestinal inflammation is associated with increased myeloperoxidase (MPO) enzyme activity in the ileum; this enzyme is a key component of the O_2-dependent microbial activity of phagocytes that, in turn, induces polymorphonuclear neutrophil and macrophage infiltration and results in the production of high levels of pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α) [11]. Cell oxidative stress is another event that is closely linked to tissue inflammation. Nutritional, or dietary, oxidative stress denotes a disturbance in the redox state resulting from excess oxidative load or inadequate nutrient supply, favouring pro-oxidant reactions [12]. Reactive oxygen species (ROS) production by immune and non-immune cells in response to an obesogenic diet could be a primary event in the intestinal inflammation associated with obesity [13]. Among all genes, the expression of the inducible isoform of nitric oxide synthase (iNOS) been widely studied in relation to intestinal inflammation [14, 15]. During the last decade, it has become increasingly clear that nitric oxide (NO) overproduction by iNOS is deleterious to intestinal function [16], thus contributing significantly to GI immunopathology. In addition, gut dysfunction in obesity is also associated with alterations in intestinal permeability. The paracellular pathway is one of the main pathways mediating the transmembrane transfer of specific intestinal components and is perturbed

upon disruption of the intestinal barrier. The paracellular pathway is integrated by tight junctions (TJs), a cluster of junctional proteins that selectively regulate the transport of ions, solutes, and peptides from the lumen to the intestinal mucosa and bloodstream. Occludin (OCLN) and claudin family members and junctional adhesion molecules are linked to the actin cytoskeleton by cytoplasmic rafts formed by zonulin/zonula occludens (ZO)-1,-2, and -3 [17]. During inflammation, persistent high circulating levels of inflammatory cytokines, which are often observed in obese patients, may cause impairment of intestinal barrier function by altering the structure and localization of TJs [18, 19]. Altogether, this induces an increase in intestinal permeability, which leads to the leakage of water and proteins into the lumen and to translocation of intraluminal solutes such as bacterial endotoxins into the systemic circulation [20]. However, a new path regulating the structure and function of the intestinal epithelial barrier through the binding of the adipocyte-derived hormone leptin to its long form leptin receptor (LepR) was recently identified [21–23]. Thus, the disruption of TJ-mediated paracellular transport and the overproduction of ROS, as well as alterations in the immune and inflammatory responses and leptin signalling pathways, could be relevant to the obesity phenotype and have not been fully explored in the context of the intestine.

Taking all this into consideration, we hypothesized that the CAF diet, as a model diet induced obesity (DIO), could alter intestinal function, and not only cause an increase in nutrient absorption but also activate the inflammatory and oxidative stress responses in the gut and alter TJs. We also hypothesized that these changes would be proportional to the length of nutritional intervention, with different degrees of intestinal inflammation induced by the CAF diet. Such effects were compared with a genetic model of obesity, *fa/fa* rats, which develop increased adiposity, hyperphagia, hyperinsulinaemia, hyperlipidaemia, and multiple endocrine abnormalities early in life.

The aim of this study was to examine the impact of the CAF diet on gut health and the time course of these changes in healthy Wistar rats in addition to comparing these changes with LepR–deficient obese (*fa/fa*) Zucker rats as a genetic obesity (GO) model. Specifically, we aimed to evaluate the time course of the alterations that occur during DIO development: obesity degree and metabolic alterations with respect to gut dysfunction, namely, inflammation, oxidative stress and TJs alterations in dietary intervention *versus* GO.

Methods

Experimental animal procedures

A total of forty-eight female Wistar rats weighing 230 g were purchased from Charles River Laboratories (Barcelona, Spain) and were housed in animal quarters at 22 °C with a 12 h light/12 h dark cycle (light from 08.00 to 20.00 hours). After one week of adaptation, the animals (n = 6-8 per group) were randomly selected to receive, either a standard diet (Panlab A-04, Barcelona, Spain) (CONTROL group) or a standard diet plus a CAF diet (CAF group) for 12, 14.5 or 17 weeks as a model of a high-fat/high-refined carbohydrate diet. Animals were fed ad libitum with fresh food daily. The CAF intervention consisted of bacon, sweets, biscuits with paté, cheese, muffins, carrots, and sugared milk, with an overall content of 62.2% carbohydrate, mostly in the form of simple sugars, 23% lipid and 12,8% protein with a mineral-vitamin content of 2 g/kg of diet [24]. In addition, a GO study was performed with obese (*fa/fa*) female Zucker rats (n = 10) and heterozygous (*Fa/fa*) lean counterparts (n = 10) with an initial weight of 210 g; these animals received a standard diet for 10 weeks.

At the end of each study, after an overnight fasting, animals were anesthetized with 50 mg/kg of sodium pentobarbital and killed. Heparinized blood was collected and plasma was obtained by centrifugation. The intestine and the different white adipose tissue depots (retroperitoneal (RWAT), mesenteric

(MWAT) and periovaric (PWAT)) were rapidly removed, weighed, frozen in liquid N_2 and stored at −80 °C until later analysis. All 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 procedures (permission no. 4250).

Morphometric variables

Body weight was monitored weekly, and the percentage of weight gain was calculated. Adiposity was determined with an adiposity index, which was computed for each rat as previously described (24). These variables together with the MWAT depot weight were evaluated as physiological indicators of the degree of obesity in these animals. Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) was quantified for each animal by using the glucose and insulin levels of each animal.

Biochemical assays

Plasma glucose and triglycerides (TAG) were measured with enzymatic colorimetric kits (QCA, Barcelona, Spain), and insulin levels were analysed with a rat insulin ELISA kit (Mercodia, Uppsala, Sweden). The manufacturer's protocol was followed in all cases.

Quantification of myeloperoxidase activity and reactive oxygen species in the ileum

Tissue samples were homogenized with a TissueLyser LT system (Qiagen, Hilden, Germany) in a 50-mM-potassium phosphate buffer (Panreac, Barcelona, Spain). An aliquot of the homogenate was stored for the subsequent measurement of ROS. The rest of the homogenate was centrifuged at 15000 x g for 15 min at 4 °C, and the resulting supernatant was discarded. The pellet was then homogenized with hexadecyltrimethylammonium bromide (Sigma-Aldrich, Madrid, Spain) and 50-mM-potassium phosphate buffer. The

homogenate was sonicated (20 seconds), subjected to three freeze-thaw cycles and centrifuged at 15000 x g for 10 minutes at 4 °C. For MPO activity determination, we used an adaptation of the Lenoir method [25]. The supernatant was mixed into a solution of phosphate buffer, 0.22% guaiacol (Sigma-Aldrich, Madrid, Spain) and 0.3% H_2O_2 (Sigma-Aldrich, Madrid, Spain), and absorbance was read at 470 nm. Enzyme activity was defined as the amount of MPO needed to degrade 1 µmol of H_2O_2 in 1 minute (U= µmol/minute) and was normalized to milligram of total protein content, which was measured using the Bradford method (Sigma-Aldrich, Madrid, Spain).

For the measurement of ROS levels, samples were mixed with 1-mM-EDTA buffer (Panreac, Barcelona, Spain) and centrifuged at 3000 x g for 5 minutes at 4°C, after which the pellet was discarded. The intracellular ROS was determined using the 2',7'-dichlorofluorescein diacetate method (Sigma-Aldrich, Madrid, Spain). Fluorescence was measured at λex = 485 nm and λem = 530 nm on an FLx800 Fluorescence Reader (Bio-Tek, Winooski, Vermont, USA).

Tissue RNA extraction and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from 50 mg of ileum using Trizol (Ambion, Foster City, USA) according to the manufacturer's instructions. The gene expression analysis was completed using both Taqman probes and SYBR Green primers. When TaqMan probes were used, complementary DNA (cDNA) was obtained from 1 µg of mRNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Madrid, Spain) according to the manufacturer's instructions. Quantitative reverse transcription PCR (qRT-PCR) amplification and detection of TJ genes were completed using TaqMan Universal PCR Master Mix and the corresponding specific TaqMan probes (Applied Biosystems, Madrid, Spain): Rn02116071_s1 for rat ZO-1, Rn00581740_m1 for rat claudin-

1, and Rn01420322_g1 for rat OCLN. All the results were normalized to cyclophilin E (PPIA) (Rn00690933_m1).

For the other 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 were as follows: TNF-α, Fw: 5-CCTCACACTCAGATCATCTTCTC-3, Rv: 5-TTGGTGGTTTGCTACGACGTG-3; F4/80 (EGF-like module-containing mucin-like hormone receptor-like 1 o Em1) Fw: 5-CTTTGGCTATGGGCTCCCAGTC-3, Rv: 5-GCAAGGAGGGCAGAGTTGATCGTG-3; iNOS, Fw: 5-CACCCGAGATGGTCAGGG-3, Rv: 5-CCACTGACACTCCGCACAA-3; PPIA, Fw: 5-CTTCGAGCTGTTTGCAGACAA-3, Rv: 5-AAGTCACCACCCTGGCACATG-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 $2^{-\Delta\Delta Ct}$ method, where $\Delta C_t = C_t$ 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 power analysis was carried out *a priori* during the design stage of the study. For a Student's *t* test to compare 2 groups of experimental animals, we calculated the sample size needed in each group to obtain a power of 0.80, when the effect size is moderate (0.3) and a significance level of 0.05 is used. The resulting sample size was 6 to 8 rats depending on the variables analysed.

Results are expressed as means mean with the standard errors of the mean (SEM). Effects were assessed using Student's *t* test to compare either control *versus* CAF groups for each CAF diet intervention or obese *fa/fa versus Fa/fa* for the genetic study. P-values < 0.05 were considered statistically significant. These calculations were performed using IBM-SPSS 22.0 software.

Pearson's correlation coefficient was used to test for correlations between the variables evaluated. Unsupervised Principal Component Analysis (PCA) was performed with XLSTAT 2015.5 (Addinsoft) to assess relationships between the expression of inflammatory marker, TJ proteins, oxidative stress genes in the ileum, and morphometric and metabolic variables. Variables included in the PCA were selected to obtain a Kaiser-Meyer-Olkin (KMO) index > 0.5 as a measure of sampling adequacy. After data scaling, the analysis was based on the correlation matrix, and principal components (PC) were considered significant if they contributed more than 5% to the total variance.

Results

Increased body weight and metabolic alterations are observed in nutritional and genetic obesity models

After 12, 14.5 or 17 w of consuming the CAF diet, rats showed statistically significant increases in body weight relative to controls fed a standard diet. All the CAF groups also presented a significantly higher percentage of body weight gain **(Figure 1)**, adiposity and mesenteric fat mass compared with the control animals, with this increase being more dramatic at 17 weeks **(Table 1)**.

Regarding the genetic model, all the previously mentioned parameters were significantly higher in *fa/fa* obese rats than the *Fa/fa* control group. The percentage of body weight gained by *fa/fa* animals after 10 weeks was higher than that gained by the CAF group after 17 weeks. In contrast, the adiposity degree was lower in *fa/fa* animals, as was mesenteric fat weight **(Table 1)**.

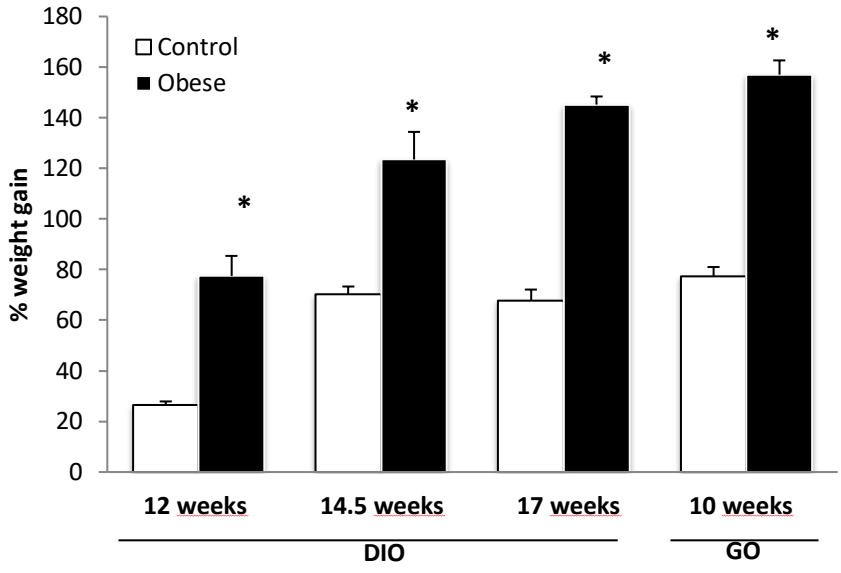


Figure 1. Weight gain during the different periods of cafeteria diet consumption and in a rat genetic model of obesity. Values are means with the SEM. n=6-10 in each experimental group. CONTROL: rats fed a standard diet or lean (*Fa/fa*) rats; OBESE: rats fed a CAF diet (DIO) or obese (*fa/fa*) rats (genetic obesity (GO)). The statistical significance of these differences was evaluated using Student's *t* test. *P values <0.05, control *versus* obese at the same time point.

To characterize the obesity models, we also evaluated the glucidic and lipidic profiles. Although all the CAF groups presented higher glucose, insulin and HOMA index levels, the differences were only statistically significant after 17 weeks of dietary intervention **(Table 1)**. Regarding the presence of hypertriacylglycerolaemia, TAG levels were higher after 12 and 14.5 weeks of CAF diet; unfortunately, we were not able to evaluate these levels at 17 weeks. All these parameters were altered in GO *fa/fa* animals, which developed a spontaneous hyperglycaemia, hyperinsulinaemia and hypertriacylglycerolaemia after 10 weeks **(Table 1)**.

Table 1. Morphometric and metabolic parameters of each experimental group.

	Diet intervention duration							
	12 weeks		14.5 weeks		17 weeks		Genetic obesity	
	Control	CAF	Control	CAF	Control	CAF	Control (*Fa/fa*)	OBESE (*fa/fa*)
Body weight (g)	236.4±2.7	332.6±15.2**	281.0±8.8	393.8±26.9*	274.5±10.0	430.2±18.0**	218.1±4.2	399.1±8.7**
Weight gain (%)	26.5±1.4	77.5±7.9**	70.2±3.1	123.5±10.9*	67.7±4.4	145.2±3.2**	77.4±3.6	157.0±5.6**
Mesenteric fat depot (g)	3.4±0.3	18.3±3.9*	4.7±0.5	15.7±3.1*	3.9±0.4	20.2±2.6*	1.8±0.1	7.1±0.3**
Adiposity (%)	4.7±0.4	14.4±1.4**	7.0±0.9	14.5±0.9**	6.4±0.4	15.7±0.7**	2.7±0.2	6.3±0.2**
Insulin (mU/L)	27.8±7.6	63.2±16.4	23.9±5.2	56.7±21.9	25.8±5.3	134.4±19.8*	24.2±4.1	263.3±23.9**
Glucose (mM)	7.1±0.9	8.8±2.1	8.0±0.7	7.5±4.5	7.0±0.5	8.6±0.9*	7.8±2.6	9.7±1.5
HOMA-IR	9.0±2.7	20.7±6.5	8.7±2.2	19.6±7.8	8.0±1.6	52.1±8.6*	8.7±1.8	111.0±7.3**
TAG (mM)	0.4±0.1	1.0±0.4*	1.2±0.3	2.2±0.4*	NA	NA	1.4±0.9	3.7±0.6**

Intestinal reactive oxygen species production and myeloperoxidase activity increased after consumption of a cafeteria diet but not in genetic obesity

We measured ROS levels and MPO activity in the ileum to evaluate the intestinal damage after the nutritional intervention and in the genetic model. As shown in **Figure 2A**, ROS levels increased in accordance with the duration of the dietary intervention, becoming statistically significant at 17 weeks. Notably, the enhancement in MPO activity followed the same pattern and was significantly increased in all CAF groups compared with the controls at each time point **(Figure 2B)**. However, no differences were observed when we measured these parameters in genetically obese rats.

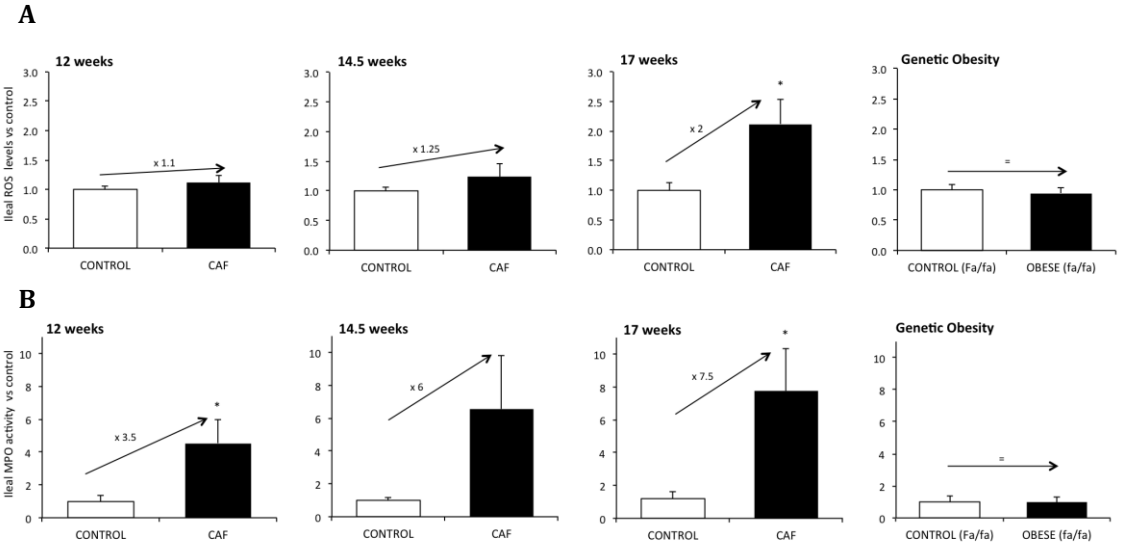


Figure 2. Ileal reactive oxygen species (ROS) levels (A) and MPO activity (B) during the different periods of cafeteria or standard diet consumption and in the rat genetic model of obesity. Values are means with the SEM. n = 6-10 in each experimental group. CONTROL: rats fed a standard diet or lean (Fa/fa) rats; CAF: rats fed a cafeteria diet (diet induced obesity (DIO)) and; OBESE: obese (fa/fa) rats (genetic obesity (GO)). The statistical significance of these differences was evaluated using Student's *t* test. **P* values <0.05, control *versus* CAF or obese at the same time point.

Ileal reactive oxygen species levels and myeloperoxidase activity correlate with morphometric and metabolic characteristics after 17 weeks of cafeteria diet consumption

The Pearson correlation test was used to evaluate for potential associations between ileal ROS or MPO and body weight measures in control and CAF groups. Ileal ROS levels and MPO activity exhibited strong and significant correlations with adiposity, weight gain and mesenteric fat weight in the 17 weeks group of animals. In addition, MPO activity was also strongly correlated with body weight after 17 weeks **(Table 2)**. Similar results were obtained when we analysed the relationship between ileal ROS and MPO and the metabolic parameters evaluated. As shown in **Table 2**, some statistically significant correlations were observed after 17 weeks of dietary intervention and in controls, with ROS levels and MPO activity showing positive associations with insulin resistance indicators. However, no correlations between ROS or MPO and morphometric and metabolic parameters were observed in the genetic model **(Table 2)**.

TNF-α is over-expressed in the ileum of cafeteria diet and fa/fa groups

To evaluate the inflammatory state in the ileum, we analysed the expressions of the pro-inflammatory markers TNF-α and iNOS and the expression of F4/80, a murine macrophage infiltration marker. **Figure 3** shows the expression of these genes in the rat ileum after 14.5 and 17 weeks of the CAF diet. The expression of these markers increased over both time periods, with statistical significance only being reached for TNF-α after 14.5 weeks **(Figure 3B)**. Furthermore, TNF-α and iNOS expression were positively correlated (r=0.499, *P*=0.030). TNF-α expression was also correlated with the macrophage marker F4/80 (r=0.491, *P*=0.028), and iNOS expression also appeared to correlate with the levels of this marker (r=0.417, *P* =0.068). Interestingly, TNF-α expression

was significantly increased in the *fa/fa* group **(Figure 3B)**, but no changes were observed in the rest of genes.

Table 2. Correlation coefficients for ileal reactive oxygen species (ROS) levels or myeloperoxidase (MPO) activity and morphometric characteristics in the control and cafeteria groups at different time points and in the genetic obesity (GO) model.

	Diet intervention duration			GO
	12 week	**14.5 week**	**17 week**	**10 week**
ROS levels				
Body weight (g)	0.277	0.620	0.570	- 0.141
Weight gain (%)	0.320	0.139	**0.592***	- 0.014
Mesenteric fat depot (g)	0.304	**0.795***	**0.688***	- 0.194
Adiposity (%)	0.381	0.472	**0.662***	- 0.145
MPO activity				
Body weight (g)	0.344	0.679	**0.798***	0.042
Weight gain (%)	0.350	0.295	**0.740***	0.078
Mesenteric fat depot (g)	0.174	0.605	**0.812***	- 0.117
Adiposity (%)	0.501	0.437	**0.778***	- 0.085
ROS levels				
Insulin (mU/L)	0.275	0.504	**0.649***	-0.159
Glucose (mM)	0.030	0.147	0.413	0.228
HOMA-IR	0.163	0.531	**0.603***	-0.122
TAG (mM)	0.267	0.284	NA	-0.100
MPO activity				
Insulin (mU/L)	-0.036	0.437	**0.757***	-0.178
Glucose (mM)	-0.010	0.169	**0.814***	0.403
HOMA-IR	-0.042	0.425	**0.792***	-0.099
TAG (mM)	0.507	0.667	NA	0.129

HOMA-IR, homeostasis model assessment of insulin resistance; NA, not avaialable. **P* values < 0.05 were considered statistically significant. The number of animals (n = 12-20) used in each time period in the control and CAF groups together is shown.

Ileal inflammation is associated with degree of obesity and metabolic alterations

As inflammation has been linked to obesity and associated insulin resistance, we tested for correlations between ileal TNF-α, iNOS and F4/80 expressions and the metabolic and morphometric characteristics measured over two time periods, 14.5 and 17 weeks.

As shown in **Table 3**, at 14.5 weeks TNF-α expression was positively associated with measures of body weight and adiposity, as well as with TAG and insulin levels. Importantly, iNOS expression was also correlated with glucose metabolism parameters and TAG levels. F4/80 also correlated positively with TAG levels at 14.5 weeks. At 17 weeks of diet intervention **(Table 3)**, TNF-α expression was positively associated with adiposity, and iNOS expression correlated positively with glucose levels.

On the other hand, as shown in the **Table 3**, TNF-α expression in *fa/fa* obese animals was positively associated with the following morphometric parameters: body weight, mesenteric fat depot and adiposity, and TAG.

The main correlations found with dietary intervention periods of 14.5 and 17 weeks were maintained even after adjustment for body weight **(**see **Supplementary Table 1)**. In the GO model, when body weight adjustment was applied, we found positive associations of macrophage infiltration (F4/80 expression) with adiposity. Also, the correlations found with TAG levels persisted. However, other associations were not present after body weight adjustment **(Supplementary Table 1)**.

Tight junctions gene expression is altered in the 17-week cafeteria diet and fa/fa groups

The expression of TJ element genes was evaluated as an approach to measure ileal permeability. The results indicate that the expression of ZO-1 and claudin-1 were strongly reduced after 17 weeks of the CAF diet **(Figure 3D** and **3F**, respectively**)**. In addition, ZO-1 gene expression was negatively associated with insulin levels **(Table 3)**, and more interestingly, ZO-1 maintained a strong negative correlation with HOMA-IR even after body weight correction **(Supplementary Table 1)**. However, OCLN expression levels over both time periods were similar to the levels observed in control rats **(Figure 3E)**.

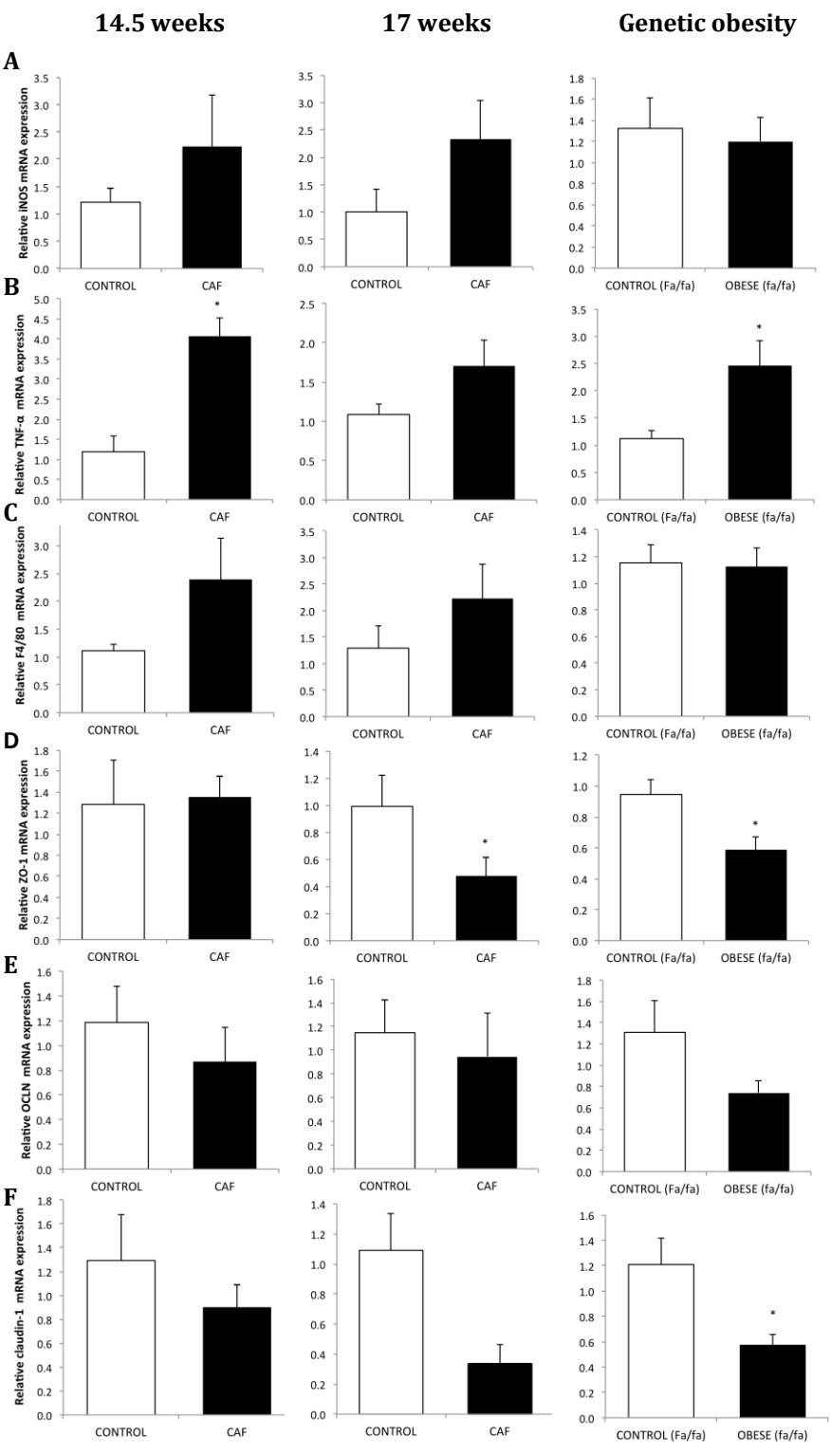

14.5 weeks
17 weeks
Genetic obesity
A
Relative iNOS mRNA expression
B
Relative TNF-α mRNA expression
C
Relative F4/80 mRNA expression
D
Relative ZO-1 mRNA expression
E
Relative OCLN mRNA expression
F
Relative claudin-1 mRNA expression
CONTROL
CAF
CONTROL (Fa/fa)
OBESE (fa/fa)
*

Figure 3. Ileal mRNA expression of isoform of nitric oxide synthase (iNOS) (A), TNF-α (B), EGF-like module-containing mucin-like hormone receptor 1 (F4/80) (C), zonula occludens-1 (ZO-1) (D), occludin (OCLN) (E) and claudin-1 (F) during the different periods of cafeteria or standard diet consumption and in the rat genetic model of obesity. Values are means with the SEM. n = 6-10 in each experimental group. CONTROL: rats fed a standard diet or lean (Fa/fa) rats; CAF: rats fed a cafeteria diet (DIO) and OBESE: obese (fa/fa) rats (genetic obesity (GO)). The statistical significance of these differences was evaluated using Student's *t* test. **P* values<0.05, control *verus* CAF or obese at the same time point.

In the case of the GO *fa/fa* group, the ZO-1 and claudin-1 genes were significantly down regulated in the ileum with respect to the *Fa/fa* control group **(Figure 3D and 3F)**, and OCLN expression showed a negative trend, as shown in **Figure 3E**. ZO-1 and claudin-1 levels were negatively correlated with morphometric parameters, HOMA index and TAG. Claudin-1 was also negatively associated with high insulin levels.

Principal Components Analysis

We used a model that reduces a matrix of data to the lowest dimension of the most significant components, or principal components, or PC, to analyse the overall distribution and associations between the variables evaluated. With this method, the PCs retained are generated sequentially, meaning the variance explained by the first component is removed before the second factor is generated to maximally explain the remaining variance in the matrix (this process is continuous with successive components). This tool is useful for identifying various combinations of variables that could reflect possible biological mechanisms, especially in association with various other health outcomes.

Table 3. Correlation coefficients for ileal gene expression and metabolic and morphometric characteristics in rats subjected to 14.5 and 17 weeks of diet intervention and in a rat genetic model of obesity and respective controls.

	Diet intervention: 14.5 weeks					
	iNOS	**TNF-α**	**F4/80**	**ZO-1**	**OCLN**	**Claudin-1**
Morphometric variables						
Body weight (g)	0.463	**0.825****	0.254	0.028	-0.415	-0.164
Weight gain (%)	0.193	**0.946****	0.478	0.244	-0.372	-0.081
Mesenteric fat depot (g)	0.304	0.697	0.152	-0.031	-0.457	-0.199
Adiposity (%)	0.384	**0.890****	0.417	0.173	-0.275	-0.182
Metabolic variables						
Insulin (mU/L)	**0.768****	**0.675***	0.192	-0.159	-0.113	-0.103
Glucose (mg/dL)	0.076	-0.183	-0.301	-0.300	0.206	-0.172
HOMA-IR	**0.749***	0.638	0.151	-0.185	-0.101	-0.131
TAG (mM)	**0.658***	**0.937****	**0.578***	0.127	-0.092	-0.162

n = 12 animals. * *P* values < 0.05 ***P* values <0,001 were considered statistically significant.

	Diet intervention: 17 weeks					
	iNOS	**TNF-α**	**F4/80**	**ZO-1**	**OCLN**	**Claudin-1**
Morphometric variables						
Body weight (g)	0.429	0.332	0.354	-0.266	-0.129	**-0.630***
Weight gain (%)	0.450	0.410	0.418	-0.283	-0.180	**-0.640***
Mesenteric fat depot (g)	0.347	0.382	0.362	-0.347	-0.078	**-0.632***
Adiposity (%)	0.428	**0.523***	0.421	-0.352	-0.142	**-0.615***
Metabolic variables						
Insulin (mU/L)	0.286	0.391	0.171	**-0.447***	-0.036	-0.575
Glucose (mM)	**0.684***	0.274	-0.056	-0.454	0.072	-0.531
HOMA-IR	0.357	0.370	0.102	-0.430	0.078	-0.559
TAG (mM)	NA	NA	NA	NA	NA	NA

n = 12 animals. **P* values< 0.05 are considered statistically significant. NA: not available.

	Genetic obesity					
	iNOS	**TNF-α**	**F4/80**	**ZO-1**	**OCLN**	**Claudin-1**
Morphometric variables						
Body weight (g)	- 0.116	**0.514***	-0.128	**-0.614***	-0.384	**-0.588***
Weight gain (%)	0.008	0.426	-0.097	**-0.529***	-0.294	**-0.580***
Mesenteric fat depot (g)	0.002	**0.511***	0.033	**-0.508***	-0.370	**-0.587***
Adiposity (%)	0.011	**0.491***	0.080	-0.465	-0.320	**-0.649***
Metabolic variables						
Insulin (mU/L)	0.075	0.410	0.104	-0.422	-0.366	**-0.521***
Glucose (mM)	-0.281	0.060	-0.367	-0.196	-0.252	-0.194
HOMA-IR	0.002	0.400	0.010	**-0.507***	-0.404	**-0.533***
TAG* (mM)	-0.176	**0.699***	-0.209	**-0.744***	-0.365	**-0.704***

n = 20 animals. * *P* values< 0.05 are considered statistically significant.

Table 4 shows the PCA results for all the variables analysed in the dietary intervention and the GO experiment. The projections in the plane of ileal gene expressions of inflammatory markers, TJ proteins, oxidative stress, and morphometric and metabolic variables defined by the first two PC of the 14.5 and 17 weeks diet intervention and GO model are shown in **Figures 4A, 4B** and **4C**, respectively (left panel). On the other hand, the distribution of animals in the space based on the different variables represented in the corresponding right panel.

The results obtained after 14.5 weeks of CAF intervention indicate that the first two PC explain 77.86% of the total variance. The first and the second PC are responsible for 45.49% and 32.37% of the total variance, respectively. Overall, PC1 was clearly characterized by the following variables: body weight and mesenteric weight, insulin levels and HOMA index, ileal MPO activity and ROS levels and ileal iNOS mRNA levels **(Table 4)**. As shown in **Figure 4a**, all these variables are grouped in the right side of the graphic, indicating a positive association between the degree of obesity in these animals and insulin pathway metabolic alterations and intestinal inflammation. On the other hand, PC2 is characterized by the percentage of adiposity, weight gain, plasma leptin levels and ileal TNF-α gene expression, which are located in the upper part of the graphic, and by the glucose levels, which are located in the lower zone. With respect to animal distribution, PC2 discriminates between the control and CAF groups. Unlike the control group, the CAF group is located on the top of the graph and shows a higher percentage of adiposity, greater weight gain, higher plasma levels of leptin and higher ileal TNF-α expression than the control group. However, PC1 does not discriminate between these groups.

In the 17-week CAF consumption group **(Table 4)**, PC1 and PC2 explain 76.56% of the total variance (43.80% and 32.76%, respectively). With respect to PC1, body weight, mesenteric weight, HOMA index, insulin, glucose and

leptin levels, MPO activity and iNOS gene expression in the ileum are negatively associated with ileal ZO-1 mRNA levels. In addition, PC2 is described by adiposity, weight gain, ROS levels, and claudin-1, TNF-α and F4/80 mRNA levels. In this context, claudin-1 expression is negatively associated with adiposity, insulin resistance and inflammatory variables. Based on the distribution of the animal groups, PC1 and PC2 discriminate between the control and CAF groups. As expected, most of the individuals in the CAF group are located in the upper right area of the space that is related to diet-induced-obesity associated metabolic alterations, intestinal inflammation and lower levels of the TJ protein **(Figure 4B)**.

Regarding GO model analysis, the first two PC explain 64.48% of the total variance. The first PC, which is responsible for 47.83%, is characterized by the morphometric variables, including body weight, mesenteric weight, weight gain, and adiposity, in addition to the following variables: insulin level, HOMA index and ileal TNF-alpha gene expression, which are all negatively associated with ileal ZO-1 and OCLN mRNA levels. In addition, PC2 is responsible for 16.65% of the total variance and is composed of ROS levels, MPO activity in the ileum and plasma glucose levels, which are negatively associated with iNOS and F4/80 mRNA levels. Moreover, PC1 discriminates between the control and CAF groups. The right panel shows that obese animals are located on the right side of the space that is defined by a higher degree of obesity, altered insulin function, disrupted inflammatory markers and the down-regulation of TJ proteins with respect to the control group **(Figure 4C)**.

Discussion

The GI tract has energetic, immune and barrier functions that serve to protect the body against infection and to absorb nutrients.

Table 4. Loadings for the first two principal components.

	Diet intervention duration				GO	
	14.5 weeks		17 weeks		10 weeks	
Variable	D1	D2	D1	D2	D1	D2
ROS	**0.605**	0.123	0.352	**0.658**	-0.225	**0.529**
Weight	**0.827**	0.536	**0.728**	0.637	**0.959**	0.142
Mesenteric weight	**0.768**	0.539	**0.722**	0.652	**0.975**	-0.050
Adiposity	0.633	**0.724**	0.688	**0.703**	**0.929**	-0.061
Insuline	**0.961**	0.036	**0.781**	0.534	**0.928**	-0.116
Glucose	0.406	**-0.779**	**0.914**	0.246	0.425	**0.655**
Leptin	0.640	**0.734**	**0.764**	0.625		
Weight gain	0.166	**0.954**	0.648	**0.691**	**0.935**	0.150
HOMA IR	**0.971**	-0.009	**0.826**	0.480	**0.958**	0.009
MPO	**0.633**	0.283	**0.835**	0.284	-0.112	**0.787**
iNOS mRNA	**0.542**	0.119	**0.612**	0.101	-0.112	**-0.453**
TNF mRNA	0.506	**0.785**	0.122	**0.630**	**0.607**	-0.144
F4/80 mRNA			-0.302	**0.900**	0.007	**-0.801**
ZO-1 mRNA			**-0.651**	0.144	**-0.673**	-0.054
Claudin-1 mRNA			-0.436	**-0.628**		
OCLN mRNA					**-0.481**	-0.266

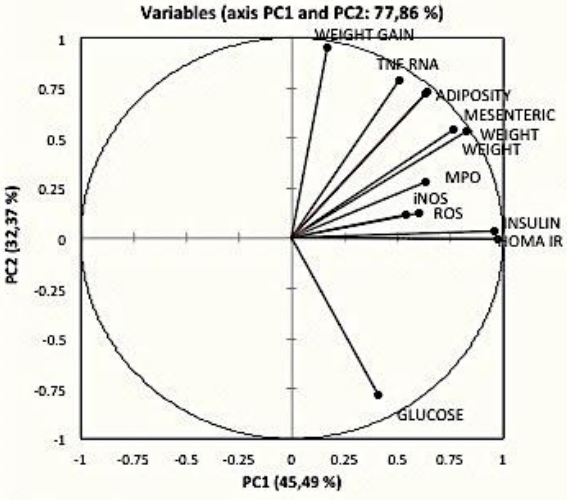
ROS, reactive oxygen species; HOMA-IR, homeostasis model assessment of insulin resistance; MPO myeloperoxidase; iNOS, isoform of nitric oxide synthase; F4/80, EGF-like module-containing mucin-like hormone receptor 1; ZO-1, zonula occludens-1; OCL, occludin.

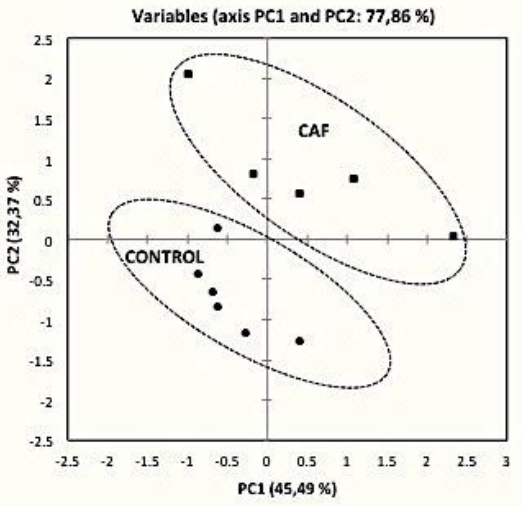
Specific parts of the GI tract, particularly the ileum of the small intestine, are specialized in both nutrient absorption and immune defence. Thus, the organs and tissues involved in the regulation of the metabolism, in addition to providing the nutrients necessary to trigger and sustain the immune response, contain resident populations of immune cells, suggesting that the immune system is poised to respond to nutrient-derived signals.

It was recently noted that the GI tract and specifically the gut is an important target organ to be considered in obesity outcome because of the effects of diet on this organ. As expected, the chronic consumption of a high-energy diet for 12, 14.5 or 17 weeks induces a time-dependent increase in body weight that is accompanied by an increase in adiposity. Insulin resistance and hypertriacylglycerolaemia, typical symptoms of the metabolic syndrome, are reached at the 17-week time point. In agreement with the literature [25], the obesity level reached in the genetic model at 10 weeks was comparable with the results obtained after 17 weeks of CAF diet consumption. In fact, Zucker obese rats became hyperglycaemic at 13–15 weeks of age [26], with hyperinsulinaemia and hypertriacylglycerolaemia appearing after 12–14 weeks of age [27], suggesting a good model of early-onset obesity.

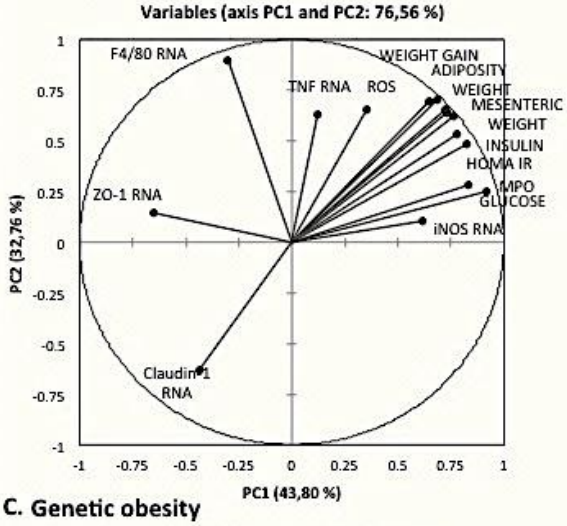
Regarding the status of the gut, our results showed that ROS levels in the ileal fraction of the small intestine were slightly increased during the first few weeks, reaching significance only at 17 weeks. Different epidemiological, animal and clinical studies have associated obesity with redox disruption [28]. A high-fat/high-refined carbohydrate diet and continuous hypernutrition can increase oxidative stress through the activation of intracellular pathways such as the nitrogen oxide pathways, oxidative phosphorylation pathway in the mitochondria, glycoxidation pathway, protein kinase C pathway and the polyol pathway [12, 29–31].

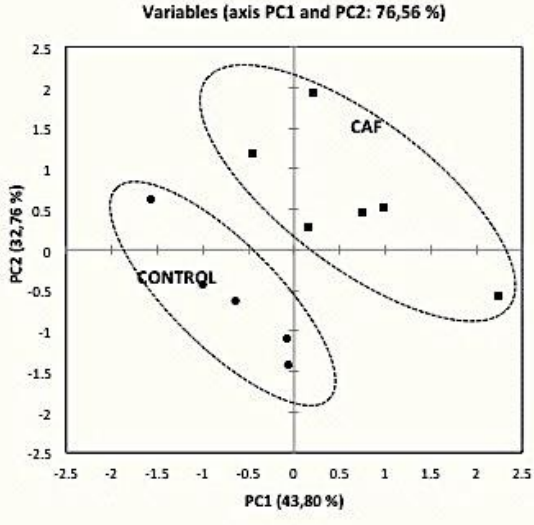
A. 14.5 weeks



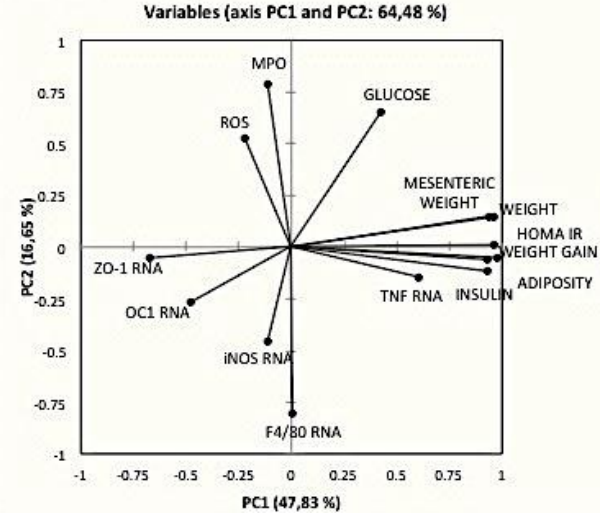


B. 17 weeks





C. Genetic obesity



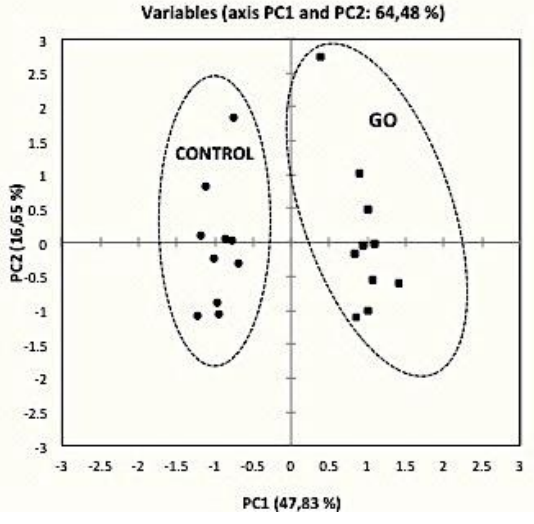


Figure 4. Principal component analysis (PCA) of all the variables analysed during the different periods of cafeteria or standard diet consumption and in the rat genetic model of obesity. The left panel represents the projection of the variables defined in the plane by the two first principal components (PC), and the right panel shows the PCA score plot of the groups analysed after 14.5 (A) or 17 weeks (B) of CAF intervention or genetic obesity (GO) (C). CAF, cafeteria diet; ROS, reactive oxygen species; MPO myeloperoxidase; iNOS, isoform of nitric oxide synthase; HOMA-IR, homeostasis model assessment of insulin resistance; F4/80, EGF-like module-containing mucin-like hormone receptor 1; ZO-1, zonula occludens-1; OCL, occludin.

However, oxidative stress can be the cause as well as the outcome of obesity. In this study we demonstrated that an obesogenic diet is able to induce oxidative stress in the ileum at later time points, suggesting that oxidative damage in this tissue is the result of a severe state of obesity. Moreover, no changes were observed in *fa/fa* Zucker rats in our study, although the degree of obesity reached is comparable with that in the 17-week CAF diet model. This fact reinforces the above idea that the intestinal oxidative stress observed in these animals is mainly associated with the ingestion of a high-fat/high-refined carbohydrate diet and not just obesity.

In contrast to oxidative stress, inflammation within the ileum, indicated by an increase in MPO activity, was apparent at the first time point and increased to a maximal level at 17 weeks. MPO is a key enzyme in the O_2-dependent microbial activity of neutrophil granulocytes, but this molecule also acts as a pro-inflammatory mediator and can cause tissue damage during acute or chronic inflammation [32]. In this context, upon detecting inflammatory signals, neutrophils change their responsiveness to allow directed migration and enhancement of microbicidal capacity. Activated polymorphonuclear neutrophils are able to synthesize and store enzymes and ROS within cytoplasmic granules [33]. The most abundant granule enzyme is MPO, which forms cytotoxic hypochlorous acid from the reaction of chloride anions with the

hydrogen peroxide produced after the respiratory burst. Beyond its bactericidal activity, MPO affects various pathways involved in cell signalling and cell-cell interactions and thus is capable of modulating inflammatory responses [34]. Thus, the increase in MPO activity found in correlation with the obesity of these animals from the first time point might indicate that inflammation precedes the oxidative stress disturbance induced by diet in the ileum. In agreement with these results, MPO activity in Zucker rats was similar to that in the lean group, a finding that is in concordance with the absence of oxidative stress in this genetic model. This fact supports the hypothesis that the CAF diet might be responsible for the observed changes in intestinal inflammation associated with obesity.

When analysing pro-inflammatory gene expression after the consumption of the CAF diet, we only found a significant increase in TNF-α levels at the 14.5 w time point; however, the other genes indicating macrophage infiltration and activity, such as F4/80 and iNOS, showed a trend of increased expression. These results again suggest that inflammation is observed from the first time point studied, whereas oxidative stress only becomes apparent at 17 weeks. Furthermore, TNF-α expression was positively associated with measures of adiposity. Importantly, iNOS expression was also correlated with glucose levels. These results are in agreement with the hypothesis that intestinal inflammation might be linked to the development of obesity-associated pathologies [35].

Immune cells, including neutrophils, have been directly implicated in disrupting TJ barrier function. It has been postulated that the pro-inflammatory cytokine-induced opening of the intestinal TJ barrier is an important mechanism contributing to the TJ barrier defects present in various inflammatory conditions of the gut [36]. The same pattern is reflected by the expression of the permeability genes ZO-1 and claudin-1, whose expression only decreases significantly after 17 weeks of the CAF diet. Thus, altered permeability is

suspected to appear at later stages, concomitant with a high degree of oxidative stress and sustained inflammation. When the correlations between these parameters were analysed, a strong positive association was found between ROS and ileal inflammatory state after 17 weeks of the CAF diet, suggesting a close link between ROS and inflammation. Moreover, the intestinal dysfunction found in the gut (oxidative stress and inflammation) in response to an obesogenic diet was correlated with the degree of obesity and insulin resistance. Taken together, our results suggest that first, the CAF diet stimulates gut inflammation and secondly, in addition to the sustained inflammatory state, there is an increase in oxidative stress and relaxation of the permeability barrier. Furthermore, this intestinal dysfunction might be linked to the development of obesity-associated pathologies.

The most surprising results obtained in this study were observed in the genetic model, in which the TJ elements ZO-1 and claudin-1 are also underexpressed. Such effects have not been described previously in this animal model. Similarly, previous investigations have demonstrated that leptin increases intestinal permeability [37, 38]. In particular, the chronic secretion of high levels of leptin by visceral adipose tissue in the context of ageing or obesity permanently impacts the structure and function of the intestinal epithelial barrier by binding LepR on basolateral side of intestinal epithelial cells and stimulating the RhoA/ROCK (Ras homolg gene family, member A/rho-kinase) pathway [37]. Upon LepR activation, the small GTPase RhoA and its effector ROCK are important modulators of actin cytoskeleton organization in response to physiological and pathological stimuli [39, 40]. Disruptions in this pathway have been demonstrated to contribute to obesity and insulin resistance in mice fed a high-fat diet. Despite the fact that obese Zucker rats display markedly elevated circulating leptin levels compared with their lean counterparts, they are LepR–deficient, meaning that the changes in intestinal permeability cannot be explained by this mechanism. Taking the fact that these animals were fed

standard chow diet into account, the modulation of intestinal permeability through the hyperphagic and obesogenic effects of the CAF diet could also be discarded. Unexpectedly, TNF-α was significantly upregulated in obese Zucker rats with respect to lean animals. Again, the pro-inflammatory cytokine-induced opening of the intestinal TJ barrier might be an important mechanism contributing to the TJ barrier defects present in these rats [36]; however, additional mechanistic studies are needed to confirm this hypothesis. Overall, the maintenance of intestinal barrier homeostasis requires complex interactions between host molecules and other environmental factors such as diet, as well as the microbiome. Commensal bacteria have also been proposed as key modulators of intestinal barrier function [41]. Other data suggest that certain bacterial species produce metabolites that can influence intestinal permeability and integrity [42]. Therefore, it could be possible that obese Zucker rats might have a different microbiome than lean animals, which would explain the changes in the expression of key genes involved in maintaining intestinal barrier integrity observed in obese animals. However, a study performed in Zucker rats found no significant differences in the intestinal bacterial profiles of the two Zucker rat genotypes at either the phylum or the family level in taxon-based analyses [43]. Thus, it is unclear which pathways could be responsible for these changes, and more studies are needed to address this topic in the future.

In summary, in high-fat/high-refined carbohydrate diet models, the deregulation of gut homeostasis in response to chronic lipid and carbohydrate overload leads to impaired metabolic homeostasis, gut inflammation, oxidative stress and changes in TJs in a time-dependent manner. Interventions that limit the intestinal alterations induced by a CAF diet may protect against obesity and insulin resistance.

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Conflict of Interest

None.

Authorship

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

Supplementary Table 1. Correlation coefficients between ileal gene expression with metabolic and morphometric characteristics in all the rats from 14.5 and 17 weeks of diet intervention and in genetic obesity rats corrected by body weight.

	Diet intervention: 14.5 weeks					
	iNOS	**TNF-α**	**F4/80**	**ZO-1**	**OC1**	**Claudin-1**
Morphometric variables						
Weight gain (%)	-0.368	**0.954***	0.426	0.297	-0.061	0.029
Mesenteric fat depot (g)	**-0.618***	-0.516	**-0.689***	-0.189	-0.048	-0.129
Adiposity (%)	-0.035	**0.603***	0.437	0.330	0.183	-0.081
Metabolic variables						
Insulin (mU/L)	**0.697***	-0.038	-0.036	-0.329	0.252	0.078
Glucose (mM)	0.263	-0.143	-0.281	-0.299	0.088	-0.201
HOMA-IR	**0.665***	-0.101	-0.98	-0.358	0.232	0.103
Triglycerides (mM)	**0.574***	**0.810***	**0.676***	0.184	**0.569***	-0.049

n = 12 animals. * *P* values< 0.05 are considered statistically significant.

	Diet intervention: 17 weeks					
	iNOS	**TNF-α**	**F4/80**	**ZO-1**	**OC1**	**Claudin-1**
Morphometric variables						
Weight gain (%)	0.150	0.316	0.278	-0.101	-0.177	-0.180
Mesenteric fat depot (g)	-0.326	0.265	0.087	-0.383	0.312	-0.092
Adiposity (%)	0.073	0.712*	0.296	-0.334	-0.060	-0.085
Metabolic variables						
Insulin (mU/L)	-0.306	0.231	-0.358	-0.527	0.386	-0.070
Glucose (mM)	**0.662***	0.010	**-0.707***	-0.440	0.343	0.021
HOMA-IR	-0.112	0.174	**-0.505***	**-0.491***	**0.486***	-0.016
Triglycerides (mM)	NA	NA	NA	NA	NA	NA

n = 12 animals. **P* values< 0.05 are considered statistically significant.

	Genetic obesity					
	iNOS	**TNF-α**	**F4/80**	**ZO-1**	**OC1**	**Claudin-1**
Morphometric variables						
Weight gain (%)	0.364	-0.202	0.077	0.210	0.230	-0.098
Mesenteric fat depot (g)	0.357	0.089	**0.487***	0.283	-0.020	-0.103
Adiposity (%)	0.260	0.085	**0.420***	0.197	0.059	-0.345
Metabolic variables						
Insulin (mU/L)	0.317	-0.036	0.399	0.199	-0.088	-0.063
Glucose (mM)	-0.257	-0.178	-0.353	0.091	-0.102	0.000
HOMA-IR	0.254	-0.168	0.305	0.183	-0.144	-0.016
Triglycerides (mM)	-0.136	**0.554***	-0.169	**-0.543***	-0.124	**-0.483***

n = 20 animals. **P* values< 0.05 are considered statistically significant.

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2. MANUSCRIPT 3

Results

Chronic supplementation with dietary proanthocyanidins protects from diet-induced intestinal alterations in obese rats

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Chronic supplementation with dietary proanthocyanidins protects from diet-induced intestinal alterations in obese rats

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Abstract

Scope: Increased attention has been paid to the link between altered intestinal function and elevated incidence of metabolic disorders, such as in obesity. This study investigated in obese rats the role of grape seed proanthocyanidin extract (GSPE) chronic treatment, taken in a low, moderate or high dose, on obesity-associated intestinal alterations in response to a cafeteria diet (CAF).

Methods and results: To evaluate the degree of intestinal inflammation, reactive oxygen species (ROS) production and myeloperoxidase (MPO) activity were measured as well as the expression of inflammatory-related genes. The barrier integrity was assessed by quantifying the gene expression of tight-junction components and measuring the plasma lipopolysaccharides. GSPE decreased the ROS levels and MPO activity, without substantial differences among the doses. The supplementation with moderate and high GSPE doses significantly decreased iNOS expression compared to the CAF group, and the same pattern

was observed in the low-dose animals with respect to IL-1β expression. Moreover, the results show that GSPE significantly increases zonulin-1 expression with respect to the CAF animals.

Conclusion: This study provides evidence for the ameliorative effect of a proanthocyanidin extract on high-fat/high-carbohydrate diet-induced intestinal alterations, specifically reducing intestinal inflammation and oxidative stress and suggesting a protection against a barrier defect.

Keywords: cafeteria diet, gut, inflammation, obesity and proanthocyanidins.

Introduction

It is now widely accepted that obesity is associated with low-grade chronic inflammation and that this situation contributes to an increased risk of insulin resistance and type 2 diabetes mellitus as well as other detrimental health consequences [1]. In this respect, the gastrointestinal tract has been recently described as a potential source of inflammation that could be associated with diet and/or obesity-related pathologies [2]. Increased attention has been paid to the link between the gut microbial composition and obesity. Components that originate from gut microbiota, such as lipopolysaccharides (LPS), can cause immune system activation [3]. Under normal conditions, the presence of this endotoxin in the intestinal lumen does not cause negative health effects. However, some factors can favour the transfer of LPS into the circulatory system and cause metabolic endotoxemia [4]. In this respect, recent studies have shown that rodents that were fed a high fat diet (HFD) display alterations in the gut microbiota that increase the intestinal permeability through gut barrier dysfunction [5]. Indeed, LPS can transfer into the circulatory system and

induce an inflammatory response, and consequently, a possible association between intestinal LPS and metabolic diseases has been suggested [6, 7].

The intestinal barrier plays an important role in protecting from foreign agents. Tight junctions (TJs) are the most important shield of the intestine for preventing the paracellular permeation of macromolecules into the circulation [8]. TJs are formed by the interaction of transmembrane proteins such as occludins [9], claudins [10], junctional adhesion molecules (JAMs) [11] and tricellulin [12], with intracellular plaque proteins zonulin/zonula occludens (ZO), including ZO-1, -2, and -3 [13]. In response to an inflammatory stimulus such as LPS, the classical NF-κB activation pathway leads to the induction of gene expression of many pro-inflammatory genes [14]. At the same time, inflammatory cytokines and bacterial antigens can affect the expression level and assembly of these proteins, therefore exerting an influence on TJ functions [15, 16].

Intestinal inflammation can be defined as the presence of cells that are involved in the immune defence system of the mucosa. In particular, the accumulation and infiltration of neutrophils, leukocytes and monocytes is a prominent feature in intestinal inflammation. It is believed that leukocytes are important sources of reactive oxygen species (ROS) in severe ulcerative colitis due to the infiltration of inflammatory cells in the intestinal mucosa [17]. Neutrophils could also be associated with colitis by increasing oxidative stress due to a rise in myeloperoxidase activity (MPO) (an index of neutrophil infiltration) in the intestinal mucosa [18]. Therefore, MPO could serve as a useful marker in the evaluation of the presence of intestinal inflammation [19].

A growing body of evidence suggests that some bioactive compounds that are present in food, in particular flavonoids, and the metabolites produced during their degradation in the colon might have significant effects on the intestinal environment. These effects mainly occur in situations of metabolic disruption

such as obesity, which may lead an important influence on the physiology and biochemistry of the gut. These molecules could play a protective role in obesity, due to their effect on inflammatory mediators, cellular events that modulate inflammation and/or their effect on microbiota [20–22].

Proanthocyanidins are a specific class of flavonoids that are oligomers of flavan-3-ol subunits, i.e., (+)-catechin and (−)-epicatechin (EC), and are present in high concentrations in cocoa, red wine, cranberries, and apples [23–25]. The above-mentioned evidence supports the concept that relevant amounts of proanthocyanidins can reach the intestine and exert anti-inflammatory, anticarcinogenic, or other beneficial effects within the gastrointestinal tract.

Interestingly, the current literature indicates that some flavonoids can reduce the intestinal inflammatory processes, protect the barrier integrity and modulate the composition of the microbiota [26, 27]. Nevertheless, there are very few reported studies that relate the role of proanthocyanidins on intestinal inflammatory modulation in obesity [28]. Due to the well-reported effects of proanthocyanidins as anti-inflammatory agents in other metabolic tissues [29–31], we hypothesize that these natural compounds might be effective at modulating obesity-associated intestinal alterations. In this report, we aimed to evaluate the possible ameliorative effects of orally administered proanthocyanidins from grape seed on obesity-associated intestinal impairment in response to a nutritional challenge, as is the cafeteria (CAF) diet, with a specific focus on intestinal inflammation, barrier permeability and oxidative stress.

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 has been previously analysed by Quiñones *et al*. [32] and is described in **Table 1**.

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.* [33]. 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.

Animals and experimental design

Female Wistar rats that were 36 weeks of age were purchased from Charles River (Barcelona, Spain). The rats were housed in the animal quarters at 22°C with a 12-h light/12-h dark cycle (light from 08:00 to 20:00 hours) and were fed ad libitum a standard chow diet (Panlab 04, Barcelona, Spain) and tap water. After 1 week of adaptation, the rats were fed *ad libitum* a standard chow diet (adapted from Baselga *et al*. [33]) for the entire experimental procedure (standard diet group, Control, n=6) or were fed ad libitum a CAF for 15 weeks. The CAF diet was a high energy diet and had additional amounts of fat and

sucrose [33], being a highly palatable diet that induces voluntary hyperphagia. After 15 weeks, the animals that were fed the CAF were randomly divided into four groups (n=6) and were maintained on this diet with GSPE supplementation at 5 mg/kg (GSPE dose less than the dietary intake of humans) (CAF+5), 25 mg/kg (GSPE dose similar to the dietary proanthocyanidin intake in humans) (CAF+25), or 50 mg/kg (GSPE dose greater than the dietary intake of humans) (CAF+50), or without GSPE (CAF control group, CAF) for 3 additional weeks. Water-diluted (1:1) condensed milk (composition per 100 g: 0.4 g fat, 8.9 g protein, 60.5 g carbohydrates, 1175 kJ (281 kcal)) was used as a vehicle for administering GSPE. Both the control group and the control CAF group were given only condensed milk and the rest of groups were given the corresponding dose of GSPE per body weight mixed with the same volume of condensed milk. The treatment was administered every day at 9:00 AM. The rats were fasted for 12 hours and were anesthetized with ketamine (70 mg/kg body weight; Parke-Davis, Grupo Pfizer, Madrid, Spain) plus xylazine (5 mg/kg body weight; Bayer, Barcelona, Spain) at 8:00 AM.

The rats were 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. The intestine and 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. All of 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 of the procedures (permission number: 4249).

Morphometric variables and blood plasma assay

Body weight was monitored weekly, and the percentage of weight gain was calculated. Adiposity was determined with an adiposity index, which was computed for each rat as previously described [33]. These variables together with the MWAT depot weight were evaluated as physiological indicators of the degree of obesity in these animals.

Plasma glucose and triglycerides (TAG) were measured with enzymatic colorimetric kits (QCA, Barcelona, Spain). The plasma LPS level was measured using Limulus Amebocyte Lysate Assay (Associates of Cape Cod, E. Falmouth, MA). The manufacturer's protocol was followed in all cases.

Quantification of MPO activity and ROS in the ileum

Tissue samples were homogenized with a TissueLyser LT system (Qiagen, Hilden, Germany) in a 50 mM potassium phosphate buffer (Panreac, Barcelona, Spain). An aliquot of the homogenate was stored for the subsequent measurement of ROS. The remainder of the homogenate was centrifuged at 15,000 x g for 15 min at 4°C, and the resulting supernatant was discarded. The pellet was then homogenized with a hexadecyltrimethylammonium bromide (HTBA) (Sigma-Aldrich, Madrid, Spain) and 50 mM potassium phosphate buffer. The homogenate was sonicated (20 seconds), subjected to three freeze-thaw cycles, and centrifuged at 15,000 x g for 10 minutes at 4°C. For MPO activity determination, we used an adaptation of the Lenoir method (25). The supernatant was mixed into a solution of phosphate buffer, 0.22% guaiacol (Sigma-Aldrich, Madrid, Spain) and 0.3% H_2O_2 (Sigma-Aldrich, Madrid, Spain), and the absorbance was read at 470 nm.

For the measurement of ROS levels, the samples were mixed with 1 mM EDTA buffer (Panreac, Barcelona, Spain) and centrifuged at 3000 x g for 5 minutes at 4°C, after which the pellet was discarded. The intracellular ROS were

determined using the DCFH-DA method (2',7'-dichlorofluorescein diacetate) (Sigma-Aldrich, Madrid, Spain). Fluorescence was measured at λex = 485 nm and λem = 530 nm on an FLx800 Fluorescence Reader (Bio-Tek, Winooski, Vermont, USA).

Tissue RNA extraction and gene expression analysis by qRT-PCR

Total RNA was extracted from 50 mg of ileum using Trizol (AMbion, USA) following the manufacturer's instructions. Gene expression analysis was performed using both TaqMan probes and SYBR Green primers. When the TaqMan probes were used, cDNA was obtained from 1 μg of mRNA using the High capacity cDNA Reverse Transcription kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions. Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) amplification and detection were performed using the TaqMan Universal PCR Master Mix (Applied Biosystems, Madrid, Spain), and the respective specific TaqMan probes (Applied Biosystems, Madrid, Spain) were used: Rn00580432_m1 for rat interleukin (IL)-1β, Rn00587615_m1 for rat IL-13, Rn02116071_s1 for rat ZO-1, Rn01420322_g1 for rat occludin, Rn00581740_m1 for rat claudin-1, and Rn00587389_m1 for rat JAM-A. All of the results were normalized with respect to cyclophilin E (PPIA) (Rn00690933_m1). When SYBR Green sequences were used, cDNA (5 ng/mL) was subjected to qRT-PCR amplification using the SYBR Green PCR Master Mix (Applied Biosystems, Madrid, Spain). The primers were as follows: IL-10 forward (Fw), 5'-GCAGGACTTTAAGGGTTACTTGG-3' and reverse (Rv), 5'-GGAGAAATCGATGACAGCGT-3'; complement component (C)3 Fw, 5'-CTGGACCCATTCGGGAGTTC-3' and Rv, 5'-CTGGGAGCTACCGACCATTG-3'; tumor necrosis factor (TNF)-α Fw, 5'-CCTCACACTCAGATCATCTTCTC-3' and Rv, 5'-TTGGTGGTTTGCTACGACGTG-3'; the monocyte/macrophage marker F4/80 Fw, 5'-CTTTGGCTATGGGCTCCCAGTC-3' and Rv, 5'-GCAAGGAGGGCAGAGTTGATCGTG-3'; inducible nitric oxide synthase (iNOS) Fw,

5'-CACCCGAGATGGTCAGGG-3' and Rv, 5'-CCACTGACACTCCGCACAA-3'; and PPIA Fw, 5'-CTTCGAGCTGTTTGCAGACAA-3' and Rv, 5'-AAGTCACCACCCTGGCACATG-3'. Gene expression was normalized to PPIA. The 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 = C_t gene of interest - C_t cyclophilin and $\Delta\Delta C_t$ = ΔC_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 the ANOVA test to compare the control and CAF groups on the one hand and the CAF with the CAF plus GSPE groups on the other hand. Tukey's honest significant difference test was used to identify the significant differences between pairwise comparisons. P-values < 0.05 were considered to be statistically significant. These calculations were performed using IBM-SPSS 22.0 software. Pearson's correlation coefficient was used to test for correlations between the variables that were evaluated. Unsupervised Principal Component Analysis (PCA) was performed, using XLStat (Adinsoft), to assess the relationships between ileum inflammatory markers, TJs proteins, LPS plasma levels, oxidative stress in the ileum, and morphometric and metabolic variables. The variables included in the PCA were selected to obtain a KMO index of > 0.5 as a measure of Sampling Adequacy. After data scaling, the analysis was based on the correlation matrix (which consisted of 15 variables), and a principal component (PC) was considered to be significant if it contributed more than 5% to the total variance.

Results

Metabolic and morphometric parameters

All of the morphometric values that were evaluated at the end point of the study were significantly increased in the CAF compared to the lean control **(Table 2)**. Neither the morphometric nor metabolic parameters were altered after GSPE supplementation. Although no significant differences were observed in the weights of the mesenteric WAT between the CAF group and the groups supplemented with GSPE, the results show decreased mesenteric WAT weight when the groups were treated with GSPE. Additionally, the TAG measurement tended to be lower in the GSPE-supplemented groups **(Table 2)**.

Table 2. Metabolic and morphometric parameters of rats fed with a standard diet (CONTROL) or a cafeteria diet (CAF), either with or without proanthocyanidin supplementation.

Variables	CONTROL	CAF	CAF+5	CAF+25	CAF+50
Body weight (g)	261.7 ± 7.42	360.2 ± 31.7 *	336.5 ± 6.6	350.8 ± 19.8	336.5 ± 20.3
Mesenteric WAT weight(g)	2.5 ± 0.2	9.3 ± 2.3 *	6.3 ± 0.5	5.6 ± 0.5	7.6 ± 1.3
Adiposity	1.9 ± 0.1	5.8 ± 0.6 *	5.0 ± 0.2	4.7 ± 0.2	5.3 ± 0.8
% Weight gain	51.5 ± 3.8	91.9 ± 2.7 *	91.1 ± 1.6	95.8 ± 11.1	90.4 ± 9.1
Glucose (mg/dL)	157.6 ± 27.0	194.4 ± 16.4	179.8 ± 10.0	205.8 ± 22.8	164.2 ± 16.2
TAG (mg/dL)	95.9 ± 4.5	249.2 ± 70.5	218.0 ± 60.7	180.2 ± 35.9	173.7 ± 37.1

CONTROL: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; CAF + 5: rats fed a cafeteria diet plus 5 mg/kg of GSPE; CAF + 25: rats fed a cafeteria diet plus 25 mg/kg of GSPE; caf + 50: rats fed a cafeteria diet plus 50 mg/kg of GSPE; GSPE: grape seed proanthocyanidin extract; WAT: white adipose tissue. Values are means ± SEM. *P < 0.05 compared to negative control (CONTROL). # P < 0.05 compared to cafeteria diet (CAF).

GSPE supplementation reduces MPO activity and ROS levels in the ileum

Oxidative stress was measured by intracellular ROS production in rat ileum. After 18 weeks of diet intervention, the CAF group showed an increase in ROS production compared to the control group. Moreover, supplementation with 25 mg/kg and 50 mg/kg of GSPE for 3 weeks reduced ROS levels compared to the CAF group **(Figure 1A)**. MPO activity was used as an inflammatory and neutrophil accumulation indicator in the rat ileum. After 18 weeks, the CAF produced a significant increase in MPO activity compared to the control group. Furthermore, supplementation with all of the concentrations of GSPE decreased the MPO activity compared to the CAF group **(Figure 1B)**.

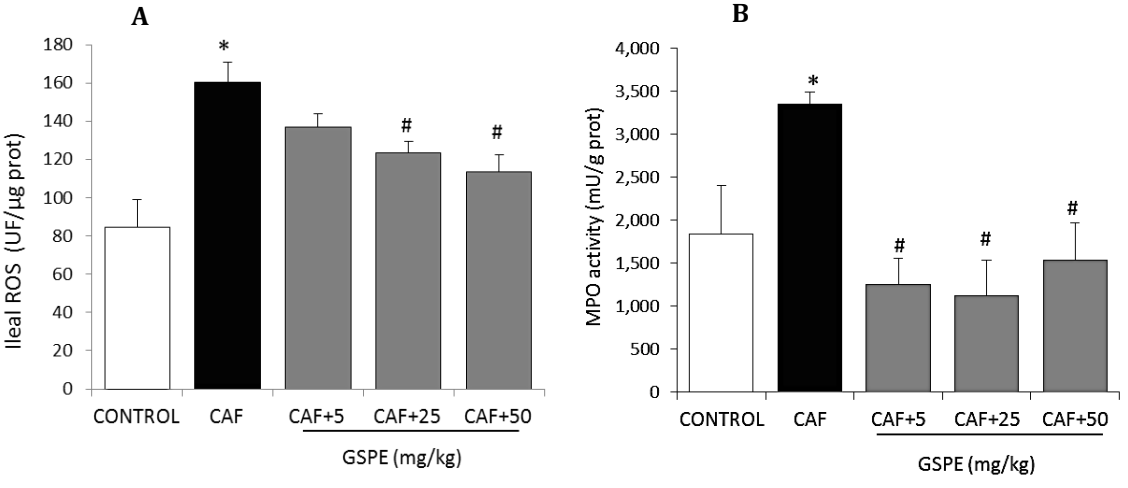

Figure 1. Levels of oxidative stress and neutrophil infiltration markers in the ileum of the CONTROL, CAF diet and CAF+GSPE-supplemented groups. A) Ileal ROS production and B) MPO activity after cafeteria diet and GSPE supplementation at different concentrations. CONTROL: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; CAF + 5: rats fed a cafeteria diet plus 5 mg/kg of GSPE; CAF + 25: rats fed a cafeteria diet plus 25 mg/kg of GSPE; CAF + 50: rats fed a cafeteria diet plus 50 mg/kg of GSPE; GSPE: grape seed proanthocyanidin extract; MPO: myeloperoxidase; ROS: reactive oxygen species; mU: milliunits; UF: fluorescence units. Values are means ± SEM. **P* < 0.05 compared to the negative control (CONTROL). # *P* < 0.05 compared to the cafeteria diet (CAF).

Metabolic endotoxemia

Plasma LPS was measured to detect the expected increased transport of LPS through the intestinal wall in obese rats and the potential protective role of GSPE. The results did not show any significant difference between the CAF and control group and also did not show any significant difference between the CAF and GSPE-supplemented groups **(Figure 2)**.

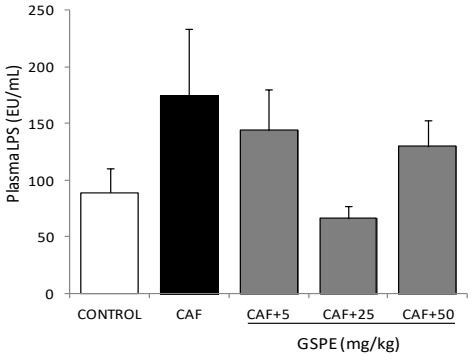

Figure 2. Plasma LPS concentration after CAF diet and GSPE supplementation. CONTROL: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; CAF + 5: rats fed a cafeteria diet plus 5 mg/kg of GSPE; CAF + 25: rats fed a cafeteria diet plus 25 mg/kg of GSPE; CAF + 50: rats fed a cafeteria diet plus 50 mg/kg of GSPE. Values are means ± SEM. **P* < 0.05 compared to control (CONTROL). # *P* < 0.05 compared to cafeteria diet (CAF).

GSPE effect on the gene expression of inflammatory-related genes

To investigate the possible pathways involved in the development of intestinal inflammation that is affected by oral GSPE treatment, we measured the gene expression of TNF-α, iNOS, an enzyme generating nitric oxide, F4/80 as a marker of macrophage infiltration, IL-1β, C3 and IL-10 and IL-13, as anti-inflammatory molecules **(Figure 3)**. None of the genes that were studied were significantly altered by the CAF compared to the standard diet. In contrast, GSPE supplementation at 25 and 50 mg/kg decreased iNOS expression compared to the CAF group. In addition, the lowest dose of GSPE was able to reduce IL-1β expression in the ileum.

GSPE effect on TJ gene expression

To evaluate whether the state of intestinal inflammation was related to an alteration in the permeability of the intestinal barrier, we analysed the expression of ZO-1, occludin, claudin-1 and JAM-A, which are structural proteins of tight junctions **(Figure 4)**. ZO-1 expression was decreased in the CAF rats with respect to the control group.

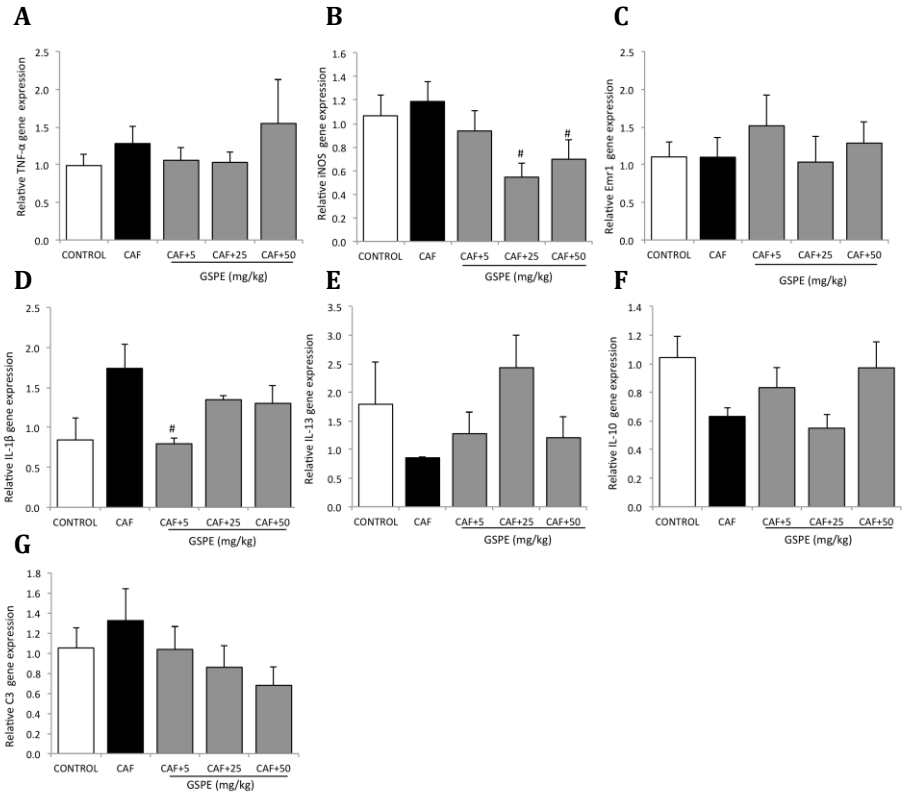

Figure 3. Effect of the CAF diet and GSPE supplementation on the gene expression of inflammatory related genes. A) TNF-α, B) iNOS, C) F4/80, D) IL-1β, E) IL-13, F) IL-10 and G) C3 gene expression. CONTROL: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; CAF + 5: rats fed a cafeteria diet plus 5 mg/kg of GSPE; CAF + 25: rats fed a cafeteria diet plus 25 mg/kg of GSPE; CAF + 50: rats fed a

cafeteria diet plus 50 mg/kg of GSPE. Values are means ± SEM. *P < 0.05 compared to negative control (CONTROL). # P < 0.05 compared to cafeteria diet (CAF).

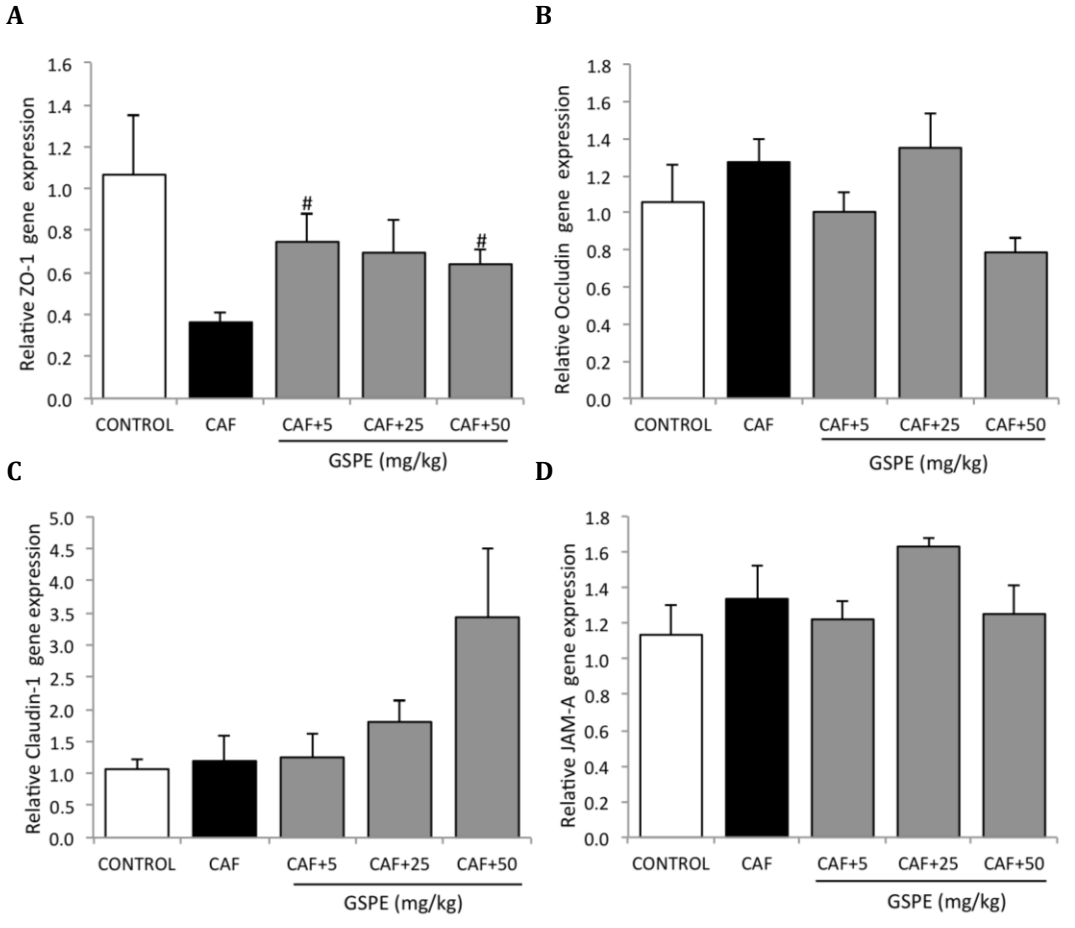

Figure 4. Effect of GSPE on the expression of structural genes of TJs. A) ZO-1, B) Occludin, C) Claudin-1 and D) JAM-A gene expression. CONTROL: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; CAF + 5: rats fed a cafeteria diet plus 5 mg/kg of GSPE; CAF + 25: rats fed a cafeteria diet plus 25 mg/kg of GSPE; CAF + 50: rats fed a cafeteria diet plus 50 mg/kg of GSPE. Values are means ± SEM. *P < 0.05 compared to negative control (CONTROL). # P < 0.05 compared to cafeteria diet (CAF).

In addition, GSPE was able to reverse this situation. The results showed that both 5 and 50 mg/kg of GSPE significantly increased ZO-1 expression compared to the CAF group; however, no differences were observed between any of the GSPE groups and CAF for occludin, claudin or JAM-A gene expression.

Associations between the ileum gene expression of inflammatory markers, TJ proteins, LPS plasma levels, and oxidative stress in the ileum, with morphometric and metabolic variables

To identify whether the different parameters that were measured in the ileum are associated with metabolic and morphometric variables, we used the Pearson's correlation test to evaluate all of the groups adjusted for body weight **(Table 3)**. MPO activity in the ileum was positively associated with mesenteric WAT weight, and the LPS plasma levels were associated with adiposity.

Interestingly, TNF-α mRNA expression in the ileum was strongly positively associated with weight gain and adiposity. Additionally, C3 expression showed a positive correlation with weight gain, and F4/80, the marker of macrophage infiltration, was associated with TAG levels. Interestingly, the expression of the anti-inflammatory interleukin IL-13 was negatively correlated with mesenteric WAT weight and adiposity. Our results also show that negative correlations were present between the expression of TJ proteins and the metabolic parameters. In concrete terms, ZO-1 presented a negative correlation with triglyceride levels. In addition, expression of JAM-A and occludin in the ileum was negatively associated with the adiposity index and triglyceride levels **(Table 3)**.

To identify whether the different parameters measured in the ileum are associated between them, we performed another correlation analysis and found that some of the inflammatory markers, oxidative stress, TJ proteins and macrophage infiltration markers significantly correlated with one another **(Figure 5)**. Interestingly, the expression of some of the pro-inflammatory

markers and LPS levels were negatively associated with ZO-1 and JAM-A, respectively.

Principal Components Analysis (PCA)

To analyse globally the distribution and associations between the variables analysed, we used a model that reduces the matrix of data to the lowest dimension of the most significant components, or principal components (PCs). This approach is useful for identifying various combinations of variables that could reflect possible biological mechanisms, especially in association with various other health outcomes. The results of performing PCA on all of the variables analyzed are shown in **Table 4**.

The first four PCs explained 63.13% of the total variance. The first and second PCs were responsible for 43.22% of the total variance, constituting 27.13 and 16.09%, respectively. PC3 explained 12.95% of the variability, and PC4 explained 9.96% of the variability. Variables with absolute loadings that were greater than or equal to +/−0.40 on a given PC were used to describe the retained PC and provide an interpretation. The projection of ileum gene expression of inflammatory markers, TJ proteins, LPS plasma levels, oxidative stress in the ileum, and morphometric and metabolic variables in the plane defined by the first two PCs is shown in **Figure 6** (right panel).

Overall, PC1 was clearly characterized by body weight, mesenteric WAT weight, glucose and TAG levels, with ileal ROS levels and ileal IL-1β mRNA expression on the right side and ZO-1 mRNA expression in the ileum on the left side **(Figure 6)**. All of the metabolic and morphometric variables together with the ileum ROS levels and IL-1β ileal expression were grouped in opposition to the ZO-1 expression levels **(Figure 6, right panel)**. Therefore, PC1 indicates a negative association between diet-induced obesity-associated metabolic alterations and intestinal inflammation, and the TJ protein ZO-1.

Table 3. Correlation coefficients between the ileal gene expression of inflammatory-related genes and TJs, LPS plasma levels, ROS levels and MPO activity with anthropometric and metabolic variables in control, CAF and CAF+GSPE rats.

Variables	Mesenteric WAT weight (g)	Adiposity (%)	Weight gain (%)	Glucose (mg/dL)	TAG (mg/dL)
ROS (UF)/ug prot	0.149	0.162	0.108	-0.132	-0.015
MPO activity (mU/g prot)	**0.445**	0.052	-0.246	0.055	-0.074
Plasma LPS (EU/mL)	0.244	**0.369**	0.227	0.055	0.197
Ileal gene expression					
Inflammatory related genes					
TNF-α	0.066	**0.520**	**0.427**	-0.143	-0.225
F4/80	-0.137	-0.022	-0.195	0.018	**0.396**
IL-13	**-0.454**	**-0.321**	-0.287	-0.007	0.042
C3	0.022	0.029	**0.546**	-0.052	-0.038
Tight junctions					
JAM-A	-0.191	**-0.487**	-0.075	0.106	**-0.406**
ZO-1	-0.165	-0.273	0.240	-0.274	**-0.352**
Occludin	**-0.304**	**-0.469**	-0.064	-0.221	**-0.434**

Pearson's correlation coefficient after adjustment for body weight. Values in bold show significant correlations ($P<0.05$).

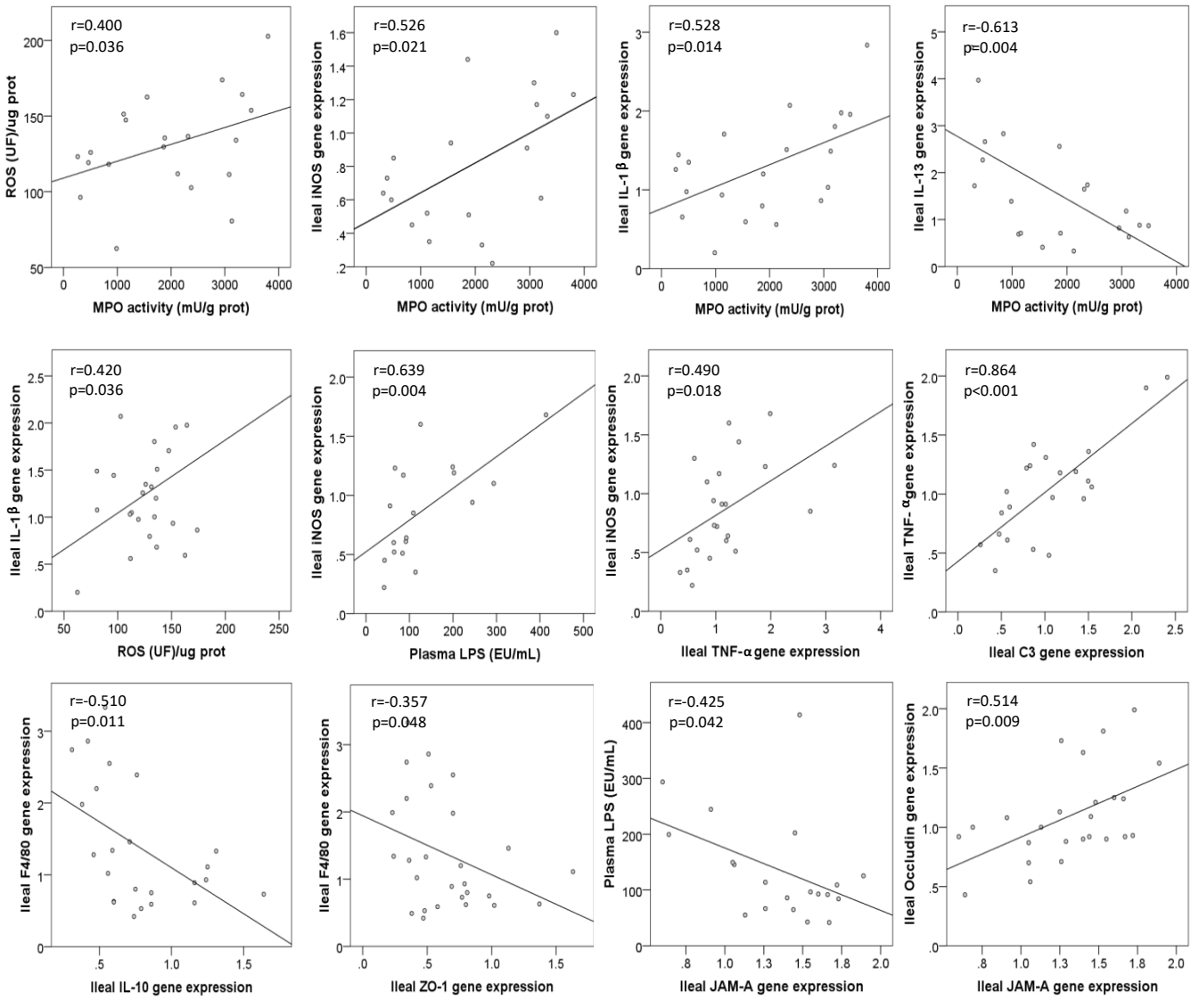
r=0.400
p=0.036
ROS (UF)/ug prot
MPO activity (mU/g prot)
r=0.526
p=0.021
Ileal iNOS gene expression
MPO activity (mU/g prot)
r=0.528
p=0.014
Ileal IL-1 β gene expression
MPO activity (mU/g prot)
r=-0.613
p=0.004
Ileal IL-13 gene expression
MPO activity (mU/g prot)
r=0.420
p=0.036
Ileal IL-1β gene expression
ROS (UF)/ug prot
r=0.639
p=0.004
Ileal iNOS gene expression
Plasma LPS (EU/mL)
r=0.490
p=0.018
Ileal iNOS gene expression
Ileal TNF-α gene expression
r=0.864
p<0.001
Ileal TNF- αgene expression
Ileal C3 gene expression
r=-0.510
p=0.011
Ileal F4/80 gene expression
Ileal IL-10 gene expression
r=-0.357
p=0.048
Ileal F4/80 gene expression
Ileal ZO-1 gene expression
r=-0.425
p=0.042
Plasma LPS (EU/mL)
Ileal JAM-A gene expression
r=0.514
p=0.009
Ileal Occludin gene expression
Ileal JAM-A gene expression

Figure 5. Plot of the significant associations between the expression of inflammatory-related markers (TNF-α, iNOS, IL-1β, IL-13, F4/80 and C3); TJ proteins (JAM-A, ZO-1 and occludin); ROS levels and MPO activity in the ileum; and LPS plasma levels from control, CAF and CAF+GSPE rats. $P < 0.05$, inset corresponds to Pearson's r correlation and the corresponding *P* value**.**

Table 4. Loadings for the first four PC.

Variables	PC1	PC2	PC3	PC4
Body weight (g)	**0.824**	0.137	0.290	0.236
Mesenteric WAT weight (g)	**0.845**	0.315	0.147	0.043
Glucose (mg/dL)	**0.474**	0.060	0.318	-0.280
TAG (mg/dL)	**0.678**	0.171	0.396	-0.330
ROS (UF/µg prot)	**0.663**	0.002	-0.287	-0.188
MPO activity (mU/g prot)	0.448	0.180	**-0.739**	-0.160
LPS (EU/mL)	0.121	**0.762**	-0.143	-0.160
Gene expression				
TNF-α	0.179	**0.582**	0.168	**0.585**
iNOS	0.227	**0.647**	-0.306	0.371
ZO-1	**-0.571**	-0.015	-0.293	0.352
Occludin	0.318	**-0.519**	-0.465	0.429
IL-1β	**0.676**	-0.223	-0.263	0.096
IL-13	-0.130	-0.406	**0.601**	0.298
JAM-A	0.454	**-0.513**	0.063	0.412
C3	0.012	**0.536**	0.267	0.333

Figure 6 (left panel) shows the PCA score plot of the control, CAF and GSPE-supplemented groups. PC1 clearly discriminates between the lean (control) and obese (CAF) groups. The CAF group is located on the right side. As expected, they present higher body weight-related parameters than the control group. Furthermore, they show higher levels of ROS and IL-1β expression in the ileum than the control group. PC1 also defines that ZO-1 ileal expression is higher in the control group than in the CAF group. Regarding the GSPE treatment, only the CAF+25 animals were grouped together, and they were located between the control and CAF animals, showing a slight improvement in the parameters

mentioned above. The other GSPE doses (5 and 50 mg/kg) were not different from the CAF group.

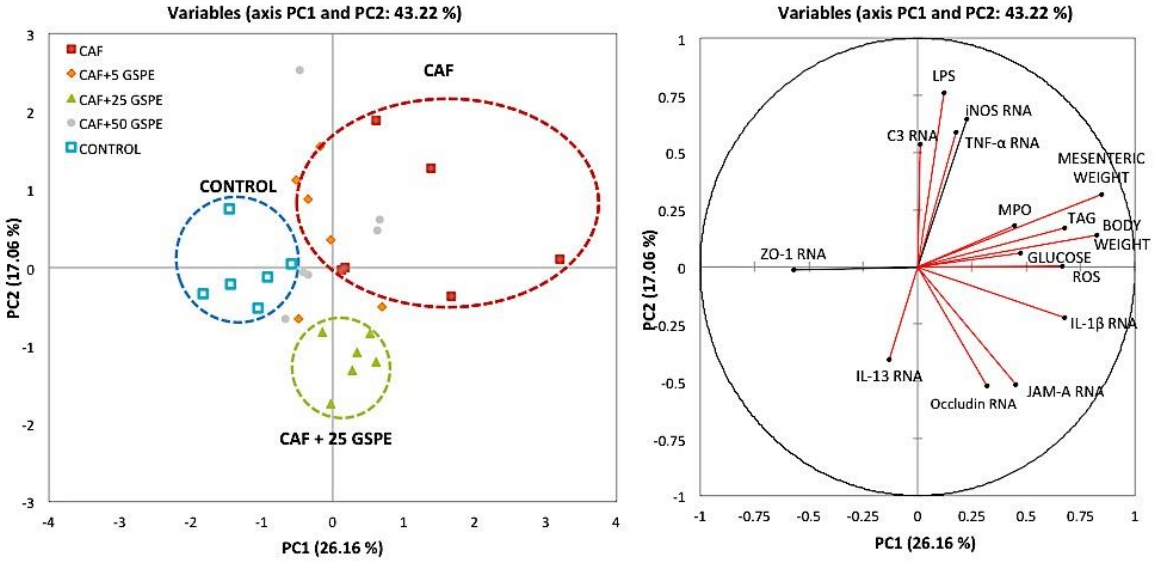

Figure 6. PCA score plot of the groups analyzed (left panel) and projection of the variables evaluated in the plane defined by the two first PCs (right panel).

PC2 was mainly characterized by the expression of inflammatory markers and LPS circulating levels located in the upper part of the graphic and by the ileal expression of TJs and the anti-inflammatory IL-13 located in the lower part. PC2 did not discriminate between the control and CAF groups; however, PC2 discriminated between the CAF and CAF+25 treated group. CAF was mainly located on the upper side, and CAF+25 was located on the lower side. Therefore, the CAF+25 group presented lower expression levels of the inflammatory markers in the ileum, lower circulating LPS and higher expression of the TJ components **(Figure 6, left panel).**

Discussion

There is increasing evidence to suggest that intestinal alterations can influence

obesity and metabolic health. Concretely, several recent studies have provided compelling new evidence to suggest that changes in the epithelial barrier function and intestinal inflammation are associated with and could even lead to altered regulation of body weight and glucose homeostasis [34]. The main consequence of the gut barrier dysfunction has been proposed to be the entry of toxins from the intestinal lumen, which can trigger local inflammation or gain access to the circulation and induce systemic inflammation through cytokine release [3]. In this sense, some food bioactive compounds might increase the success of dietary regimens designed to ameliorate risk factors related to obesity and other metabolic disorders. Therefore, identifying selective natural compounds that can modulate the development of obesity and its associated conditions, as a consequence of ameliorating the intestinal inflammation and barrier function, represents a realistic therapeutic strategy for future development. For this study we designed a long-term experiment to evaluate in the intestine the effect of GSPE dose less than the dietary proanthocyanidin intake in humans (i.e., 5mg/kg in rats) an equivalent dose (i.e., 25 mg/kg in rats) and greater dose (i.e., 50 mg/kg in rats). Using a conversion system of rodent to human doses [35] and considering the daily intake for a 70-kg human model, these doses are equivalent to intakes of 57, 284, and 560 mg of proanthocyanidins per day, realistic intakes for humans in a normal diet [36–38]. In this sense, 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. However, the beneficial health effects of flavonoids are also attributed to the compounds derived from their metabolism [39].

This study provides evidence for the ameliorative effect of a GSPE extract, which is rich in proanthocyanidins, on high-fat/high-sucrose diet-induced intestinal alterations in rats, including the reduction of intestinal inflammation and oxidative stress, which suggests a prevention of barrier defects. Although

the ameliorative effects of other polyphenols, such as curcumin [40], quercetin [40], naringenin [41], or grape seed extract [42], on experimental severe intestinal inflammation (inflammatory bowel disease and colitis) have been examined (reviewed in [43]), to our knowledge, there is little information on the effects of proanthocyanidins on diet-induced intestinal alterations *in vivo*.

The gastrointestinal tract is prone to oxidative damage due to its exposure to the luminal oxidants present in foods [44, 45]. In this sense, increased free radical production and impaired antioxidant defences have been related to the progression of intestinal chronic inflammation [44, 45]. In this experiment, a high-fat/high-sucrose diet for 18 weeks produced marked obesity, but it also produced clear increased intestinal oxidative stress as measured by ROS levels and MPO activity in the ileum. Interestingly, GSPE treatment significantly reduced ROS levels at some of the doses tested.

MPO is an enzyme marker of inflammation and neutrophil infiltration in the intestinal mucosa. MPO catalyses the production of hypochlorous acid from hydrogen peroxide and chloride anion during the neutrophil's respiratory burst, and thus, it is a potent oxidative inductor [46]. We found that the activity of MPO in the ileum from obese rats was increased and that GSPE, in addition to its ROS-lowering properties, was able to reduce MPO activity. Taken together, these findings indicate that GSPE could act as an anti-oxidant in the ileum of obese rats. Previous studies in our group have demonstrated these effects in other tissues and cell models [47–49]. They proposed that the GSPE defence mechanisms to counteract the oxidative stress involved the modulation of anti-oxidant enzymes, mitochondrial function and ROS scavenging properties [47–49]. Further studies are needed to identify the specific anti-oxidant mechanisms of GSPE's modulation of intestinal oxidative stress.

The role of intestinal inflammation with obesity-associated systemic inflammation and gut barrier function is unclear. As stated before, gut

inflammation as measured with MPO is increased in rats after 18 weeks on a cafeteria diet, and the pro-inflammatory cytokine (TNF-α) expression in the ileum was shown to be positively correlated with adiposity and measures of body weight. These results are in agreement with previously published reports in rats [50] and mice [2, 28]. Interestingly, the anti-inflammatory interleukin IL-13 showed a strong negative association with mesenteric adipose tissue weight and adiposity. Taken together, these results support the hypothesis that diet-induced intestinal inflammation is involved in the development of obesity [2]. With regard to the potential anti-inflammatory effects of proanthocyanidins, it has been described that flavonoids can inhibit the intestinal expression of TNF-α, iNOS, interleukin-6, and COX-2 in chemically induced intestinal inflammation animal models [51, 52]. Our results indicate, for the first time, that GSPE was able to reduce iNOS and IL-1β gene expression in the ileum of rats that were fed a high-fat/high-sucrose diet. The relevance of these findings is highlighted by the fact that IL-1β plays a central role in the intestinal inflammatory process, and recent studies show that IL-1β causes increased intestinal TJ permeability [53]. Furthermore, the expression of iNOS has been widely studied in relation to intestinal inflammation because sustained high nitric oxide production mediated by iNOS plays a role in the pathology of chronic inflammatory bowel disease [54, 55]. Due to the potential link between intestinal inflammation and obesity, GSPE anti-inflammatory effects on the intestine can be prominent for diet interventions that limit intestinal inflammation induced by an obesogenic diet, and the GSPE anti-inflammatory effects could protect against obesity and its consequences.

Another point of interest in this study was to evaluate whether GSPE could modulate the alterations in the permeability of the intestinal barrier that are related to the state of intestinal inflammation. Our findings indicate that the TJ proteins were negatively associated with measures of adiposity and with the circulating levels of TAG. These are not causal associations, but they suggest

that increased adiposity is accompanied by lower expression of TJ components, which is in agreement with the hypothesis that obesity and a HFD are associated with increased intestinal permeability [56]. Then, given the importance of having a healthy barrier function, dietary interventions that can modulate the intestinal permeability might afford an effective tool for the prevention and treatment of metabolic diseases associated with obesity. In this regard, our results indicated that obese rats presented lower expression of ZO-1, and at the same time, GSPE significantly increased its expression compared to the CAF group. Peng *et al*. also found that puerarin, an isoflavone administered at 90 and 180 mg/kg for 5 weeks, up-regulated intestinal ZO-1 expression in male Sprague-Dawley rats, which presented chemically induced intestinal inflammation [57]. With regard to the more specific effects of a proanthocyanidin-rich extract, Goodrich *et al*. [58] found that chronic administration of dietary grape seed extract increases the expression of the TJ protein occludin in the proximal colon of healthy lean Wistar rats. There is very little information with regard to flavonoid effects on TJs in vivo [8], but very recently, Masumoto *et al*. [28] found that apple procyanidins improved intestinal permeability by increasing ZO-1 and occludin expression in obese mice. In addition, some in vitro experiments on enterocyte cell lines have suggested that procyanidins were able to prevent a TJ decrease and then protect against intestinal permeability alterations [59–61]. However, the molecular mechanisms that underlie these effects remain unclear.

Metabolic endotoxemia is defined as increased levels of circulating LPS found during a fat-enriched diet [62–64]. Although the results did not show any significant differences between the CAF and control groups, or between the CAF and GSPE-supplemented groups, we observed a clear tendency to increase plasma LPS in the diet-induced obese rats compared to lean rats. In this study, we did not perform a direct measurement of the altered permeability *in vivo*, and for this reason, we cannot ensure that the presence of LPS in the

bloodstream is caused by a deficiency in the gut barrier function [65]. Nevertheless, the results suggested that some alterations in permeability occurred, but further *in vivo* studies are needed to confirm the potential GSPE protective effects on LPS diffusion into the bloodstream.

Finally, to analyse globally the distribution and associations between morphometric and metabolic variables, and inflammatory markers, oxidative stress and barrier components, we used PCA. Using this model, we found that the PC1 plot was characterized by an opposite distribution of measures of body weight and adiposity, together with ileum ROS levels, with respect to ZO-1 mRNA expression in the ileum. Furthermore, using pair-wise correlation analysis, we found that ZO-1 and occludin expression was negatively associated with the expression of F4/80, which is a marker of the macrophage population. Taken together, these findings are in agreement with previously published reports, which indicate that an altered intestinal barrier function is correlated with obesity [3] and with obesity-associated intestinal inflammation [65, 66]. On the other hand, PC2 was mainly characterized by the expression of inflammatory markers and LPS circulating levels. This component discriminated between HFD rats and rats that were treated with a moderate dose of GSPE and showed that this GSPE-treated group had lower expression levels of the inflammatory markers in the ileum and lower circulating LPS. Therefore, our results highlight the fact that compounds that can improve gut health, including barrier function and intestinal inflammation, might be able to ameliorate obesity-associated alterations.

In conclusion, our findings indicate that orally administered GSPE modulates the intestinal inflammation, oxidative stress and possibly the barrier function. Based on these findings, our data suggest that nutritional and/or therapeutic interventions focused on gut health and modulation of the intestinal permeability should be extensively explored in the context of obesity.

Author contributions

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

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Conflict of Interest

None.

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3. MANUSCRIPT 4

Results

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

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

In preparation

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

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Abstract

Scope: 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.

Methods and results: 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) and the expression of tight junction (TJ) genes in the small and large intestines were monitored. The CAF diet increased OVA, LPS 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.

Conclusions: 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.

Keywords: cafeteria diet, gut, permeability, obesity and proanthocyanidins

Introduction

Obesity and metabolic disorders have recently become a major health public challenge [1]. Environmental factors, such as a sedentary lifestyle and hypercaloric diet, are the main causes of the increasing prevalence of obesity in the global population [2]. Animal models have correlated obesity with particular physiological changes, such as impaired intestinal barrier function [3, 4], and similar changes have been observed in human studies [3, 5, 6].

The intestinal epithelium is the first site where the diet, microbes and host interact [7]. Intestinal epithelial cells are distributed in a single layer of cells 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 [8–10] and a barrier that prevents the passage of harmful intraluminal entities, including foreign antigens and their toxins [11, 12]. 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 [8–10]. 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 [13]. Contact between intestinal epithelial cells includes three components: desmosomes, adherens junctions (AJs) and tight junctions (TJs) [14]. TJs are the most important shield of the intestine and prevent the paracellular permeation of macromolecules into the circulation [15]. TJs are formed by the interaction of transmembrane proteins, such as occludin (OCLN) [16], claudins [17], and junctional adhesion molecules (JAMs) [18], with intracellular plaque-forming zonulin/zonula occludens (ZO) proteins [19].

As mentioned above, the intestinal epithelium acts as a barrier that prevents the translocation of microbial components, but some endogenous or exogenous process may alter this protective function. According to the literature, and previous results from our group, rodents fed a cafeteria (CAF) diet, as a model of high-fat/high-carbohydrate diet, for extended periods exhibit diet-induced obesity (DIO), which has been proposed to disrupt the intestinal barrier function. This disruption in barrier function is associated with increased bacteria-derived lipopolysaccharide (LPS) absorption across the intestinal barrier, increasing its plasma levels by two- to three-fold through a process referred to as metabolic endotoxemia [20–24]. Due to the link between intestinal alterations and the detrimental consequences of obesity, the possibility of modulating these specific physiological functions in the intestine has become a highly interesting top. As shown in our previous study, chronic supplementation with a grape seed proanthocyanidin extract (GSPE) seems to protect against this intestinal defect by TJ modulation [24]. Proanthocyanidins represent one of the most abundant groups of plant secondary metabolites and are present in high concentrations in cocoa, red wine, cranberries, and apples [25, 26]. Chemically, proanthocyanidins are a specific class of oligomeric flavonoids, and their basic structural elements include polyhydroxyflavan-3-ol

units linked together by carbon–carbon bonds [27]. In our previous study [24], we evaluated the protective effects of three dietary doses of GSPE (5, 25 and 50 mg/kg bw), which are equivalent to human consumption of proanthocyanidins in a normal diet [28–30]. We observed interesting beneficial effects of these bioactive compounds on intestinal dysfunction, when they were administered in a corrective way for 21 days. Therefore a study of other doses administered at different time points might be interesting to obtain insights into the mode of action of these molecules.

TJs gene expression and protein localization studies provide important data about intestinal permeability, but are simultaneously limited in describing the potential mechanisms by which the paracellular pathway might be altered [31]. *In vivo* intestinal permeability tests have been useful to estimate the impairments in intestinal permeability in obese subjects [5, 32]. However, more in-depth studies are needed to identify the site of the defect. In the present study, *ex vivo* experiments based on the Ussing chamber technique, were performed to compare changes in the different sections of the small and large intestines induced by the dietary interventions.

The main aim of this study was to investigate the temporal relationship between obesity and barrier dysfunction in the small and large intestines (intestinal permeability and metabolic endotoxemia) of rats fed a cafeteria diet (CAF diet) as a model of a high-fat/high-carbohydrate diet by evaluating the development of this loss of function throughout the experiment. Using this experimental design, we also aimed to compare the effectiveness of a pharmacological dose of GSPE (500 mg/kg bw) administered at two different time points with respect to the start of the obesogenic diet at preventing possible intestinal alterations associated with the CAF diet.

Materials and Methods

Proanthocyanidin extract

The grape seed extract enriched in proanthocyanidins (GSPE) was kindly provided by *Les Dérivés Résiniques et Terpéniques* (Dax, France). The following GSPE composition used in this study has been previously analysed by Margalef *et al.* [33] and contains: monomers of flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5-13 units; 31.7%) of proanthocyanidins.

Animal model

Forty 7-week-old, female Wistar rats, each weighing 240-270g, were purchased from Charles River Laboratories (Barcelona, Spain). The 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 for 10 days. During this period one group of animals (PRE-CAF group) received a preventive treatment with proanthocyanidins (500 mg GSPE/Kg bw) whereas the rest of animals received just the vehicle. When the pre-treatment finished one group of animals 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 plus 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 diet was: 1.77 % fibre, 14.98 % protein, 20.69 % fat and 62.55 % sugar. The GSPE was dissolved in water and was orally gavage administered to the animals for each treatment in a volume of 500 μL.

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 [34]. 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 [35]. 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.

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 [23]. 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). 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; 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 the Student's t-test to compare the control and CAF group on the one hand and ANOVA to compare the CAF with the CAF plus GSPE treated groups on the other hand. P-values < 0.05 were considered to be statistically significant. These calculations were performed using IBM-SPSS 22.0 software. 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 1)**. 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 1)**.

Table 1. 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.

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. Although lower OVA levels were detected in the PRE-CAF group than in the CAF group, the difference was not statistically significant, and the pre-treatment with GSPE lost its early protective effect after 17 weeks of diet intervention **(Figure 1)**.

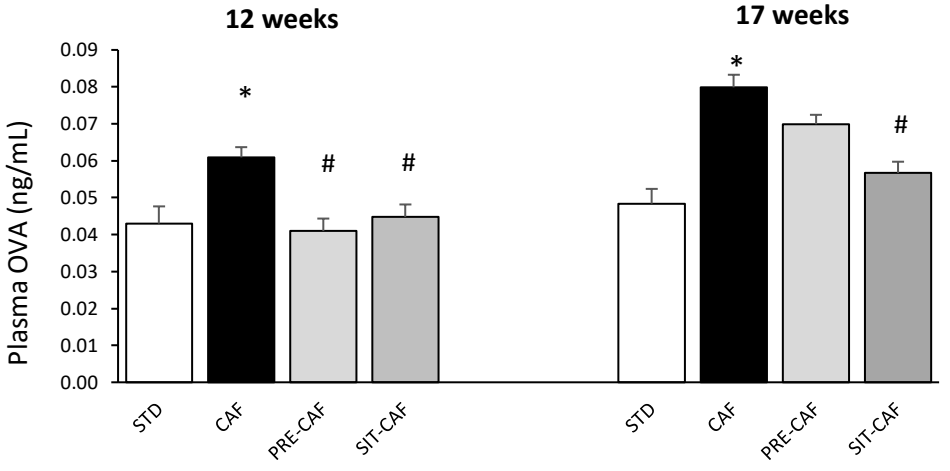

Figure 1. OVA intestinal permeability test at weeks 12 and 17 of CAF diet consumption. STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a pre-treatment with 500 mg/kg GSPE; SIT-CAF: rats fed a cafeteria diet simultaneously with a 500 mg/kg 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.

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 weight gain, MWAT weight and the final body weight. After 17 weeks, these correlations were stronger and a new positive correlation with the adiposity index was established **(Table 2)**.

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 2A)**. 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 2B)**.

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 2)**. 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 3)**.

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 3A)**.

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 3B)**.

LPS levels were positively correlated with adiposity at week 8 and with weight gain and final weight and adiposity at the end of the CAF diet intervention **(Table 2)**. In addition, 17 week LPS levels were positively correlated with 12 and 17 weeks OVA levels **(Table 3)**. As expected, TNF-α levels were positively correlated with adiposity at 12 weeks and with MWAT and adiposity at the end point **(Table 2)**. In addition, TNF-α levels at 12 and 17 weeks were positively correlated with OVA and TEER values **(Table 3)**.

Table 2. Correlation coefficients for TEER values and plasma OVA, TNF-α and LPS levels compared with the morphometric variables.

Variables	Morphometric variables			
	Weight gain (%)	**MWAT weight (g)**	**Adiposity index (%)**	**Final body weight (g)**
8 weeks of diet intervention				
OVA (ng/mL)	-	**-**	-	-
LPS (EU/mL)	0,228	0,351	**0,389***	0,267
TNF-α (pg/mL)	-0,285	-0,189	-0,109	-0,119
12 weeks of diet intervention				
OVA (ng/mL)	**0,475***	**0,418***	0,334	**0,500***
LPS (EU/mL)	0,209	0,124	0,315	0,093
TNF-α (pg/mL)	0,073	0,165	**0,327***	0,156
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.*

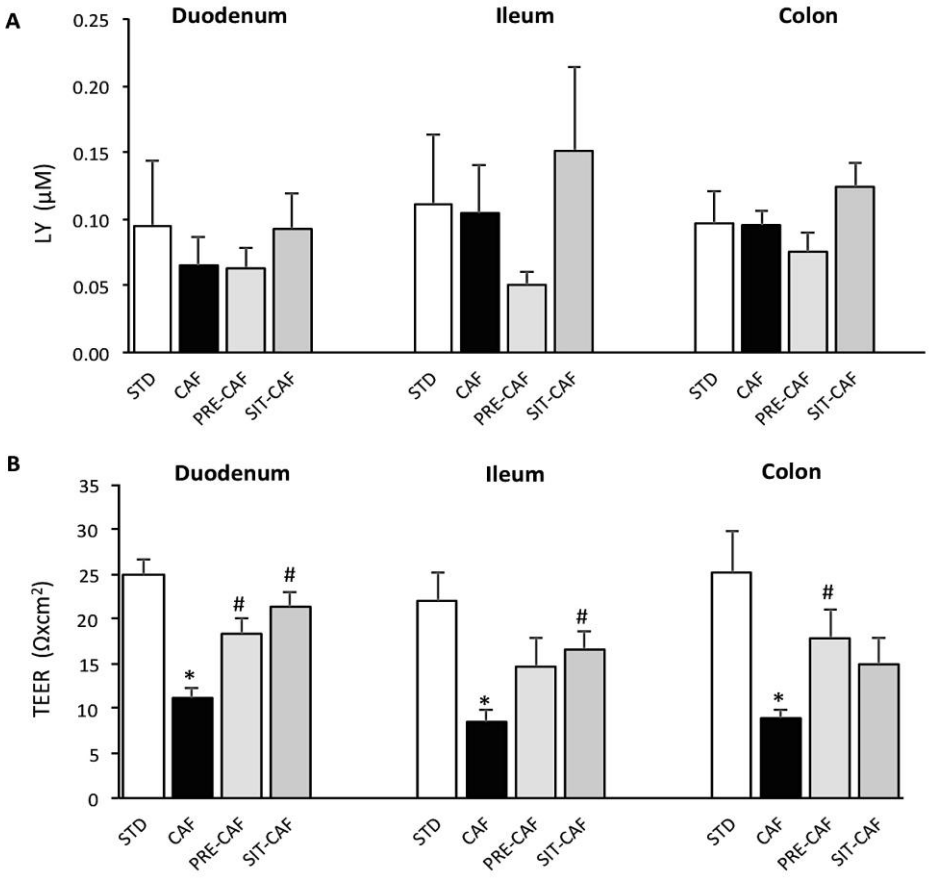

Figure 2. Measurement of the integrity of the intestinal mucosa using LY staining (A) and TEER (B). STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a 500 mg/kg GSPE pre-treatment; SIT-CAF: rats fed a cafeteria diet simultaneously with a 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.

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

The paracellular pathway is constituted by TJs, consisting of ZO-1, 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 expression in the ileum was decreased by the CAF intervention, and the SIT-CAF treatment increased its expression. We did not

observe any other changes in the expression pattern of TJ genes in the intestine **(Table 4)**.

Table 3. 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	**OVA 17w**	**TNF-α 8w**	**TNF-α 12w**	**TNF-α 17w**
OVA 12w	1	**0,551***	0,326	**0,497***	**0,367***
OVA 17w	**0,551***	1	0,038	0,174	0,346
TNF-α 8w	0,326	0,038	1	**0,622***	-0,023
TNF-α 12w	**0,497***	0,174	**0,622***	1	**0,354***
TNF-α 17w	**0,367***	0,346	-0,023	**0,354***	1
LPS 8w	0,051	0,064	-0,032	0,142	0,232
LPS 12w	0,117	0,342	0,324	0,389	0,301
LPS 17w	**0,398***	**0,415***	0,179	0,205	0,120
TEER duodenum	-0,283	**-0,794***	-0,164	-0,316	**-0,686***
TEER ileum	**-0,493***	**-0,627***	-0,118	0,015	**-0,495***
TEER 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.*

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 4)**.

Discussion

A causal relationship between the pathophysiological changes in the intestinal epithelium and the onset of obesity has been suggested in animal models [36] and humans [37]. Therefore, studies aiming to identify selective natural

compounds that modulate the development of obesity and its associated comorbid conditions, by ameliorating the intestinal barrier dysfunction will be instrumental in the future developing of interesting and promising therapeutic strategies. In this study, we determined the temporal relationship between obesity and impaired intestinal barrier function (intestinal permeability alterations and metabolic endotoxemia) in rats fed a CAF diet, and compared the effectiveness of the GSPE extract administered at two different time points.

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 [38]. This diet induces voluntary hyperphagia, resulting in rapid weight gain and an increase in the fat pad mass [38]. 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 [38]. In addition, the SIT-CAF supplementation with GSPE drastically reduced the body weight and adiposity index. As shown in our previous publication, these results were at least partially derived from the inhibition of energy intake and increase in energy expenditure (article under revision) and are consistent with the results obtained by Serrano *et al.* at using the same dose of GSPE administered to healthy rats [39].

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. At the 12th and 17th weeks, animals were submitted an oral OVA test, which is useful as an *in vivo* intestinal permeability marker.

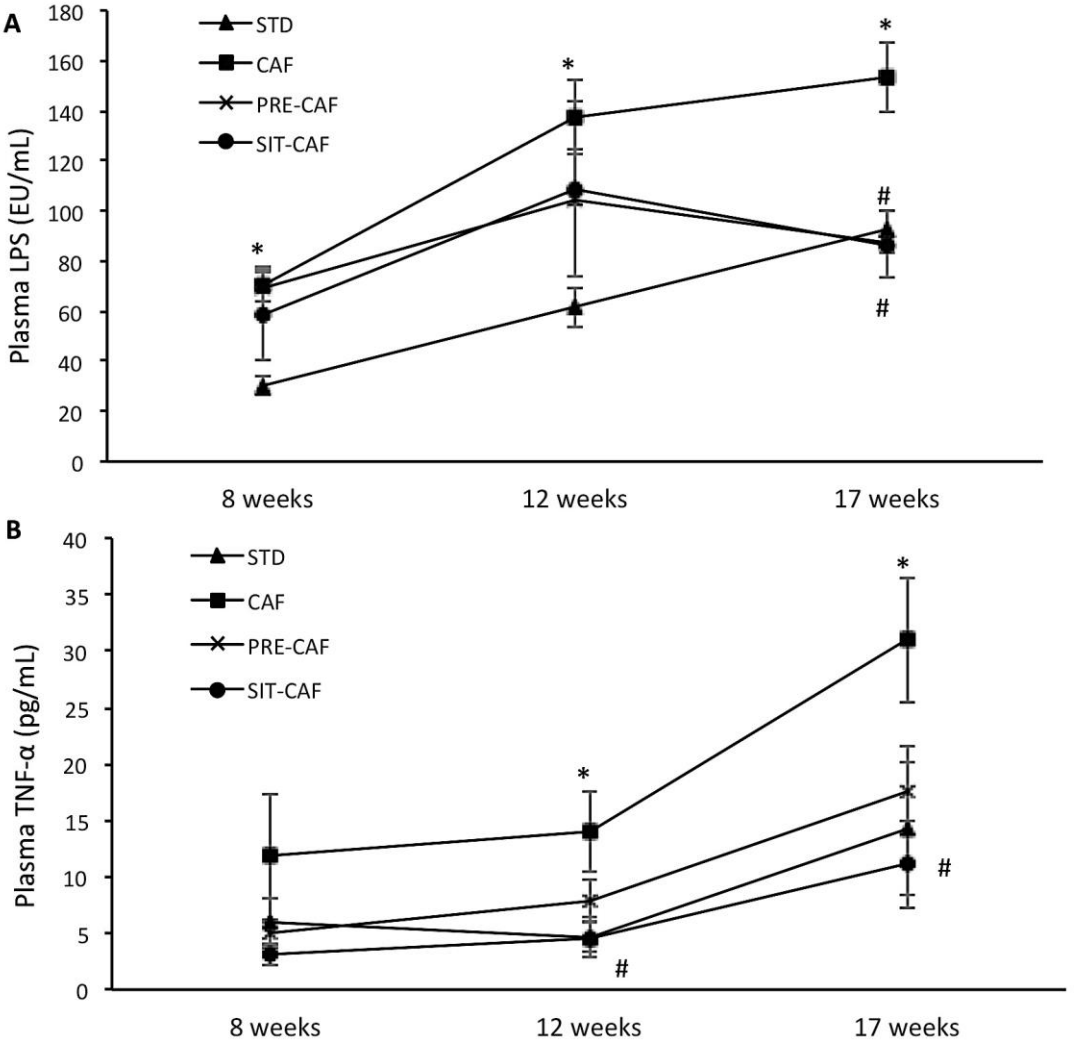

Figure 3. LPS (A) and TNF-α plasma levels (B) at weeks 8, 12 and 17 of CAF diet consumption. STD: lean rats fed a standard chow diet; CAF: rats fed a cafeteria diet; PRE-CAF: rats fed a cafeteria diet plus a 500 mg/kg GSPE pre-treatment of; SIT-CAF: rats fed a cafeteria diet simultaneously with a 500 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.

Table 4. Expression of TJ-related mRNAs in the duodenum, ileum and colon of rats fed a STD diet or a CAF diet either with or without GSPE supplementation.

	Duodenum						
	claudin-1	**claudin-2**	**claudin-3**	**ZO-1**	**OCLN**	**JAM-A**	**MLCK**
STD	n.d	n.d	1.26±0.23	1.12±0.19	1.09±0.15	1.35±0.28	0.96±0.18
CAF	n.d	n.d	1.38±0.37	1.44±0.34	1.33±0.32	1.13±0.23	1.13±0.12
PRE-CAF	n.d	n.d	0.78±0.11	0.92±0.16	1.10±0.13	1.34±0.29	0.98±0.17
SIT-CAF	n.d	n.d	1.06±0.15	0.99±0.08	1.10±0.26	1.37±0.22	1.57±0.40
	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
	Colon						
	claudin-1	**claudin-2**	**claudin-3**	**ZO-1**	**OCLN**	**JAM-A**	**MLCK**
STD	1.21±0.26	0.94±0.13	1.03±0.09	1.03±0.09	1.03±0.08	1.08±0.14	1.48±0.42
CAF	2.39±0.60	0.51±0.11	1.01±0.15	0.96±0.19	1.19±0.13	0.91±0.14	0.76±0.22
PRE-CAF	2.14±0.41	0.63±0.21	1.37±0.20	1.29±0.20	1.59±0.26	1.23±0.17	0.94±0.36
SIT-CAF	1.66±0.22	0.81±0.17	1.11±0.11	1.19±0.15	1.31±0.15	1.06±0.18	1.15±0.29

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; n.d: not detected. Values are presented as the means ± SEM. *P < 0.05 compared to the STD group. # P < 0.05 compared to the CAF group.

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. At the end of the experiment, intestinal permeability in the small (duodenum and ileum) and large intestines was evaluated *ex vivo* using the Ussing chamber system. Interestingly, 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 proanthocyandins on the intestinal permeability throughout the intestine of an obesity model *ex vivo.* We are the first group to show that both PRE-CAF and SIT-CAF proanthocyanidin treatments were able to normalized 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.* [40]. 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. PPs are isolated and aggregated lymphoid follicles that constitute the

gut-associated lymphoid tissue (GALT), which is one of the largest lymphoid organs and contains up to 70% of the immunocytes in the body [41]. 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 [42, 43]. Therefore, once the beneficial role of proanthocyanidins in the regulation of barrier function has been described, further studies should focus on ascertaining the inflammatory mechanisms involved in the effect of proanthocyanidins on the intestine, particularly in the ileum and the GALT.

Although a meaningful systemic impairment in intestinal permeability was obtained from the 12th week of the CAF intervention to the end of the experiment and proanthocyanidins exerted a significant protective effect, 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. Only 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 [24]. Thus, proanthocyanidins behave differently, depending on the dose administered and the time of administration. Moreover, due to the important effect of GSPE on intestinal permeability, we hypothesised that in addition to modulating TJ proteins, proanthocyanidins might exert their effects on other mechanisms of transport, such as the transcellular pathways. In fact, intact OVA is absorbed across intestinal epithelial cells through the paracellular as well as transcellular pathways [44]. Therefore, the observed changes in intestinal permeability following the administration of a CAF diet might be attributed to both pathways. Altogether, these findings provide new insights into the mechanisms

regulating of intestinal permeability. However, additional studies are needed to elucidate the mechanisms underlying the effects of the proanthocyanidins at the molecular level.

Increased intestinal permeability has been closely linked to systemic endotoxemia [20, 21, 45]. 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 gut 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. Under normal conditions, the presence of this endotoxin in the intestinal lumen does not cause negative effects on health. However, rodents fed a high-fat diet for extended periods exhibit DIO, increasing bacteria-derived LPS absorption across the intestinal barrier and enhancing its plasma levels by two- to three-fold through a process a known as metabolic endotoxemia [20, 21, 45]. Obesogenic diets may promote intestinal LPS absorption through different molecular pathways. One possibility is that some components of the diet promote paracellular leakage of LPS across the intestinal epithelium. This hypothesis is supported by observations that intestinal-epithelial TJ integrity is compromised in obese mice [40] and experimental exposure of the intestinal lumen to some fatty acids [46] or fructose [47] causes small intestinal epithelial damage. An alternative possibility is that LPS enters the bloodstream by transcellular transport through intestinal epithelial cells. This transport method could occur through the so called intestinal epithelial microfold cells (M-cells), which are permeable to bacteria and macromolecules and facilitate sampling of gut antigens by the underlying lymphoid tissue [48]. A third possibility is the

absorption and transport of LPS through mesenteric lymph nodes following chylomicrons formation [49]. Considering the results obtained in the present and our previous studies, any of the mechanisms identified as contributing to the detrimental effect of the CAF diet and the protective mechanism of action of proanthocyanidins should not be excluded.

Metabolic endotoxemia is also related to systemic inflammation. Upon entering the circulation, LPS induces the activation of toll-like receptors (TLR) 2 and 4, and the CD14 LPS receptor, leading to increased activation of inflammatory pathways through cytokine release and contributing to the state of systemic inflammation present in obese subjects [50–52]. In the present study, the CAF diet increased TNF-α levels from the 12th 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 consumption 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 suggests a key role of the gut-adipose tissue axis in the development of obesity and metabolic disorders. Additionally, our results highlight the fact that consumption of proanthocyanidins improves gut health, including intestinal permeability and

metabolic endotoxemia. In this sense, our study provides fundamental data regarding possible nutritional treatments for obesity, demosntrating the effectiveness of proanthocyanidins consumed in a SIT way. Although, this treatment has been reported to be the most effective strategy, a PRE-CAF treatment interestingly maintains a positive effect until the 12th week of the obesogenic diet intervention. Based on these findings, nutritional and therapeutic interventions based on gut 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.

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Conflict of Interest

None.

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4. MANUSCRIPT 5

Results

Protective effect of proanthocyanidins in a rat model of mild intestinal inflammation and impaired intestinal permeability induced by LPS

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Protective effect of proanthocyanidins in a rat model of mild intestinal inflammation and impaired intestinal permeability induced by LPS

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Abstract

Scope: Intestinal tight junction (TJ) disruption and increased bacterial endotoxins flux play key roles in inflammatory bowel diseases. Proanthocyanidins are anti-inflammatory agents that can act as intestinal modulators. This study analyzed the protective effects of two oral doses of a grape seed proanthocyanidin extract (GSPE) in a rat model of mild intestinal inflammation and impaired intestinal permeability induced by lipopolysaccharides (LPS).

Methods and results: TNF-α and LPS levels were measured to assess systemic inflammation and endotoxemia. Intestinal inflammation was determined using myeloperoxidase (MPO) and cyclooxygenase-2 (COX-2) activity assays, and also the gene expression of inflammation related genes. Intestinal permeability was

determined by ovalbumin assay and the gene expression of TJs. LPS administration increased plasma TNF-α and intestinal MPO and COX-2 activity accompanied by the overexpression of inflammatory genes and increased intestinal permeability; however, GSPE played a protective role by normalizing most of these parameters.

Conclusions: A solid model of intestinal inflammation and altered intestinal permeability is offered in the present study. Both doses of GSPE prevent endotoxin induced-intestinal inflammation and permeability; however, the inflammatory response and the effect of the GSPE are different in each intestinal section. These results should be considered for inflammation and altered permeability associated diseases.

Keywords: intestine, inflammation, permeability, proanthocyanidins and lipopolysaccharide

Introduction

Intestinal dysfunction is a common feature of several diseases, including inflammatory bowel disease (IBD) [1] and obesity [2, 3]. Intestinal dysfunction is based on a pro-inflammatory state in the intestine and by a defective intestinal barrier function that, in its turn, allows the flux of luminal endotoxins through the epithelial cell to the underlying lamina propria and inner layers [4].

Intestinal inflammation is characterized by the activation of inflammatory cells and polymorphonuclear neutrophils and the infiltration of macrophages [5, 6], stimulating the production of high levels of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α) [7]. Intestinal inflammation is also associated with increased myeloperoxidase (MPO) enzyme activity in the ileum [8]. This enzyme is a key component of the oxygen-dependent microbial activity of

phagocytes and neutrophils [9]. Other pro-inflammatory enzymes, such as cyclooxygenase-2 (COX-2), are also relevant in intestinal inflammation [10]. Although the gastrointestinal (GI) tract expresses both COX-1 and COX-2 isoforms, the inducible form COX-2 is the one that is relevant to the inflammatory response and COX-1 seems constitutive in the absence of stimuli [11].

In addition, intestinal dysfunction is also associated with alterations in intestinal permeability [12, 13]. The paracellular pathway is one of the main pathways mediating the transport of molecules through the intercellular spaces between epithelial cells and it is perturbed upon disruption of the intestinal barrier [14]. This mechanism is regulated by intercellular complexes localized at the apical-lateral membrane junction, highlighting the role of tight junctions (TJs) [15]. Claudin and occludin (OCLN) as well as family members and junctional adhesion molecules (JAM) are linked to the actin cytoskeleton by cytoplasmic rafts formed by catenins and zonulin/zonula occludens (ZO)-1,-2, and -3 constituting TJs complex [16].

Several studies have shown lipopolysaccharides (LPS) to be an important contributing factor to intestinal dysfunction [17–19] . LPS at low doses does not cause cell death but can cause an increase in pro-inflammatory markers. In normal healthy individuals, plasma LPS concentrations are up to 0.2 ng/mL and proceed from the intestinal lumen [20, 21]. Physiological factors, such as a high fat diet, can increase LPS levels in plasma to as high as 1-2 ng/ml [22]. However, the presence of intestinal permeability disorders, such as Crohn's disease, can also lead to elevated plasma levels as high as 2 ng/mL, which are clinically relevant at 10 ng/mL [21, 23].

For a long time now there have been studies of animal models of intestinal dysfunction, however, these models are generally very aggressive and mainly induced by chemicals but not physiologic inducers, such as TNBT or DSS [24,

25]. In contrast, mice exposed to LPS at low doses intraperitoneally (ip) can develop the features of a mild intestinal inflammation and increased intestinal permeability [26]. They thus resemble a state similar to that found in humans with intestinal inflammation, like in obesity. However, this model has not yet been fully explored in rats.

Several natural compounds from the flavonoid family have been described as anti-inflammatory agents that are also able to prevent increased intestinal permeability [27]. Amongst them, proanthocyanidins are one of the most interesting compounds [28]. Proanthocyanidins are phenolic compounds (flavan-3-ols) that are found widely in fruits and are associated with beneficial effects on health, like anti-inflammatory activities [29]. Although their effect on intestinal dysfunction has been little studied [30, 31] their properties make them good candidates for affecting intestinal physiology. In this study, we hypothesize that proanthocyanidins ingested preventively can protect against intestinal inflammation and barrier disruption derived from LPS administration.

Materials and Methods

Grape seed proanthocyanidin extract

Grape seed proanthocyanidin extract or GSPE was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer the proanthocyanidin profile of the GSPE was composed 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. A chromatographic separation was performed for the quantification. The GSPE composition used in this study has been previously analysed and published in Margalef et al [32].

Animals and experimental design

Eight-week-old male Wistar rats (Charles River Laboratories, Barcelona, Spain)

were housed individually in a 22ºC temperature-controlled room with a 12-h light/dark cycle. The animals were fed *ad libitum* with a standard diet (SCD, Panlab A-04, Barcelona, Spain) and tap water, which was renewed daily. After an adaptation period, rats were randomly distributed into four experimental groups (n=6). The first two groups received a preventive treatment with proanthocyanidins (75 mg GSPE/Kg bw and 375 mg respectively) for fifteen days. When the treatment finished, animals were injected with 300 ug/kg bw of LPS ip every 24 hours for the last 5 days of the experimental period. The third group received the vehicle and the ip LPS injection. The control group received skimmed condensed milk as the vehicle and an ip saline solution **(Figure 1)**. The bacterial endotoxin used for this study was isolated from E.coli 0111:B4 and was purchased from Sigma Aldrich (Madrid, Spain).

Intestinal permeability was assessed using the ovalbumin (OVA) test [33]. Animals were previously fasted overnight. On the day of the sacrifice, OVA (Sigma-Aldrich, Madrid, Spain) was administered to the rats by orally gavage at a concentration of 250 mg/Kg bw. One hour after gavage, rats were euthanized by exsanguination under anaesthesia. Blood was collected from the abdominal aorta, heparinized and centrifuged (10 min, 12,000*g*, 4 °C). The plasma was stored at -80 °C for analysis of OVA by ELISA, with a detection range of 16-10,000 pg/mL (MyBioSource, Madrid, Spain).

The intestine and white adipose tissue depots (mesenteric, epididymal and retroperitoneal) were rapidly removed, weighed, frozen in liquid nitrogen and stored at -80 ºC until analysis. All the procedures were performed with the approval of the Animal Ethics Committee of the Universitat Rovira i Virgili (Tarragona, Spain).

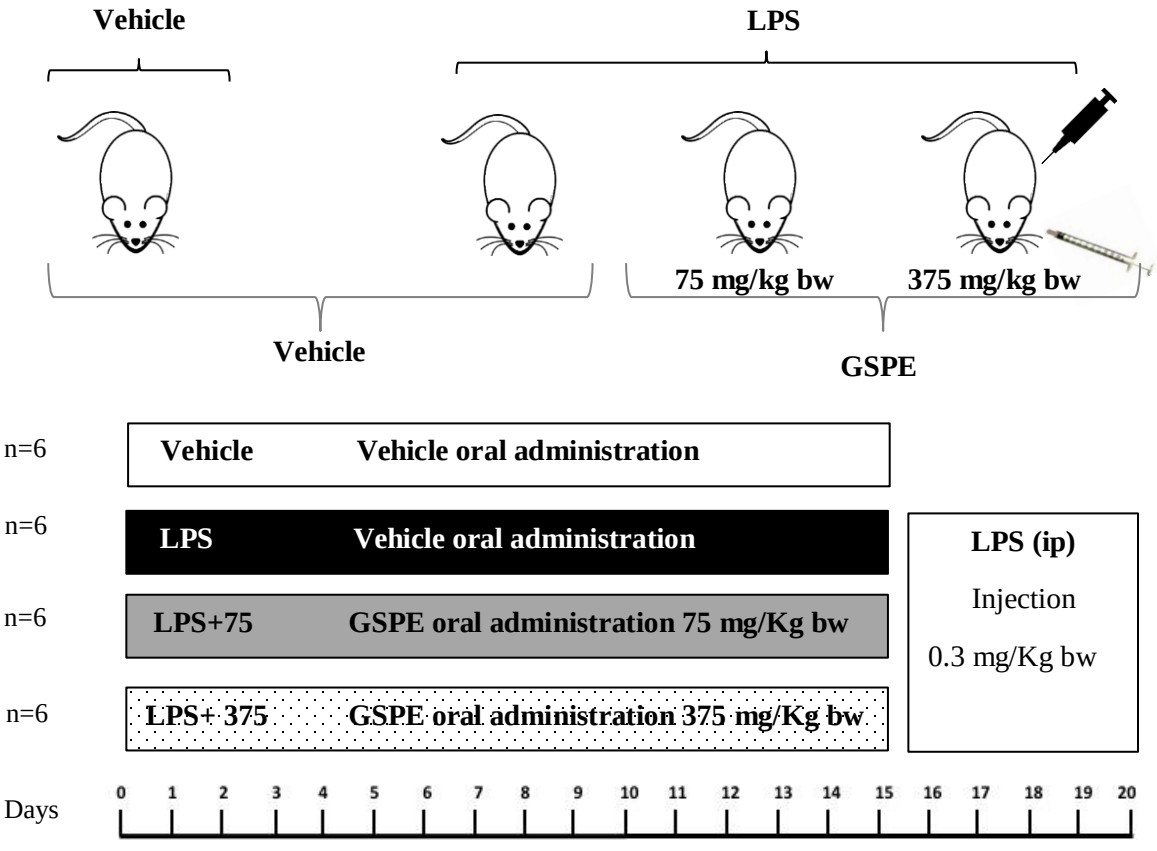

Figure 1. Schematic diagram of the experimental design. Twenty-four male Wistar rats were randomly distributed into four experimental groups (n=6): 1) The vehicle group was fed vehicle for 15-days and received an ip saline injection for 5 days more; 2) the LPS group was fed vehicle for 15-days and received an ip LPS injection for 5 days more; 3) the LPS+75 group was fed a GSPE extract at 75 mg/kg bw for 15 days and then received an ip LPS injection for 5 days more; and 4) the LPS+375 group was fed a GSPE extract at 375 mg/kg bw for 15 days and then received an ip LPS injection for 5 days more. After overnight fasting the animals were euthanized and the blood was collected from the abdominal aorta, heparinized and centrifuged. The gastrointestinal tract was sectioned into the duodenum, jejunum, ileum and distal and proximal colon and sections were rapidly removed and stored until analysis.

Determination of plasma parameters

Triglycerides (TAG), glucose levels (QCA, Barcelona, Spain) and the concentration of β-hydroxybutyrate (Materlab, Madrid, Spain) were measured

using enzymatic colorimetric methods. The non-esterified free fatty acid enzymatic colorimetric kit was purchased from WAKO (Zaragoza, Spain). An ELISA kit was used to measure the plasma concentrations of TNF-α (Merck Millipore, Madrid, Spain). The plasma LPS level was measured using Pyrochrome Lysate Mix, a quantitative chromogenic reagent (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. The manufacturer's protocol was followed in all cases.

Histological assessment of intestinal samples

Histological observations were performed with haematoxylin-eosin staining. At the end of the experiment the different sections of the small intestine (duodenum, jejunum and ileum) and the colon were fixed with formalin. After that, sections with a thickness of 5 μm were prepared with a microtome (MicroTec, Rotary microtome, CUT 4050). These slides were observed microscopically (Nikon Eclipse- TE200).

Quantification of myeloperoxidase activity and reactive oxygen species in the GI tract

Both MPO and reactive oxygen species (ROS) were analysed in the different sections of the small and large intestine following the protocol described previously [34]. For MPO analysis we used a modification of the 2-*o*-methoxyphenol (guaiacol) peroxidation assay and for ROS measurement we used the 2',7'-dichlorofluescein diacetate (DCFH-DA) method.

Quantification of COX-2 activity in the GI tract

To measure the COX-2 activity, tissue was homogenized in 1 mL of cold buffer (0.1 M Tris-HCl, pH 7,8 containing 1 mM EDTA) with the TissueLyser LT system (Qiagen, Hilden, Germany). The homogenate was then sonicated for 20 seconds and centrifuged at 10.000x g for 15 minutes at 4ºC. The COX-2 activity was

tested with a commercial colorimetric COX Activity Assay Kit (Cayman Chem., Madrid, Spain) that measures the peroxidase activity of this enzyme at 590 nm. We followed the manufacturer's protocol. The kit includes isozyme-specific inhibitor to distinguish COX-2 from COX-1 activity.

Tissue RNA extraction and gene expression analysis by Taqman Low Density Arrays and qRT-PCR

RNA isolation. Total RNA was extracted from ileum tissue using an innuPREP RNA mini kit (Analytikjena, Berlin, Germany) according to the manufacturer's instructions. RNA integrity and concentration were checked with an Agilent RNA6000 NanoKit (Agilent, Santa Clara, California). The isolated RNA was used in all card array analyses.

cDNA synthesis and array data analysis. RNA was converted into cDNA using High-Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. The cDNA obtained was diluted 10-fold and used in RT^2 Profiler PCR Arrays (Qiagen, Hilden, MD). To each 96 well plate, we added 100µl of diluted cDNA. After sealing the RT^2 Profiler PCR plates, we centrifuged and soaked the plate at room temperature for 15 min prior to loading it into a qPCR machine. The reaction was carried out in a 7500 Fast Real Time PCR (Applied Biosystems). The thermal protocol was 95°C for 10 min (holding stage); followed by 40 cycles of two steps of denaturation at 95°C for 15 sec and extension at 60°C for 1 min. Finally, the melt curve stage was 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec [35]. Data was exported to a Microsoft Excel file spreadsheet and matched with the array pattern.

Ileum RNA was used for the Taqman Low Density Arrays, and 84 genes related to the inflammation process were analysed, including: interleukins, chemokines, chemokine receptors and receptor ligands. In addition, 12 reference and control genes (Applied Biosystems, Rome, Italy) were used.

The results of the gene expression were analysed using the LPS treated group as the control. A Student's t-test was used for comparisons with a $P< 0.05$. Fold change (2^(- Delta Delta C_t)) is the normalized gene expression (2^(- Delta C_t)) in the Test Sample divided the normalized gene expression (2^(- Delta C_t)) in the Control Sample. Fold change values greater than 1.9 indicate positive or up-regulation. Fold change values less than 0.6 indicate a down-regulation [36].

Tissue RNA extraction and gene expression analysis by qRT-PCR

RNA isolation. Total RNA was extracted from 50 mg of duodenum and ileum using Trizol (AMbion, USA) following the manufacturer's instructions as we described previously [34]. cDNA was obtained from 1 μg of mRNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) following the manufacturer's instructions.

cDNA synthesis and array data analysis. 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): Rn02116071_s1 for rat ZO-1, Rn01420322_g1 for rat OCLN, Rn02063575_s1 for rat claudin-2, Rn00581751_s1 for rat claudin-3, Rn00587389_m1 for rat JAM-A. All the results were normalized with respect to cyclophilin E (PPIA) (Rn00690933_m1).

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 at 95 °C for 15 seconds and at 60 °C for 2 minutes.

Statistical analysis

The results are expressed as the mean value ± standard error of the mean (SEM). The effects of LPS and GSPE were assessed using an ANOVA test with its appropriate post hoc test. P-values < 0.05 were considered statistically

significant. These calculations were performed using the IBM-SPSS 21.0 software.

Results

The effect of LPS and proanthocyanidins on biochemical parameters

We measured general biochemical parameters in plasma to assess glucose and lipid metabolism states, as well as ketogenesis, after the preventive supplementation with 75 or 375 mg/kg bw of GSPE and LPS injection in the circulation of experimental animals.

Neither the GSPE nor LPS administration modifies the general biochemical parameters in an abnormal way. However, there were some slight modifications: glucose levels were slightly decreased in the 75 mg/kg bw GSPE group. The β-hydroxybutyrate levels were slightly decreased in the LPS and high dose GSPE treated group; however, these values are included in the normal range, indicating that ketogenesis was not present in any experimental group. With respect to NEFAs, the LPS and the high dose GSPE group showed decreased the NEFA levels significantly; no changes were observed in TAG levels **(Table 1)**.

The effect of LPS and proanthocyanidins on systemic inflammation and intestinal permeability

Plasma LPS levels were analysed as a measure of endotoxemia 24h after LPS injection. There were no significant changes in plasma LPS levels with respect to the vehicle group or with respect to the GSPE treated groups **(Figure 2A)**. TNF-α levels were analysed in plasma as a marker of systemic inflammation. A significant increase in TNF-α was found in the plasma of the LPS group, as expected. However, GSPE administration at two different doses did not counteract the effect of LPS on TNF-α levels **(Figure 2B)**.

Table 1. Biochemical parameters.

	Vehicle	LPS	LPS + 75	LPS + 375
Glucose (mM)	10.87 ± 0.15 a	9.59 ± 1.02 ab	9.78 ± 0,26 b	10.97 ± 0.85 a
β-hydroxybutyrate (mM)	1.06 ± 0.09 a	0,71 ± 0.00 b	0,81 ± 0.04 ab	0.76 ± 0.07 b
NEFAs (mM)	3.92 ± 0.08 a	2.85 ± 0.02 bc	3.34 ± 0.15 ab	2.32 ± 0.23 c
TAG (mM)	0.63 ± 0.09 a	0.51 ± 0.06 a	0.60 ± 0.06 a	0.52 ± 0.06 a

The statistical significance among groups was evaluated using an ANOVA test ($P<0.05$). Different letters indicate significant differences between treatments. n=6 in each experimental group. Vehicle: rats received skimmed condensed milk as the vehicle and an ip saline solution; LPS: rats received the vehicle and the ip LPS; LPS + 75: rats received a preventive treatment with proanthocyanidins (75 mg GSPE/Kg bw) plus LPS and LPS + 375: rats received a preventive treatment with proanthocyanidins (375 mg GSPE/Kg bw) plus LPS**.**

The LPS and/or GSPE effects on intestinal epithelial permeability were determined *in vivo* by measuring the concentration of OVA in plasma after its oral administration. The intestine of the LPS group was demonstrated to be more permeable to macromolecules, such as OVA compared to the vehicle group **(Figure 2C)**. Interestingly, the GSPE treatment at a dose of 75-mg/kg bw decreased the OVA flux, evidencing a protective effect against barrier disruption **(Figure 2C)**.

We analysed each part of the small and large intestine by general histology under haematoxylin and eosin staining to assess whether the LPS treatment could have produced intestinal damage. The duodenum, jejunum and colon in LPS-treated rats appeared normal in all the animals and sections; however, a light morphologic disorganization was observed in the ileum **(Figure 2 D)**.

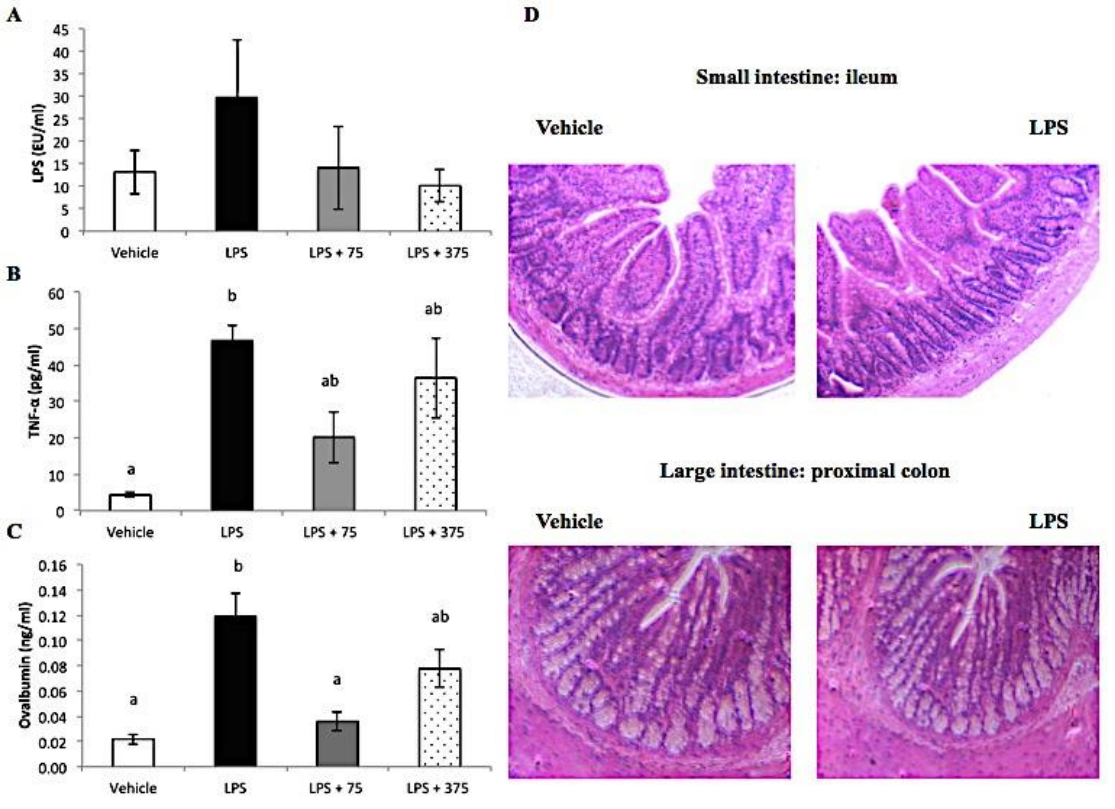

Figure 2. Endotoxin and TNF-α plasma levels, ovalbumin intestinal permeabilitytest and histologic assessment in rats with ip LPS administration and the effect of the proanthocyanidin extract. A: Effect of LPS and proanthocyanidins on plasma LPS levels. B: Effect of LPS and proanthocyanidins on plasma TNF-α levels. C: Ovalbumin intestinal permeability test. D: Effect of LPS administration and proanthocyanidin treatment on intestinal tissue. Vehicle: rats received skimmed condensed milk as the vehicle and an ip saline solution; LPS: rats received the vehicle and the ip LPS; LPS + 75: rats received a preventive treatment with proanthocyanidins (75 mg GSPE/Kg bw) plus LPS and LPS + 375: rats received a preventive treatment with proanthocyanidins (375 mg GSPE/Kg bw) plus LPS. Graphs (A-C) show the mean and SEM. n = 6 in each experimental group. An ANOVA test was used to evaluate significance between groups (P<0.05). Hematoxilin and eosin stain was used in graph D, original magnification, x40.

Effects of LPS and proanthocyanidins on local intestinal inflammation

To determine and describe the impairment induced by LPS along the gastrointestinal tract as well as the effect of the treatment with GSPE, we determined the activities of two enzymes that are indicators of inflammation (MPO and COX-2) in the different sectioned parts of the small intestine (duodenum, jejunum and ileum) and the large intestine (proximal and distal colon) in all the animals.

The duodenum showed the highest MPO activity of the intestinal sections of the small intestine and this activity decreased as we approached the distal area of the intestine **(Figure 3A-D)**. In terms of the effect of LPS on the intestine, all the sections showed the highest levels of MPO activity in the LPS group; however, these values were only significant in the ileum section **(Figure 3C)**. Regarding the effect of GSPE, in the duodenum and ileum sections of the small intestine, the GSPE treatment had a significantly decreased effect on MPO activity **(Figure 3A and C)**. In the duodenum, both the high and low doses of proanthocyanidin extract significantly reverted the inflammatory damage caused by LPS, but there were no differences between the two doses. However, in the ileum the higher dose was the effective. No changes were observed in the jejunum section or in the large intestine **(Figure 3B, D and E)**.

In addition, COX-2 activity was also determined throughout the intestine. According to **Figure 4**, the administration of LPS significantly increased the activity of COX-2 in the duodenum, but not in the ileum, jejunum or colon sections. Moreover, the 75 and 375 mg/kg bw doses of GSPE administered had a significant effect by decreasing COX-2 activity in the duodenum, but there were no differences between the two doses **(Figure 4 A)**.

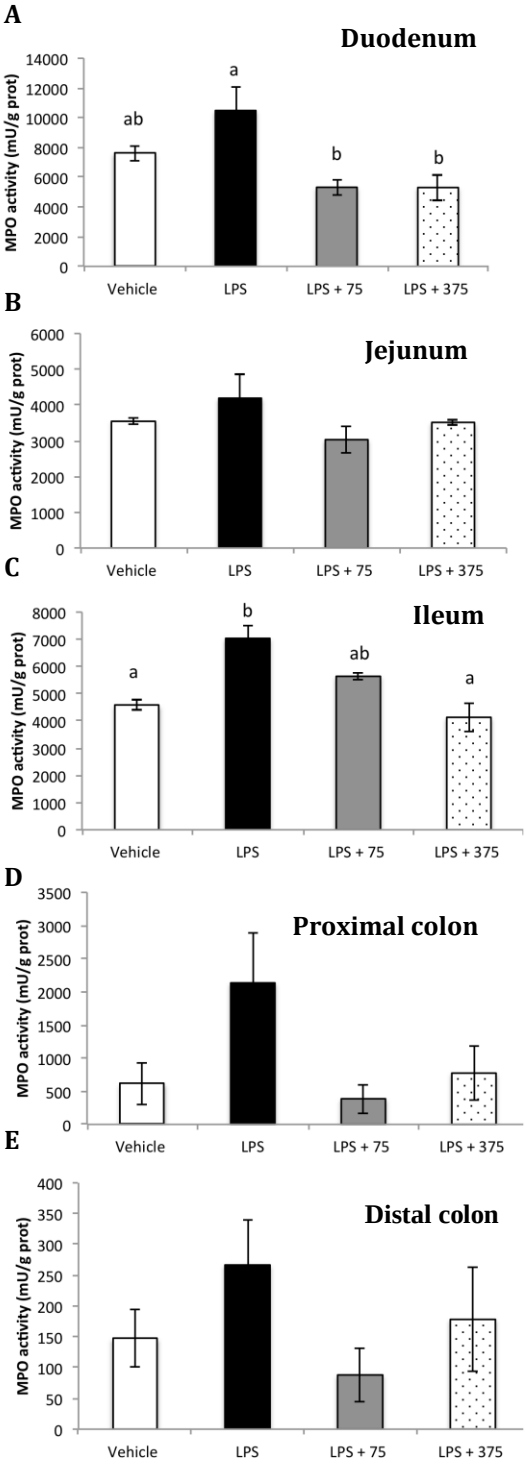

Figure 3. MPO activity in the small and large intestinal sections of rats. Effect of LPS and the proanthocyanidin treatment. A) Duodenum, B) Jejunum, C) Ileum, D) Proximal colon and E) Distal colon. Vehicle: rats received skimmed condensed milk as the vehicle and an ip saline solution; LPS: rats received the vehicle and the ip LPS; LPS + 75: rats received a preventive treatment with proanthocyanidins (75 mg GSPE/Kg bw) plus LPS and LPS + 375: rats received a preventive treatment with proanthocyanidins (375 mg GSPE/Kg bw) plus LPS. Graph shows the mean and SEM. n = 6 in each experimental group. An ANOVA test was used to evaluate significance between groups (P<0.05).

Intestinal oxidative stress assessment and the role of oral proanthocyanidins

To determine the role of oxidative stress in the gastrointestinal tract impairment induced by LPS, we determined the ROS levels in the different parts of the small intestine (duodenum, jejunum and ileum) as well as in the distal and proximal colon in all the animals. None of the small intestine sections showed an increase in the ROS levels due to the LPS treatment. Moreover, the two doses of proanthocyanidin extract did not exert any significant change in the small intestine oxidative stress **(Figure 5A, B, and C).**

When we looked for an association between ROS levels and MPO activity, we found that these parameters were positively correlated in the duodenum ($r=0.662$, $p< 0,000$) and nearly in the ileum ($r= 0.438$, $p=0,053$) but not in the jejunum section ($r=-0.104$, $p= 0.627$).

In contrast to the small intestine, the LPS group showed a significant increase in ROS levels in the proximal and distal colon. Moreover, the two treatments with GSPE normalized the ROS levels to those found in the vehicle group in the proximal colon but only the low dose in the distal colon **(Figure 5 D and E)**. The analysis of the associations between ROS levels and MPO activity showed that they tended to be correlated in the distal colon ($r=0.394$, $p< 0.057$) but not in the proximal colon ($r=0.290$, $p<0.180$).

Effect of LPS and/or proanthocyanidins on inflammation-associated protein gene expression

We analysed the gene expression of 84 genes that encode for inflammation-associated proteins in the ileum by TLDM (Taqman Low Density Microarrays) technic. The results of the gene expression were obtained considering the LPS treated group as the control, in order to establish a negative local effect in the

intestine derived by the LPS and a possible GSPE protective effect. The results are shown in **Table 2**.

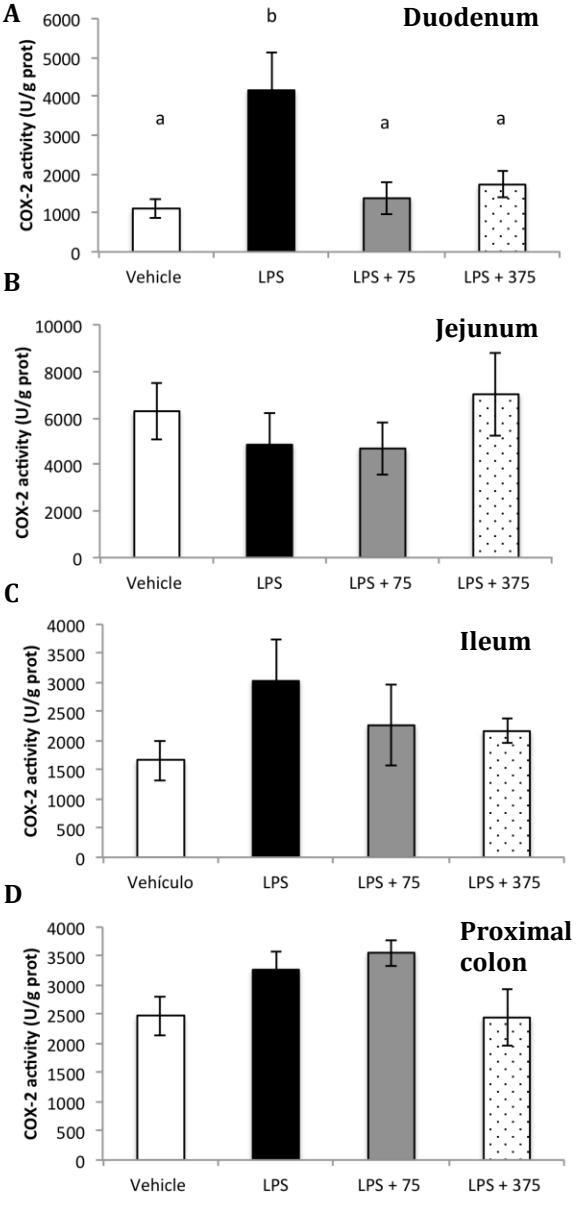

Figure 4. COX-2 activity in the small and large intestine. A) Duodenum, B) Jejunum, C) Ileum and D) Proximal colon. Effect of LPS and the proanthocyanidin treatment. Vehicle: rats received skimmed condensed milk as the vehicle and an ip saline solution; LPS: rats received the vehicle and the LPS ip; LPS + 75: rats received a preventive treatment with proanthocyanidins (75 mg GSPE/Kg bw) plus LPS and LPS + 375: rats received a preventive treatment with proanthocyanidins (375 mg GSPE/Kg bw) plus LPS. Graph shows the mean and SEM. n = 6 in each experimental group. An ANOVA test was used to evaluate significance between groups (P<0.05).

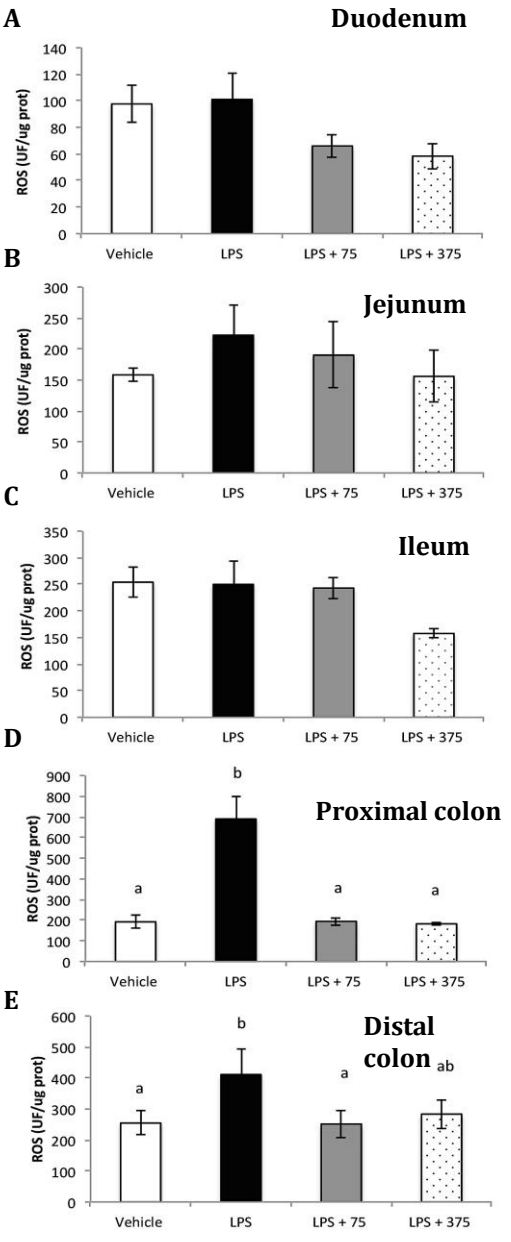

Figure 5. ROS levels in the small and large intestine of rats treated with LPS and/or proanthocyanidins. A) Duodenum, B) Jejunum, C) Ileum, D) Proximal colon and E) Distal colon. Vehicle: rats received skimmed condensed milk as the vehicle and an ip saline solution; LPS: rats received the vehicle and the ip LPS injection; LPS + 75: rats received a preventive treatment with proanthocyanidins (75 mg GSPE/Kg bw) plus LPS and LPS + 375: rats received a preventive treatment with proanthocyanidins (375 mg GSPE/Kg bw) plus LPS. Graph shows the mean and SEM. n = 6 in each experimental group. An ANOVA test was used to evaluate significance between groups (P<0.05).

LPS treatment induced the up-regulation of 54 genes **(Table 2)**, most of them associated with a pro-inflammatory immune response. They mainly include chemokines pertained CC family, like MCP-1, and their respective receptors, CXC chemokines and their receptors, interleukins, like IL-1β, and TNF ligands **(Table 2)**. In this analysis, we also found that the low dose of GSPE was not associated with significant variations of this profile with the exception of CCL9

and 19 suppression and CCR5 overexpression. In contrast, the high dose of GSPE (375 mg/kg bw) had a stronger effect in the gene expression profile compared to the LPS group showing the down-regulation of CCL20 and the overexpression of 13 genes including CC and CXC chemokines and interleukins. In addition, some gene promoting inflammation, such as IL-17B and CCL6, were up-regulated **(Table 2)**. Noteworthy, IL-13, which is considered strongly anti-inflammatory, was also upraised **(Table 2)**.

Effect of LPS and/or proanthocyanidins on tight junction-associated protein gene expression

To evaluate the role of proanthocyanidins in the differential gene expression in the LPS-induced intestinal permeability in Wistar rats, an analysis of gene expression in the duodenum and ileum was performed. These two sections were analysed because they have been shown to be more susceptible to the inflammatory effect of LPS in the intestine. The gene expression of some representative transmembrane proteins that form TJ: JAM-A, OCLN, claudin-2 and 3, and the gene expression of ZO-1, which is an intracellular plaque protein that anchors TJ to the actin component of the cytoskeleton, were evaluated.

In the duodenum, none of the selected genes showed any response to LPS or the treatment except JAM-A, which had a significant overexpression in the GSPE 375-mg/kg bw group with respect to the other groups **(left panel, Figure 6)**. In the ileum **(right panel, Figure 6)** the administration of LPS significantly increased the gene expression of claudin-2 and both doses of GSPE normalized these results. In addition, no effect of LPS was observed with respect to the expression pattern of the rest of the genes; however, the GSPE treatment resulted in a significant down-regulation in the ileum gene expression of ZO-1, JAM-A and OCLN.

Table 2. Taqman Low Density Array of inflammatory related genes in the ileum compared to LPS group.

VEHICLE			
Gene Symbol	**Other aliases**	**Fold change**	**Protein biological function**
CCL2	MCP-1	0.4785	Chemotactic activity for monocytes and basophils
CCL3	MIP-1-α	0.5486	Inflammatory activity
CCL4	MIP-1-β	0.5216	Chemokinetic and inflammatory activity
CCL7	MCP-3	0.5026	Chemotactic activity for monocytes and eosinophils
CCL9	MIP-1-γ	0.3048	Chemotactic activity for dendritic cells
CCL11	Eotaxin-1	0.5636	Chemotactic activity for eosinophils
CCL12	MCP-5	0.5190	Murine monocyte chemotactic/activating factor
CCL17	TARC	0.5289	Chemotactic activity for T lymphocytes
CCL19	MIP-3-β	0.4437	Chemokine role in homing of lymphocytes
CCL20	MIP-3-α	0.3533	Chemotactic activity for lymphocytes
CCL22	STCP-1	0.5290	Chemotactic activity for monocytes, dendritic cells and natural killer
CCL24	Eotaxin-2	0.5594	Chemotactic activity on resting T lymphocytes
CCR1	MIP-1-αr	0.5602	Chemokine receptor
CCR2	MCP-1r	0.4808	Chemokine receptor
CCR3	CMKBR3	0.4825	Chemokine receptor
CCR4	CMKBR4	0.5024	Chemokine receptor
CCR5	CMKBR5	0.4857	Chemokine receptor
CCR6	CMKBR6	0.5440	Chemokine receptor
CCR8	CMKBR8	0.5530	Chemokine receptor
CD40LG	TNFr ligand 5	0.4483	TNF receptor ligand
CSF1	MCSF	0.5832	Macrophage stimulating activity
CSF3	GCSF	0.5495	Granulocytes stimulating activity
CXCL1	GRO-α	0.5966	Chemoattractant activity for neutrophils
CXCL2	MIP-2-α	0.5159	Immunoregulatory and inflammatory activity
CXCL6	GCP-2	0.5209	Chemotactic activity for neutrophil granulocytes

CXCL9	CMX	0.5793	Chemoattractant activity for lymphocytes
CXCL11	SCYB11	0.5894	Chemotactic activity for interleukin-activated T-cells
CXCR1	IL-8αr	0.5479	Chemokine receptor
CXCR2	IL-8βr	0.4936	Chemokine receptor
CXCR5	BLR1	0.5320	Chemokine receptor
IFN-γ	Interferon γ	0.5551	Immunoregulatory activity
IL-1β	Interleukin-1β	0.5899	Pro-inflammatory cytokine
IL-3	Interleukin-3	0.5144	Granulocyte/macrophage colony-stimulating factor
IL-4	Interleukin-4	0.5210	Pleiotropic cytokine
IL-5	Interleukin-5	0.5502	Growth and differentiation factor for B cells and eosinophils
IL-11	Interleukin-11	0.5290	Stimulation of hematopoietic stem cells proliferation
IL-13	Interleukin-13	0.5943	Inhibition of inflammatory cytokine production
IL-17A	Interleukin-17A	0.4870	Pro-inflammatory cytokine
IL-17B	Interleukin-17B	0.4903	Stimulation of TNF-α and IL-1β
IL-17F	Interleukin-17F	0.4507	Stimulation of cytokines production
IL-21	Interleukin-21	0.4804	Immunoregulatory activity
IL-33	Interleukin-33	0.5763	Maturation of Th2 cells
LTα	Lymphotoxin α	0.5476	Modulation of inflammatory and immunostimulatory responses
LTβ	Lymphotoxin β	0.5386	Inflammatory activity
MIF	Macrophage migration inhibitory factor	0.5304	Lymphokine involved in cell-mediated immunity and inflammation
OSM	Oncostatin-M	0.5092	Regulation other cytokines production
PF4	Oncostatin-A	0.5077	Chemotactic activity
RGD1561905	C5	0.5101	Component of the complement system
SPP1	Secreted phosphoprotein 1	0.5027	Cytokine that upregulates IFN-γ and IL-12 expression
TNFRSF1B	TNF receptor superfamily member 11B	0.5926	TNF-receptor superfamily
TNFSF4	TNF superfamily member 4	0.4232	TNF ligand
TNFSF11	TNF superfamily member 11	0.4647	TNF ligand
TNFSF13B	TNF superfamily member 13	0.4336	TNF ligand
TNFSF14	TNF superfamily member 14	0.4924	TNF ligand

LPS 75 mg/kg bw			
Gene Symbol	**Other aliases**	**Fold change**	**Protein biological function**
CCL9	MIP-1-γ	0.5006	Chemotactic factor for dendritic cells
CCL19	MIP-3-β	0.5894	Chemokine role in homing of lymphocytes

LPS 375 mg/kg bw			
Gene Symbol	**Other aliases**	**Fold change**	**Protein biological function**
CCL20	MIP-3-α	0.4481	Chemotactic activity for lymphocytes
CCL2	MCP-1	2.3247	Chemotactic activity for monocytes and basophils
CCL6	MRP-1	5.1205	Rodent chemokine
CXCL1	GRO-α	2.1194	Chemoattractant activity for neutrophils
CXCL2	MIP-2-α	2.4229	Immunoregulatory and inflammatory activity
CXCL6	GCP-2	2.3258	Chemotactic activity for neutrophil granulocytes
IL-1α	Interleukin-1α	3.297	Immune pleiotropic activity
IL-2rg	IL-2r subunit γ	2.5027	Signalling component of interleukin receptors
IL-5	Interleukin-5	2.9474	Growth and differentiation factor for B cells and eosinophils
IL-13	Interleukin-13	2.4391	Inhibition of inflammatory cytokine production
IL-17B	Interleukin-17B	2.8163	Stimulation of TNF-α and IL-1β
IL-17F	Interleukin-17F	2.2887	Stimulation of cytokines production
IL-27	Interleukin-27	2.1215	Pro- and anti-inflammatory properties
IL-6r	Gp80	2.4593	Interleukin receptor

Gene expression results were analysed using the LPS treated group as the control. A Student's t-test was used for comparisons with a P< 0.05. Fold change (2^(- Delta Delta C_t)) was the normalized gene expression (2^(- Delta C_t)) in the Test Sample divided the normalized gene expression (2^(- Delta C_t)) in the Control Sample. Fold change values greater than 1.9 indicate a positive or up-regulation. Fold-change values less than 0.6 indicate a down-regulation.

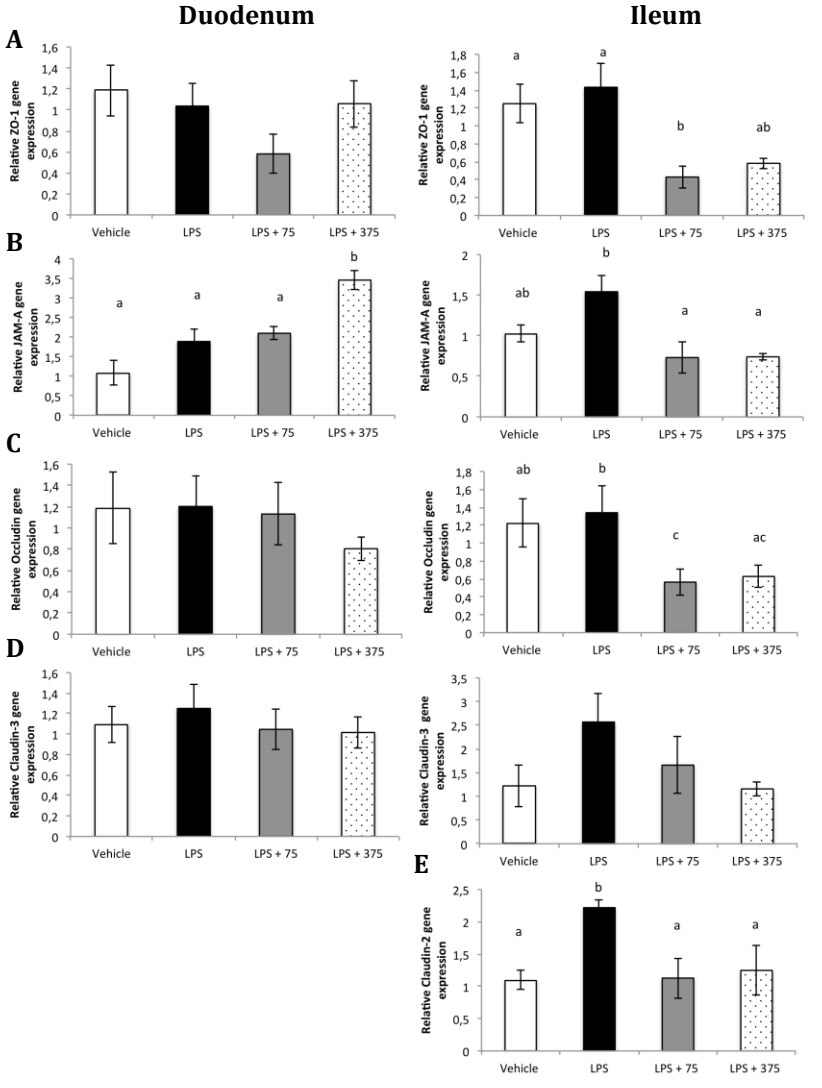

Figure 6. Effect of LPS and proanthocyanidins on tight junction-associated protein gene expression in the duodenum and ileum. A) ZO-1, B) JAM-A, C) Occludin, D) Claudin-3 and E) Claudin-2 only in the ileum, this gene was not expressed in the duodenum. Vehicle: rats received skimmed condensed milk as the vehicle and an

ip saline solution; LPS: rats received the vehicle and the ip LPS injection; LPS + 75: rats received a preventive treatment with proanthocyanidins (75 mg GSPE/Kg bw) plus LPS and LPS + 375: rats received a preventive treatment with proanthocyanidins (375 mg GSPE/Kg bw) plus LPS. Graph shows the mean and SEM. n = 6 in each experimental group. An ANOVA test was used to evaluate significance between groups ($P<0.05$).

Discussion

In the present study, we first evaluated whether the ip administration of LPS endotoxin to Wistar rats can induce intestinal inflammation and intestinal permeability impairment without signs of evident and excessive aggression to the intestinal epithelium. This would make it a strategic good model for studying early intestinal disease events in rats, as Guo and colleagues previously demonstrated in mice [26]. Parallel, we compared and determined whether the previous oral consumption of a high nutritional (75 mg/kg bw) and a pharmacological (375 mg/kg bw) GSPE dose was able to prevent this situation, in order to select the most effective dose required for this prevention. Although both GSPE doses can not be considered dietary doses, they did not previously demonstrated to cause any toxicity after long-term chronic exposition [37]; in contrast, they showed to possess anti-inflammatory effects induced by LPS administration [38] and also to have a desired effect in some intestinal functions [39].

In general, animals showed normal biochemical values in plasma. Increased TNF-α levels in the LPS treated group is a clear sign that ip LPS administration has a pro-inflammatory effect. Although LPS plasma levels did not show differences in concentration among groups, this is probably due to the time of sampling and because LPS would be cleared after 24 h [13]. The administration of LPS did not exert any sign of mucosal damage as has been observed at higher doses of LPS, or any sign of enterocolitis or modifications in the rats' behaviour.

This makes it a less aggressive model and more physiological than those obtained from chemical inducers such as TNBT or DSS [25].

MPO is an enzyme involved in the oxygen-dependent microbial activity of phagocytes [40] and it has also been associated with tissue damage in a state of acute or chronic inflammation [8]. Beyond its oxidative effects, MPO affects various processes involved in cell signaling and cell-cell interactions and is capable of modulating inflammatory responses [41]. The analysis of the pro-inflammatory activity of this enzyme along the intestine as an indicator of more local intestinal inflammation showed that each intestinal section *per se* had different levels of enzymatic activity and that when inflamed the immune response was also section specific. The small intestine was the most inflamed part. This is expectable because the small intestine is the intestinal section with most GALT and PPs. Although the gastrointestinal tract contains the largest immune system in the body, the greatest density of immune cells resides in the small intestine, which is densely populated with many different types of immune cells [42]. The GSPE had an anti-inflammatory effect in the small intestine, specifically in the duodenum and ileum sections, without any difference between doses; however, in the large intestine the MPO activity was not affected by GSPE ingestion.

Moreover, COX-2 activity was increased by LPS treatment in the duodenum. The COX enzymes are responsible for the conversion of free arachidonic acid into prostaglandins and thromboxanes. COX-1 generally contributes to maintaining the gastric mucosa. In contrast, COX-2 is an immediate-early response gene normally absent from most cells but is induced mainly in response to inflammatory stimuli, including cytokines such as IL-1β and TNF-α [43, 44]. In previous studies, LPS has also been demonstrated to induce COX-2 in rat intestinal epithelial cells *in vivo* and *ex vivo* [45]. In addition, different studies have also associated COX-2 with intestinal inflammation. For example,

COX-2 enzyme activity is induced in large intestinal epithelium in active human IBD and in inflamed tissues of a IL-10 deficient rodent model of IBD [10, 46]. Furthermore, more genetic approaches showed that COX-2 deficient mice have increased sensitivity to chemically-induced colitis [47]. Our data support that LPS could stimulate COX-2 activity in the duodenum fraction and this activity was inhibited by GSPE. In a previous study, Nunes et al. observed that pre-treatment with a red wine extract rich in catechins, oligomeric procyanidins and anthocyanidins inhibited COX-2 cytokine-induced expression in HT-29, a human epithelial colorectal adenocarcinoma cell [48]. Other authors have demonstrated the same beneficial effect of flavonoids reducing TNF-α-induced COX-2 expression in HT-29 cells pre-treated with pomegranate juice, which is rich in anthocyanidins and catechins [49].

Inflammation and oxidative stress are closely connected. In this sense, the recruitment and activation of additional phagocytic leukocytes (PMNs, eosinophils, monocytes) have been observed in IBD, accompanied by the overproduction of ROS [50]. In this study, we measured ROS levels in the different parts of the intestine and we expected these values would be highly influenced by the GSPE treatment. The results showed that there were more ROS in the intestine as we move along the small to large intestine, and the effect of pro-oxidant LPS is more pronounced in the colon. We observed that proanthocyanidins have an antioxidant effect with the low and high concentrations in both the proximal and distal colon. However, we do not know whether it is a direct effect of the proanthocyanidin extract or from proanthocyanidins derived from metabolites after colonic microbiota transformation. Probably, this colonic GSPE antioxidant effect is related to the higher retention time of proanthocyanidins in this part of the intestine, but more studies are needed to elucidate this.

The gene expression of inflammation related genes was analysed in the ileum. LPS up-regulated 54 genes mainly associated with a pro-inflammatory response, including: CC and CXC chemokines, their respective receptors, interleukins and TNF ligands. These results together with the physiological results and the increased pro-inflammatory enzymes activity obtained in the ileum, reinforce ip administered LPS as a good animal model of intestinal inflammation. This analysis also gives light to the potential pathways affected and whether the GSPE extract may protect against LPS-induced alterations. The administration of 75 mg/kg bw dose of GSPE on animals following LPS pro-inflammatory treatment did not have a strong effect, with the exception of CCL9 and 19 suppression and CCR5 overexpression. Therefore, the low dose of GSPE was not associated with neither positive nor negative significant variations of this inflamed phenotype; altogether it might indicate that this dose is not able to counteract the effect caused by LPS in the intestine. In contrast, the 375 mg/kg bw dose of GSPE had a stronger effect in the gene expression profile mainly showing an overexpression of chemokines and interleukins but also the induction of the potent anti-inflammatory molecule IL-13. Altogether, this could indicate a much stronger immune response in the ileum to this high dose of proanthocyanidins. However, considering that MPO activity levels were lower due to the high dose GSPE pre-treatment, the global effect of this dose in the ileum might not be pro-inflammatory. Further studies are necessary to confirm the present hypothesis.

Intestinal inflammation is usually linked with impaired intestinal permeability. We found that rats treated with LPS had higher levels of plasma OVA, which translates into an increase in intestinal permeability. This means that peritoneal LPS could affect intestinal performance. To describe these results in a molecular approximation, we decided to analyse the gene expression pattern of TJ proteins in the duodenum and ileum, which were previously shown to be the intestinal sections most affected by LPS. According with our results,

claudin-2 was overexpressed in the LPS group in the ileum section. Claudins can be divided into two classes: pore-forming claudins, which form specific permeability pathways, and claudins that reduce permeability (sealing or barrier-forming claudins: claudin-1 and 3)[51]. Claudin-2 is a pore-forming claudin that forms high conductance, paracellular cation-selective pores that determine paracellular ion selectivity and water permeability [52]. An up-regulation of claudin-2 has been observed in a state of intestinal inflammation like IBD or ulcerative colitis, followed by changes in the structure of TJs [53, 54]. The pre-administration of the two different doses of proanthocyanidins protects against claudin-2 overexpression in the ileum but not in the duodenum, where its expression was unquantifiable. In contrast, the expression of sealing claudins, in particular claudin-3 was deceased or redistributed in a state of disease [55]. However, our results have not shown changes in the expression of claudin-3 either in the duodenum or the ileum after LPS or GSPE. Altogether, the duodenum section was less susceptible to changes in TJ expression than the ileum. In the ileal section the administration of LPS had no effect on the rest of the permeability genes; however, they were slightly susceptible to GSPE. It is necessary to emphasize that this effect of GSPE on these TJs genes does not translate in a global effect in intestinal permeability, considering the protective role of GSPE against increased OVA levels. Therefore, future research is required to elucidate why this is happening in the ileum section, determine whether these changes are restricted only to this section, as well as, elucidate the precise mechanisms of the effect of proanthocyanidins on intestinal alterations.

In summary, in this study we offer an adequate physiological rat model for studying moderate intestinal inflammation based on LPS administration to further evaluate the potential benefits of other bioactive food compounds on the intestine in a situation closer to health without biochemical alterations and without strong GI damage. Based on these findings, the two doses of GSPE (75

and 375 mg/Kg) prevent intestinal inflammation and increased permeability, both induced by endotoxin. Considering the intestine is not homogenous, the inflammatory response and the general effect of the GSPE supplementation are different in each intestinal section. Our results highlight that proanthocyanidins administered preventively could be considered to be included in nutritional and/or nutraceutic therapeutic interventions for protecting barrier dysfunction and moderate intestinal inflammation.

Author contributions

K. G.-C. and R.C. performed the laboratory work, initiated the literature search and was in charge of drafting the manuscript. Both K. G.-C. and I. G designed the figures. M.P. and A.A. revised the first drafts. F.V. collaborated in the discussion and manuscript revision. X.T. and M.B. were responsible for the final editing and content. M.B. was responsible for the experimental design and supervision. All of the authors critically reviewed the manuscript and approved the final version.

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Conflict of Interest

None.

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IV. GENERAL DISCUSSION

1. General discussion

Chronic intestinal diseases share two principal features closely linked. On one hand, chronic intestinal diseases show a defective mucosal barrier function that increases intestinal permeability. On the other hand, they are characterized by chronic intestinal inflammation. More specifically, increased intestinal permeability induces the exposition of luminal content that triggers an immunological response, altogether promoting a pro-inflammatory state in the intestine. This intestinal dysfunction is a phenotype that has also been described not only in IBD and Crohn's disease, but also in the obesity disease.

The intestine is a selective nutrient absorption system, where signal transduction exchange is produced between nutrients and the body. Consumption of obesogenic diets sends negative signals to the intestinal cells, altering intestinal homeostasis [363, 364]. In contrast, the intestine is also the target organ of bioactive compounds, remarkably flavonoids. They are secondary metabolites of plants that participate in vegetal defence. Previously, flavonoids were mostly ignored until few decades ago, when their potential in health was discovered [365].

The Mediterranean diet is an important source of these flavonoids, and proanthocyanidins are the most representative class. Previous studies in our group demonstrated an immunomodulatory and anti-inflammatory role of a grape seed extract, rich in proanthocyanidins, in obesity models [354, 355]. Other experimental evidence in our group revealed that the same extract can directly interact with the intestinal cells, modulating several metabolic processes and improving intestinal health [330, 366]. Despite that little is known about the role of proanthocyanidins in MIS and barrier function, *in vivo*, the information mentioned above together with the protective role of proanthocyanidins improving the metabolic alterations associated with DIO

[356, 367, 368] sheds new clues about proanthocyanidins as possible immunomodulators and barrier protective agents in the intestine. In this framework, the global objective of this thesis was to study the possible role of proanthocyanidins in the modulation of the intestinal inflammatory response and barrier function in two different animal models of intestinal dysfunction.

We firstly address this purpose in the obesity context. Prior to analyse the effect of proanthocyanidins, we focused on defining a chronic robust model of intestinal dysfunction associated with obesity. For this reason, the first specific objective was to analyse the impact of an obesogenic diet on intestinal health status at three time points, and to compare them with a model of genetic obesity, in order to distinguish between effects derived from the genetic background or the diet.

Impact of an obesogenic diet and genetic obesity on the intestinal health status: defining the proper model to study intestinal dysfunction

In this first study **(Manuscript 2)**, we demonstrated that 17 weeks of CAF diet are required to obtain a solid rat model of obesity-induced intestinal alterations. The administration of the CAF diet increased body weight in a time-dependent way, reaching the maximum values at 17 weeks. Additionally, these changes in body weight were associated with metabolic disturbances. Concretely, increased insulin and plasma glucose levels, typical indicators of metabolic syndrome, showed drastical increase after 17 weeks of diet intervention.

Regarding the impact of the diet intervention in the intestinal status, the CAF diet induced a time-dependent intestinal damage. Oxidative stress, quantified by ileal ROS levels, reached significancy at 17 weeks. Despite the CAF diet had a negative effect in morphometric parameters from week 12, here we show this obesogenic diet induced intestinal oxidative stress at later time points. These

results suggest that oxidative damage in this tissue is the consequence of a severe state of obesity.

Several studies reaffirm that obesity *per se* can induce systemic oxidative stress due to an increase in Nox activity and endoplasmic reticulum stress in adipocytes that promotes ROS production [369, 370]. However, high-caloric diets can stimulate nutritional oxidative stress, which results from inadequate nutrient supply [371], through multiple intracellular and biochemical mechanisms: superoxide generation from NADPH oxidases, oxidative phosphorylation, glyceraldehyde autoxidation, activation of protein kinase C, and polyol and hexosamine pathways [371–373]. Abnormal post-prandial ROS generation [374], tissue dysfunction [373], hyperleptinemia [375], low antioxidant mechanisms of defence [376], and chronic inflammation [377] are considered other factors that can induce oxidative stress.

When the associations between parameters were evaluated, oxidative stress and intestinal inflammation were shown to be closely linked with metabolic and morphometric parameters, and both of them demonstrated to be interlinked in obesity disease; however, determining the temporal sequence of their crosstalk appears to be a difficult task. The obesity-associated infiltration of phagocytes into the intestinal mucosa is increasingly believed to be a key factor in the development of intestinal inflammation [308]. Neutrophils participate in both innate and adaptive immunity. However, considering their association with numerous pro- and anti-inflammatory mediators, these immune cells can also contribute to inflammation damage. One of the principal enzymes released as consequence of the recruitment and activation of phagocytes is MPO, an enzyme that plays a key role in the various functions of neutrophils in both innate and adaptive immunity [174]. Likewise, circulating MPO levels have been associated with metabolic and inflammatory diseases. In fact, elevated MPO levels have been reported in obese patients [378, 379], in subjects with diabetes mellitus type 1 [380], with hypercholesterolemia [381]

and it is also considered an early biomarker of cardiovascular disease and inflammation [382]. According to our results, ileal MPO activity significantly increased from the week 12, without many substantial differences between weeks 14.5 and 17. MPO is an important source of pro-oxidant products generators, including hypochlorous acid/hypochlorite, tyrosyl radicals, chloramines, and nitrogen dioxide [383]. These products can react with different biological substrates (DNA, lipids and proteins) contributing to oxidative stress and tissue damage [384]. Altogether, our results suggest that an active pro-inflammatory state is necessary to reach severe oxidative stress damage.

With respect to TJs, decreased ZO-1 and claudin-1 gene expression in the ileum was observed at later stages of obesity in parallel with a high degree of oxidative stress and sustained intestinal inflammation. However, these results could mask earlier and subtler changes in intestinal permeability and in the functionally of the intestinal barrier. In this regard, de la Serre *et al*. observed increased intestinal permeability quantified *in vivo* by FITC-dextran after 10 weeks of overfeeding [8]. These results were associated with an increase in TLR4 activation in the intestinal wall and phosphorylated MLCK, altering TJs distribution and led to a pro-inflammatory state in the intestine and changes in the intestinal microbiota [8].

Despite obese Zucker rats displayed a markedly decrease in the gene expression of representative TJs proteins in the ileum, these animals did not show changes in MPO activity nor in oxidative stress, what does not translate into a global intestinal damage. Altogether, considering these results and the fact that obese Zucker rats were fed a standard chow diet we can conclude that the intestinal alterations observed in DIO animals can be attributed to the effect of the CAF diet.

From the results obtained in the first objective, we concluded that CAF diet has a negative impact on intestinal health in rats, in a time-dependent manner, reaching the greatest damage at 17 weeks of diet intervention, and these results depend on the composition of the diet and not on the genetic load. Consequently, the time sequencing proposed for intestinal damage is the following: chronic lipid and carbohydrate overload stimulates intestinal inflammation and the resulting sustained inflammatory state increases oxidative stress; the relaxation of TJs complexes accompanies all these processes.

The following part of the thesis was focused on evaluating whether the intestine is able to sense bioactive food compounds, in different doses and time conditions of administration, in a situation of obesity-associated intestinal alterations. For the second objective, a long-term experiment was designed in order to evaluate and compare in the intestine the effect of three dietary doses of GSPE on intestinal alterations in response to the CAF diet.

Effects of dietary doses of GSPE administered in a corrective way on intestinal alterations induced by the CAF diet

According to the **Manuscript 3**, obesity was induced after 18 weeks of CAF diet intervention. During the last three weeks of the study, the CAF diet was administered together with GSPE supplementation in three dietary doses: 5, 25 and 50 mg/kg bw in the treated groups. These doses are equivalent to intakes of 57, 284, and 560 mg of proanthocyanidins per day in humans [385]. Considering that the proanthocyanidin intake in Spanish adults has been estimated as around 187 mg/day [386], the present experiment was designed to test a GSPE dose similar to the human dietary proanthocyanidins intake (25

mg/kg bw in rats), and a lower and a higher GSPE dose than the dietary intake of humans (5 and 50 mg/kg bw in rats, respectively).

MPO increased activity was attenuated after GSPE administration at the three doses. Besides, it is remarkable the effectiveness of the low dose which is able to revert MPO increased activity derived from 18 weeks of CAF diet to the point of normalizing its levels regarding the control group. In accordance with these results, we found an ameliorative effect of this low dose decreasing IL-1β and a beneficial effect of the dose 25 and 50 mg GSPE/Kg bw in iNOS gene expression in the ileum.

Hereof, iNOS gene expression has been related to intestinal inflammation because of sustained high NO production, mediated by iNOS, and it plays an important role in the pathology of chronic IBD and in oxidative stress [387, 388]. In fact, NO participates in intestinal mucosal defence maintaining mucosal integrity [389]. However, increased iNOS expression has been associated with mucosal defence injury [310]. The intestine is continuously in contact with oxidants released by the intestine itself and the dietary reactive species. Here we showed that increased intestinal ROS levels together with the observed MPO up-regulation translate into clear increased intestinal oxidative stress. GSPE doses of 25 and 50 mg/Kg bw strongly modified ROS levels to the point of normalizing them. Antioxidant effects of proanthocyanidins have been observed in previous studies in our group, in other tissues and cell models [390, 391] and also, recently, in the intestine [330]. In this sense, different defence mechanisms for GSPE have been proposed to protect against oxidative stress: modulation of anti-oxidant enzymes have an effect on mitochondrial function and ROS scavenging properties, and also the action on absorptive cells and enterohormone-secreting cells [330, 390, 391].

Regarding to TJs gene expression, 18 w of CAF diet altered the expression of ZO-1 in the ileum; however, the doses of 5 and 50 mg of GSPE significantly

increased its expression. Previous *in vitro* experiments designed in enterocyte cells lines had already shown a protective effect of proanthocyanidins against intestinal permeability disruptions, positively modulating TJs gene expression [360, 392, 393]. These results are in accordance with Masumoto *et al.* study, in which they observed *in vivo* that a pre-treatment with apple procyanidins ameliorated ZO-1 and occludin altered expression in the ileum of obese mice fed a HF/HC diet intervention. In addition, they observed that proanthocyanidins administration influenced the intestinal microbiota composition, significantly increasing the proportion of *Akkermansia* [345]. *Akkermansia muciniphila* is a commensal bacteria specie positively associated with reducing weight gain, improving metabolic responses and also reversing impaired barrier function by its effect on mucus layer thickness, characteristics assigned to the lean phenotype [394].

Elevated circulating LPS plasma levels, known as endotoxemia, is associated with changes in intestinal microbiota and alterations in intestinal permeability and have been found in obese phenotype [395]. Although the results of the **Manuscript 3** did not show significant differences derived from the diet and the treatment, a clear tendency to increase plasma LPS in the DIO rats was observed. For next studies, *in vivo* techniques to directly measure obesity-derived alterations in permeability will be necessary to elucidate deficiency in the intestinal barrier function and to confirm possible GSPE beneficial effects.

Finally, we performed a PCA to globally interpret the distribution and association among all the parameters analysed. The PCA confirmed that obesity was correlated with altered intestinal permeability and elevated inflammatory response in the intestine. In contrast, the moderate dose of GSPE showed lower expression of inflammatory marker levels in the ileum as well as LPS circulating plasma levels.

Taking together all the results included in the second objective, we concluded that the corrective treatment with the three dietary doses of proanthocyanidins (5, 25 and 50 mg of GSPE/Kg bw) has ameliorative effects on CAF diet-induced intestinal alterations, including the reduction of intestinal inflammation and oxidative stress, and changes in TJs, without substantial differences among the three doses

In the **Manuscript 3**, the protective effect that the dietary doses of GSPE administered in a corrective way have shown increasing TJs gene expression in the intestine suggests a possible modulation of barrier function by proanthocyanidins, and it leads to the possibility of trying new doses and treatments. To study more in depth this issue, in the **Manuscript 4** we proceeded to apply *in vivo* and *ex vivo* technics to directly measure DIO-derived alterations in permeability and metabolic endotoxemia, in order to determine deficiency in the correct function of the intestinal barrier and to evaluate GSPE beneficial effects. For this study, a pharmacological dose of proanthocyanidins, administered in two different time-points of the experiment, was evaluated.

Effects of a pharmacological dose of GSPE on DIO-derived impaired barrier function

There is evidence linking obesity with pathophysiological changes in the intestinal epithelium and barrier function [198]. Hence, identifying bioactive natural compounds that can selectively modulate intestinal barrier presents a promising therapeutic strategy for obesity treatment.

In the literature exist different tests to assess intestinal permeability *in vivo* [56]. In the **Manuscript 4**, OVA permeability marker was orally administered to the rats at weeks 12th and 17th of the experiment. On the other hand, *ex vivo* assessment of barrier function involves the measurement of TEER by Ussing chamber technique. In the **Manuscript 4**, TEER values were measured in the

duodenum, ileum and colon at the end of the experiment. According to our results, OVA plasma levels increased in a time-dependent way by the CAF diet intervention, which translated into increased intestinal permeability. Additionally, lower TEER values were found in the small and large intestine at the end of the CAF diet intervention accompanied by an under-expression of claudin-1 gene expression in the ileum. Permeability parameters have shown to be strongly correlated with the morphometric parameters at the end of the experiment. Reinforcing this, other studies have shown a positive correlation between intestinal permeability markers and waist circumference [396]. They also positively related intestinal permeability to visceral and liver fat, but not to subcutaneous or whole-body fat. Overall, these results evidence how the intestinal barrier function is compromised in obesity disease and strongly supports a link between intestinal permeability and visceral fat accumulation in the obese phenotype [396].

Some molecular pathways have been suggested to be involved in the effect of obesogenic meals in barrier function, and TLR4 pathway overactivation is one of the most studied [8]. As we have previously mentioned, HF diets have been related to changes in the abundance of some intestinal bacteria, like *Akkermansia munichiphila*, which were associated with decreased antimicrobial peptides expression, impaired mucus production and secretion, and reduction of mucus layer thickness. Overall, these effects induce physiopathological changes, mainly in the ileum, changes that are associated with impaired barrier function [397, 398]. Other authors have associated obesity and altered intestinal permeability with the endocannabinoid system [399]. Murconi *et al.* postulated that intestinal microbiota can modulate intestinal permeability and plasma LPS levels via the endocannabinoid system, the tone of which is altered in obesity [400].

A pre-treatment (PRE-CAF) with the pharmacological dose of 500 mg/Kg bw of GSPE was able to prevent increased intestinal permeability until week 12 of overfeeding. This dose also ameliorated claudin-1 gene expression in the ileum. Additionally, the same dose of proanthocyanidins administered in a Simultaneous-Intermittent-Treatment (SIT)-CAF protected against impaired intestinal permeability during the 17 weeks of the diet intervention. These changes were accompanied by a direct effect of PRE-CAF and SIT-CAF proanthocyanidin treatments normalizing TEER values in the small intestine. In a previous study, Bitzer and colleagues described that cocoa procyanidins, being the polymeric structures the most effective, protected and preserved the intestinal membrane integrity after testing a grand variety of procyanidins with different degree of polymerization in human colon cell models of disrupted membrane integrity [401]. However, our study was the first to describe the positive effect of proanthocyanidins in an *ex vivo* model of impaired intestinal permeability.

The principal consequence of the alterations in the barrier function was an appreciable time-dependent rise in the LPS plasma levels from the week 8. At the end of the experiment LPS reached its maximum values, probably as the result of an extended impaired barrier function and a severe obesity state, considering Pearson's correlations. Previous authors have already established a positive correlation between energy intake and metabolic endotoxemia in a cohort study with healthy voluntaries [402]. The same authors reproduced these results in mice fed HF diet, observing an increase in plasma LPS levels. However when they fed animals with HC diet, the results were acute [402]. In fact, this relationship between dietary fatty acids and metabolic endotoxemia has been observed in other multiple studies [403–405]. According to proanthocyanidins role in metabolic endotoxemia, we observed that both treatments with GSPE were able to avoid greater LPS plasma levels derived from the CAF intervention. These results are in concordance with previous

studies, in which the administration for 20 weeks of apple procyanidins alleviated metabolic endotoxemia, preventing LPS increased levels in mice fed obesogenic diet [345].

Furthermore, metabolic endotoxemia has been shown to increase inflammatory markers, such as TNF-α, contributing to a state of systemic inflammation. Here we observed that CAF diet increased TNF-α plasma levels from the 12th week until the end of the experiment, whereas the SIT treatment ameliorated this situation. The positive correlation that TNF-α levels established with both OVA and TEER values in the whole intestine reaffirms the crosstalk established between barrier physiology and function, and systemic inflammation. Additionally, LPS started to increase from the 8th week, thus we can hypothesize that metabolic endotoxemia precedes systemic inflammation.

Relative to the effectiveness of both treatments, the PRE-CAF treatment interestingly maintained a positive effect until the 12th week of the obesogenic diet intervention. However, on balance the results indicated that the SIT-CAF treatment was the most effective way to counteract the effects of the CAF diet throughout the intervention.

In conclusion, the pharmacological dose of proanthocyanidins (500 mg of GSPE/Kg bw) administered before or together with the CAF diet ameliorates barrier dysfunction, improving intestinal permeability and preventing metabolic endotoxemia, being the intermittent treatment the most effective after 17 weeks of CAF diet intervention.

Considering the results collected in these three manuscripts, we postulate that the consumption of a CAF diet, as a model of HF/HC diet, leads to excessive energy harvesting and storage and increases intestinal permeability, thus

promoting LPS translocation to circulation. In parallel, immune cells infiltration is associated with the activation of pro-inflammatory response in the intestine, contributing to intestinal inflammation and systemic inflammation and finally, closing to oxidative damage in the intestine. Regarding the proanthocyanidins effect, we can conclude that proanthocyanidins behaviour on intestinal health depends on the dose of treatment and the time of administration. In this sense, the SIT treatment with the pharmacological dose of 500 mg/Kg of GSPE seems to be the most effective treatment acting in a positive manner modulating systemic inflammation, intestinal barrier function and protecting against metabolic endotoxemia that result of 17 weeks of diet intervention. It is necessary to emphasise how the PRE-CAF treatment with the same dose can maintain its positive protective role until the 12th week of the CAF diet intervention, and how the nutritional doses are beneficial against intestinal inflammation, oxidative stress and TJs alterations. Finally, 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. This fact could suggest that the beneficial health effects of proanthocyanidins may be also attributed to aromatic metabolites derived from their metabolism, thus more studies are needed to elucidate the molecules and molecular mechanisms responsible for these effects.

Once proanthocyanidins were confirmed as potent intestinal anti-inflammatory agents and modulators of barrier function in response to DIO, in the next section of the thesis we evaluated the same properties of these bioactive compounds in other model of intestinal dysfunction. For the **Manuscript 5**, we analysed the role of proanthocyanidins in a rat model of acute intestinal inflammation and impaired intestinal permeability induced by LPS injection.

Effects of proanthocyanidins on acute LPS-induced intestinal inflammation and impaired permeability

Intestinal dysfunction is not just limited to obesity, it has been also described in other animal models. Models of impaired permeability were also obtained in animals exposed to low doses of bacteria endotoxin [3, 406]. In the **Manuscript 5,** we determined the effects of a high nutritional (75mg/Kg bw) and a pharmacological (375 mg/Kg bw) dose of GSPE administered in a preventive way in an acute model of intestinal dysfunction induced by LPS.

As we expected, IP LPS injection enhanced OVA circulating levels and induced claudin-2 overexpression in the ileum, altogether corresponding to an increase in intestinal permeability without inducing epithelial cell death or intestinal mucosal damage. However, the pre-administration of the two doses of proanthocyanidins protected against claudin-2 overexpression and the low dose was also able to prevent increased OVA levels in plasma. Previous studies have revealed that LPS-induced increased permeability is modulated via an intracellular mechanism involving TLR-4–dependent up-regulation of CD14 membrane expression. Particularly, LPS-induced an increase in TLR4 expression, leading to an activation of NF-κB and consequently an up-regulation of MLCK [3, 406]. Therefore, these results show possible down-stream places of LPS pathway that can be targets of proanthocyanidins action. Results in this area have demonstrated that hexameric procyanidins, at this molecular level, inhibit NF-κB activation in intestinal epithelial cells. More specifically, procyanidins can interact with the plasma membrane of intestinal cells, delaying IκB phosphorylation and degradation, p50 and RelA nuclear translocation and the subsequent NF-κB-DNA binding [407].

Despite barrier function alterations, this model can be additionally considered a good model of acute intestinal inflammation. LPS up-regulated 54 genes mainly associated with a pro-inflammatory response include: CC and CXC chemokines,

their respective receptors, ILs and TNF ligands. However, the treatment with the low dose of GSPE was not able to prevent the up-regulation of these genes. In contrast, the pharmacological dose of GSPE had a stronger effect in the gene expression profile mainly showing an overexpression of some pro-inflammatory chemokines and ILs, but it also showed the induction of anti-inflammatory genes, thus indicating a much stronger immune response in the ileum of this high dose of proanthocyanidins. The small intestine was the most inflamed section, considering gene expression results, and MPO and COX-2 values. Here, we showed LPS increased the activity of MPO and COX-2 inflammatory markers in the duodenum and ileum. Meanwhile, GSPE had an anti-inflammatory effect in both regions, acting in MPO and COX-2 level, without any difference between doses. Although we have described before that GSPE can positively modify MPO levels in DIO-induced intestinal inflammation model, but we did not report data about the role of these compounds in COX-2 activity. However, in a previous study, Nunes *et al.* observed that a pre-treatment with a red wine extract, rich in oligomeric procyanidins, inhibited COX-2 cytokine-induced expression in colon human epithelial cells [359]. Altogether, our results reaffirmed the behaviour of proanthocyanidins in the intestine as anti-inflammatory agents.

Unlike the DIO-induced intestinal dysfunction model, in which oxidative damage was observed in the ileum, in the present model, the pro-oxidant effect of LPS was more pronounced in the colon. In this line, both doses of proanthocyanidins showed an antioxidant effect protecting the proximal and distal colon against LPS damage. It seems to indicate that colonic GSPE antioxidant effect is related to the higher retention time of proanthocyanidins in this part of the intestine.

Based on these findings, we concluded that IP administration with a low dose of LPS causes a local mild intestinal inflammation and impaired barrier function, similar to the damages found in the DIO-induced model. At the same time, our analyses revealed that the treatment with a high nutritional (75 mg of GSPE/Kg bw) dose and a pharmacological (375 mg of GSPE/Kg bw) dose of proanthocyanidins has a region-dependent protective effect, preventing against intestinal alterations induced by LPS, without significant differences between both doses.

Summarizing the four manuscripts, we concluded that DIO and LPS exposition trigger intestinal inflammation and an impaired barrier function state, and that the administration of bioactive food compounds, such as proanthocyanidins, improve intestinal health. Therefore, we propose nutritional and therapeutic interventions with proanthocyanidins, based on intestinal health modulation, which should be extensively explored in the context of intestinal dysfunction.

V. CONCLUSIONS

1. Main conclusions

The main conclusions of this thesis are:

1. **The chronic intervention with the CAF diet entails intestinal homeostasis alterations, inducing intestinal dysfunction.**
   - These alterations in the intestine include: intestinal inflammation, oxidative stress and impaired barrier function (increased permeability and metabolic endotoxemia).
   - These alterations in the intestine are in response to chronic lipid and carbohydrate overload and not to the genetic load.
   - The disruptive effect of the CAF diet in the intestine is time-dependent, getting more damage at 17 weeks of the dietary intervention.

2. **The subchronic administration of a grape seed proanthocyanidin extract ameliorates intestinal alterations induced by the CAF diet.**
   - Proanthocyanidins have anti-inflammatory, antioxidant and barrier function modulatory effects in the intestine that result in a global improvement of intestinal health.
   - The bioactive activity that proanthocyanidins show in this model of intestinal dysfunction depends on the dose of treatment and time of administration.

3. **The intraperitoneal administration with a low dose of LPS induces a similar intestinal dysfunction state to that obtained with the CAF diet.**
   - Endotoxin induces a local mild intestinal inflammation, mainly in the duodenum and ileum, colon oxidative stress and intestinal permeability impairment.

- These alterations do not show signs of severe disease or excessive aggression to the intestinal epithelium.

4. **The administration of a grape seed proanthocyanidin extract prevents intestinal LPS-induced intestinal dysfunction.**
   - The administration of a high nutritional and a pharmacological dose of proanthocyanidins have an anti-inflammatory behaviour in the small intestine, have an antioxidant effect in the colon and positively modulated intestinal permeability.
   - Proanthocyanidins show a preventive region-dependent role in this model of intestinal dysfunction, without significant differences between doses.

VI. REFERENCES

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LIST OF PUBLICATIONS

Papers included in the thesis

Gil-Cardoso, K., Ginés, I., Pinent, M., Ardévol, A., Blay, M., Terra, X. (2017) The co-administration of proanthocyanidins and an obesogenic diet prevents the increase in intestinal permeability and metabolic endotoxemia. In preparation.

Gil-Cardoso, K., Comitato, R., Ginés, I., Ardévol, A., Pinent, M., Virgili, F., Terra, X., Blay, M. (2017) Protective effect of proanthocyanidins in a rat model of mild intestinal inflammation and impaired intestinal permeability induced by LPS. Submitted in Journal of Nutritional Biochemistry.

Gil-Cardoso, K., Ginés, I., Pinent, M., Ardévol, A., Arola, L., Blay, M., Terra, X. (2017) Chronic supplementation with dietary proanthocyanidins protects from diet-induced intestinal alterations in obese rats. Molecular Nutrition and Food Research 61(8).

Gil-Cardoso, K., Ginés, I., Pinent, M., Ardévol, A., Terra, X., Blay, M. (2017) A cafeteria diet triggers intestinal inflammation and oxidative stress in obese rats. British Journal of Nutrition 117(2), 218-229.

Gil-Cardoso, K., Ginés, I., Pinent, M., Ardévol, A., Blay, M., Terra, X. (2016) Effects of flavonoids on intestinal inflammation, barrier integrity and changes in the gut microbiota during diet-induced obesity. Nutrition Research Reviews 29(2), 234-248.

Other papers

Ginés, I., **Gil-Cardoso, K.,** Serrano, J., Casanova-Martí, À., Blay, M., Pinent, M., Ardévol, A., Terra, X. (2017) Effects of an intermittent Grape-Seed Proanthocyanins (GSPE) treatment on a cafeteria diet obesogenic challenge in rats. Submitted in British Journal of Nutrition.

Ginés, I., **Gil-Cardoso, K.,** Robles, P., Arola, L., Terra, X., Blay, M., Ardévol, A., Pinent, M. (2017) Ap-to-Bas (AtB) as an intestinal dispositive to measure pig mucosa enteroendocrine secretory activity in response to apical stimulation. Submitted in Scientific Reports.

Serrano, J., Casanova-Martí, À., **Gil-Cardoso, K.,** Blay, M., Terra, X., Pinent, M., Ardévol, A. (2016) Acutely administered grape-seed proanthocyanidin extract acts as a satiating agent. Food and Function 7(1), 483-490.

Abstracts

Ginés, I., **Gil-Cardoso, K.,** Robles, P., Arola, L., Terra, X., Blay, M., Ardévol, A., Pinent, M. (2017) Ap-to-Bas (AtB), an intestinal dispositive to measure pig mucosa enteroendocrine secretory activity in response to apical stimulation. Presented at 14th NuGO week Congress, Varna, Bulgaria.

Gil-Cardoso, K., Ginés, I., Pinent, M., Ardévol, A., Terra, X., Blay, M. (2016) A rat model of moderate intestinal inflammation to study molecular bioactivity of food. Presented at The 1st International Conference on Food Bioactive and Health, Norwich, United Kingdom.

Gil-Cardoso, K., Casanova-Martí, À., Serrano, J., Ginés, I., Pinent, M., Ardévol, A., Blay, M., Terra, X. (2016) Chronic supplementation with proanthocyanidins protects from diet-induced intestinal alterations in obese rats. Presented at XXV Congrés de la Societat Catalana de Digestologia , Reus, Spain.

Gil-Cardoso, K., Casanova-Martí, À., Serrano, J., Ginés, I., A, Gual., Pinent, M., Ardévol, A., Blay, M., Terra, X. (2015) Chronic supplementation with proanthocyanidins protects from diet-induced intestinal alterations in obese rats. Presented at 12th NuGO week Congress, Barcelona, Spain.

Intestinal dysfunction is based on a pro-inflammatory state in the intestine and on a defective barrier function, both considered common features of intestinal chronic diseases. However, intestinal dysfunction has also been associated with obesity and other metabolic diet-related pathologies. In this regard, proanthocyanidins are natural bioactive compounds from the flavonoid family with anti-inflammatory and antioxidant properties that might have significant effects on the intestinal environment. In this framework, the present thesis was designed to elucidate the role of proanthocyanidins in the modulation of the intestinal inflammatory response and barrier function in complementary animal models of intestinal dysfunction.

To accomplish this global objective, firstly we examined the impact of an obesogenic diet on intestinal health status over time to then analyse the protective effect of a grape seed proanthocyanidin extract and compare the effectiveness of different doses and times of administration to protect against intestinal dysfunction. Secondly, the role of the same extract of proanthocyanidins was analysed in an animal model of acute intestinal inflammation and impaired intestinal permeability induced by lipopolysaccharides injection.

To sum up, the present thesis revealed that diet induced obesity and acute lipopolysaccharides exposition trigger similar degree of intestinal inflammation and impaired barrier function state, and more importantly, that the oral administration of proanthocyanidins improves intestinal inflammation and barrier function.

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