UNIVERSITAT ROVIRA i VIRGILI

Impact of Proanthocyanidins on Intestinal Dysfunction Induced by Nutritional or Chemical Agents

Carlos Alberto González Quilen

DOCTORAL THESIS
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Supervised by Dr. Maria Teresa Blay Olivé and

Dr. Ximena Terra Barbadora

MoBioFood Research Group

Department of Biochemistry and Biotechnology

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UNIVERSITAT ROVIRA i VIRGILI

DEPARTAMENT DE BIOQUÍMICA I BIOTECNOLOGIA
Campus Sescelades
C/ Marcel·lí Domingo, 1
43007 Tarragona
Tel. +34 977 55 84 97
Fax +34 977 55 82 32

WE STATE that the present study entitled **IMPACT OF PROANTHOCYANIDINS ON INTESTINAL DYSFUNCTION INDUCED BY NUTRITIONAL OR CHEMICAL AGENTS** presented by **CARLOS ALBERTO GONZÁLEZ QUILEN** for the award of the degree of Doctor has been carried out under our supervision at the Department of Biochemistry and Biotechnology from the Universitat Rovira i Virgili.

FEM CONSTAR que aquest treball titulat **IMPACT OF PROANTHOCYANIDINS ON INTESTINAL DYSFUNCTION INDUCED BY NUTRITIONAL OR CHEMICAL AGENTS** que presenta **CARLOS ALBERTO GONZÁLEZ QUILEN** per al'obtenció del títol de doctorat ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili.

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Doctoral thesis supervisors / Les directores de la tesi doctoral

Dr. M. Teresa Blay Olivé

Dr. Ximena Terra Barbadora

“Somewhere, something incredible

is waiting to be known.”

—Carl Sagan

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Summary

The intestinal tract is a site of interaction with microorganisms and potentially detrimental environmental factors. The high intake of fructose and saturated fats typical of the Western diet has been associated with intestinal dysfunction (disruption of barrier function and inflammation) and an increased influx of proinflammatory bacterial endotoxins into the systemic circulation. In turn, high concentrations of plasma endotoxins (metabolic endotoxemia) are a precursor to the onset of metabolic syndrome. In view of the above, the intestine is emerging as a target for disease prevention and therapy. Proanthocyanins (PACs) are naturally occurring phenolic compounds with remarkable anti-inflammatory properties in the intestinal mucosa, according to preclinical studies. Thus, PAC administration is a promising adjunctive therapeutic strategy for the treatment of intestinal dysfunction, but its efficacy in humans is yet to be confirmed. The main objective of this doctoral thesis was to evaluate the impact of a grape-seed PAC extract (GSPE) on preclinical models of intestinal dysfunction and to investigate its effectiveness in humans. We found that a long-term Western-style diet (cafeteria diet) induces intestinal dysfunction in rats, and that alterations in the permeability of the colon largely contribute to metabolic endotoxemia. These effects are partially driven by high luminal concentrations of fructose and could be effectively reversed *in vivo* by pharmacological doses of GSPE. Lastly, we compared these findings with evidence derived from an *ex vivo* human model of chemically-induced colonic dysfunction in which we were able to replicate the reduction of intestinal permeability and the amelioration of inflammatory status by means of GSPE found *in vivo*. In conclusion, the administration of GSPE results in the overall improvement of intestinal dysfunction and associated metabolic endotoxemia. Effective doses in humans are probably pharmacological and will have to be determined in clinical trials.

Resum

El tracte intestinal és un lloc d'interacció amb microorganismes i factors ambientals potencialment nocius. En aquest sentit, un alt consum de components majoritaris de la dieta occidental com la fructosa i els greixos saturats s'ha associat amb la disfunció intestinal (disrupció de la funció de barrera i inflamació) i l'entrada d'endotoxines bacterianes amb efecte proinflamatori en la circulació. Addicionalment, una alta concentració d'endotoxina en plasma (endotoxemia metabòlica) es vincula amb la síndrome metabòlica. Així, l'intestí està emergint com un blanc terapèutic per a la prevenció i tractament de malalties. Les proantocianidines (PACs) són compostos fenòlics naturals amb potent efecte antiinflamatori en la mucosa intestinal d'acord amb evidència preclínica. Per tant, l'administració de PACs és prometedora com a estratègia terapèutica complementària, però la seva eficàcia s'ha de confirmar en humans. El principal objectiu de la present tesi doctoral va ser avaluar l'impacte d'un extracte de PACs de la pinyol de raïm (GSPE) en models preclínics de disfunció intestinal i explorar la seva efectivitat en l'humà. Trobem que una dieta estil occidental (dieta de cafeteria) indueix disfunció intestinal en rates i que l'alteració de permeabilitat al còlon contribueix en gran mesura a la endotoxemia metabòlica. Aquests efectes van ser atribuïts parcialment a altes concentracions luminals de fructosa i van poder ser revertits amb dosis farmacològiques de GSPE *in vivo*. Finalment, contrastem aquests resultats amb evidència derivada d'un model humà *ex vivo* de disfunció colònica. En aquest model vam poder replicar la reducció de permeabilitat i la millora de l'estat inflamatori reportats *in vivo*. En conclusió, l'administració de GSPE pot millorar la disfunció intestinal i la endotoxemia metabòlica associada. Les dosis efectives en humans són probablement farmacològiques i hauran de ser establertes en estudis clínics posteriors.

Abbreviations

CAF, cafeteria

COX, cyclooxygenase

DP, degree of polymerization

DSS, dextran sodium sulfate

FD4, fluorescein isothiocyanate 4 kDa

GSPE, grape-seed proanthocyanidin extract

HEB, human equivalent dose

IBD, inflammatory bowel disease

IFN-γ, interferon-γ

IgA, immunoglobulin A

IKK, IκB kinase

IL, interleukin

JAM, junctional adhesion molecule

LDH, lactate dehydrogenase

LPS, lipopolysaccharides

LY, Lucifer yellow

M cell, microfold cell

MAPK, mitogen-activated protein kinase

MLCK, myosin light chain kinase

MPO, myeloperoxidase

MUC2, mucin 2

NF-κB, nuclear factor-κB

NO, nitric oxide

NOAEL, no-observed-adverse-effect level

NOS, nitric oxide synthase

OVA, ovalbumin

Papp, apparent permeability

SFA, saturated fatty acid

ROS, reactive oxygen species

TEER, transepithelial electrical resistance

Th, T helper cell

TJ, tight junction

TLR, toll-like receptor

TNBS, trinitrobenzene sulfonic acid

TNF-α, tumor necrosis factor-α

UCh, Ussing chamber

ZO, zonula occludens

I. Introduction

1. Background

The burden of metabolic disorders including abdominal obesity, dyslipidemia, hyperglycemia and hypertension—namely metabolic syndrome—is increasing all over the globe [1]. These chronic conditions are linked to the unhealthy dietary habits and lifestyle behaviors prevalent in Western societies [2]. Ultra-processed food products are the primary calorie source in the so-called Western diet, dominating the global food system of high-income countries [3,4]. In general, ultra-processed foods are energy-dense and highly palatable products, rich in fats, sugars and various metabolically active food additives [5]. Furthermore, the consumption of these foods is also increasing rapidly in middle-income countries due to their convenience and attractiveness [5,6].

The intestinal epithelium is the largest interface between the host, microbial organisms and the external environment. It also constitutes an integral component of innate immunity and therefore a primary site of immune induction [7]. To address the challenges related to the continuous exposure to antigens, the intestinal mucosa maintains a finely tuned balance between immune response and tolerance [7,8]. Dietary components can substantially alter the physiology of the intestine by modifying microbiota composition [9] or directly targeting host responses [10,11]. Studies in mammals have reported that a high intake of saturated fats and fructose increases intestinal permeability and the influx of bacterial endotoxins (lipopolysaccharides, LPS) into the systemic circulation [12–14]. The resulting metabolic endotoxemia may elicit strong proinflammatory responses and induce features of metabolic syndrome [15]. Some research has suggested that the diet-induced disruption of intestinal barrier integrity and inflammation (i.e. intestinal dysfunction) is a precursor to obesity and insulin resistance [16]. Additionally, the disruption of the intestinal barrier may precede the clinical onset of type 1 diabetes [17] and inflammatory bowel disease (IBD) in

humans [18]. In this paradigm, the intestine is emerging as an immune organ with significant influence on the etiology of serious pathologies and as a potential target for disease prevention and therapy [19,20].

Natural products derived from plants have historically been a source of therapeutic agents [21]. Proanthocyanins (PACs) are the second most abundant phenolics in nature after lignin [22] and the most important group of flavonoids in the human diet [23]. Attention has been focused on these phytochemicals due to their remarkable immunomodulatory properties, which make them promising complementary agents for therapeutic purposes in the treatment of chronic conditions [24,25]. Accumulated evidence mostly provided by *in vivo* studies in animals suggests that PACs may be beneficial in the treatment of intestinal dysfunction associated with diet-induced obesity [26,27] or caused by other detrimental factors [28–30], although their effectiveness in humans has yet to be confirmed.

2. The Western Diet: Definition and Implications for Health

Ultra-processed foods are formulations of ingredients that contain little, if any, intact food, typically combined by a series of industrial techniques and processes. The overall purpose of ultra-processing is to create convenient, attractive (hyper-palatable), accessible and durable food products [31]. Some common ultra-processed products are sugar-sweetened beverages and carbonated soft drinks, candy, sweetened breakfast cereals, snacks, mass-produced packaged breads and buns, cookies (biscuits), pastries, cakes, margarine and other spreads, and ready-to-eat meals [5]. Ultra-processed foods are cheaper to produce, transport, and store than perishable foods such as unprocessed meats or dairy products. As a result,

diets based on refined grains, added sugars and fats are more affordable than diets based on lean meats, fish, whole grains, fresh vegetables and fruits. This inverse relationship between energy density and energy cost [32] has shaped a shift from traditional diets towards a "Western" pattern diet [33].

The Western diet can be defined as a hypercaloric diet characterized by the overconsumption of saturated fats, refined sugar and corn-derived fructose syrup, salt and proteins derived from fatty and processed meats, with an associated reduced consumption of fruits and vegetables [34]. This dietary pattern represents a prominent contributing factor to the prevalence of obesity and related comorbidities [35,36]. The effects of the Western diet on human health have been accurately reproduced in rodents fed a cafeteria (CAF) diet [37]. In this model, animals are allowed free access to ultra-processed human foods, thus inducing a voluntary and sustained overfed state. The body weight of CAF-diet fed Wistar rats significantly increases as early as one week after initiating the intervention [38] and the animals manifest clear alterations in biochemical parameters (hypertriglyceridemia, hyperglycemia and hyperinsulinemia) after 17 weeks [39]. In rats, the CAF diet has also been found to shift the composition of intestinal microbiota and to decrease bacterial diversity (dysbiosis) at 12 weeks [40]. In multiple studies, alterations in the intestinal microbiota caused by high-fat diets have been correlated with two main physiological effects [41]: first, a greater microbial ability to break down food components and harvest energy from the diet, which may result in an increase in body fat; and second, an increase in intestinal LPS-bearing bacterial species and the subsequent over-activation of proinflammatory signal transduction pathways in the intestinal mucosa. Accordingly, an increased expression of proinflammatory mediators, immune cell infiltration and oxidative stress have been reported in the ileum of Wistar rats fed a CAF diet after 12–14.5 weeks of intervention [39], suggesting that intestinal dysfunction is an early event in obesity.

The dietary components invariably present in ultra-processed foods, i.e. fats and sugars, are both linked to adiposity and weight gain. Nevertheless, as stated above, the association between the components of the Western diet and health issues is substantially more complex than the simple concept of energy balance, and it involves a variety of underlying metabolic mechanisms [42]. Decades of dietary recommendations have focused on reducing total fat intake. In light of accumulated evidence identifying saturated fat intake as a risk factor for cardiovascular disease, these guidelines have been established with the goal of keeping the consumption of this type of fat to less than 10% of daily caloric intake. While recent findings shift the responsibility for the rising toll of chronic illness from fats to carbohydrates and sugars [43,44] (which is certainly not a new trend [45]), other studies highlight the need to focus on lowering the intake of specific types of nutrients and specific foods, and on changing overall dietary patterns rather than reducing the total fat/carbohydrate content of the diet [46,47].

3. Fructose Metabolism and Metabolic Syndrome

Carbohydrates provide up to 22% of total daily calories in the Western diet [48] and include complex polymers (e.g., starch), and sugars, including the disaccharides lactose and sucrose, and the monosaccharides glucose and fructose [48]. Fructose has become a major component of the Western diet due to the increasing consumption of sucrose and high-fructose corn syrup, which are widely used as sweeteners by the food industry [49,50] and represent up to 10–12% of the daily calorie intake of some populations [49]. The contribution of fructose consumption to the toll of chronic illness in humans is still under discussion [51,52] due to a lack of clinical data (the current state of the controversy has been extensively reviewed by Stanhope [53]). On the one hand, fructose consumption was indeed suggested to be beneficial in the context of diabetes since, unlike

glucose, it does not stimulate insulin secretion from pancreatic beta cells, and it has been shown to reduce glycated hemoglobin [54]. On the other hand, epidemiological and preclinical data links chronic fructose intake to non-alcoholic fatty liver disease [55–57], hypertriglyceridemia [58], and even insulin resistance [59–61]. The ability of fructose to induce insulin resistance and other features of metabolic syndrome in experimental animals does not seem to require excessive energy intake [13,62], suggesting the existence of molecular mechanisms responsible for its metabolic effects beyond body weight gain.

The absorption and metabolism of fructose differ from those of glucose. Fructose is transported across the apical membrane of the intestinal epithelial cells by GLUT5 [63]. After a meal, most of the fructose is not metabolized in the intestine, but rather passes via the portal vein to the liver. There, it is rapidly converted to fructose 1-phosphate by fructokinase (ketohexokinase, KHK) and then into the trioses glyceraldehyde 3-phosphate and dihydroxyacetone to enter glycolysis [64]. Unlike glucose, fructose bypasses the major rate-limiting step of glycolysis (phosphofructokinase) and stimulates an accelerated *de novo* fatty acid synthesis. Thus, excessive fructose consumption may contribute both to hepatic steatosis and to increased circulating triglyceride levels in the form of very low-density lipoprotein (VLDL) [64]. The metabolism of large loads of fructose has been associated with hepatotoxicity [65,66]. Furthermore, some *in vivo* studies have linked the intake of large amounts of fructose to intestinal dysfunction [62,67], raising the possibility that the metabolism of fructose in the intestinal mucosa contributes to metabolic endotoxemia and to the associated chronic low-grade inflammation in the context of the Western diet.

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4. PACs for Intestinal Dysfunction: An Approach for Disease Prevention and Treatment

Manuscript 1, presented below, is a comprehensive literature review comprising a series of relevant topics that can be grouped into two main subjects.

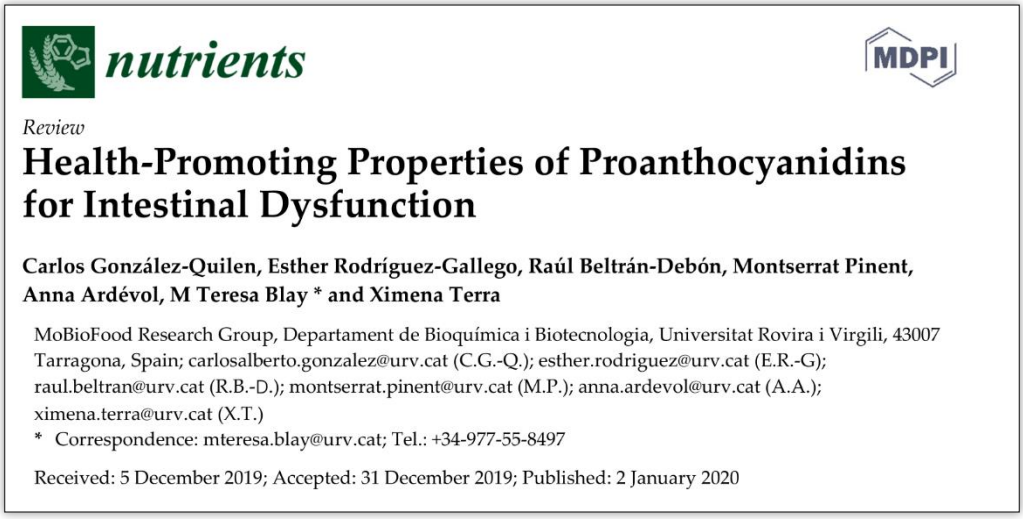
The first subject includes basic notions of intestinal physiology, the definition of intestinal dysfunction and the role of environmental factors in its pathophysiology. This subject is covered in the following sections of the manuscript:

- The Intestinal Barrier
- Intestinal Inflammatory Response: The Critical Role of TLRs and NF-κB
- Cytokine-Induced Regulation of Tight Junctions
- Intestinal Dysfunction: Pathophysiological Basis and Environmental Triggers

The second subject focuses on the generalities of PACs, the accumulated evidence of their effects on intestinal dysfunction with an emphasis on grape-seed PAC extract (GSPE) and the mechanisms underlying the therapeutic properties of these bioactive compounds on intestinal health. This subject is covered in the following sections of the manuscript:

- PACs: Chemical Structure, Occurrence, and Intake
- The Fate of PACs after Ingestion
- Studies on the Benefits of PACs for Intestinal Dysfunction
- Biochemical and Molecular Mechanisms Underlying the Barrier Protective and Anti-Inflammatory Properties of PACs in the Intestine

4.1. Manuscript No. 1



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Review

Health-Promoting Properties of Proanthocyanidins for Intestinal Dysfunction

Carlos González-Quilen, Esther Rodríguez-Gallego, Raúl Beltrán-Debón, Montserrat Pinent, Anna Ardévol, M Teresa Blay * and Ximena Terra

MoBioFood Research Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, 43007 Tarragona, Spain; carlosalberto.gonzalez@urv.cat (C.G.-Q.); esther.rodriguez@urv.cat (E.R.-G); raul.beltran@urv.cat (R.B.-D.); montserrat.pinent@urv.cat (M.P.); anna.ardevol@urv.cat (A.A.); ximena.terra@urv.cat (X.T.)

* Correspondence: mteresa.blay@urv.cat; Tel.: +34-977-55-8497

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Health-Promoting Properties of Proanthocyanidins for Intestinal Dysfunction

By Carlos González-Quilen, Esther Rodríguez-Gallego, Raúl Beltrán-Debón, Montserrat Pinent, Anna Ardévol, M Teresa Blay and Ximena Terra

MoBioFood Research Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, 43007 Tarragona, Spain

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Abstract: The intestinal barrier is constantly exposed to potentially harmful environmental factors, including food components and bacterial endotoxins. When intestinal barrier function and immune homeostasis are compromised (intestinal dysfunction), inflammatory conditions may develop and impact overall health. Evidence from experimental animal and cell culture studies suggests that exposure of intestinal mucosa to proanthocyanidin (PAC)-rich plant products, such as grape seeds, may contribute to maintaining the barrier function and to ameliorating the pathological inflammation present in diet-induced obesity and inflammatory bowel disease. In this review, we aim to update the current knowledge on the bioactivity of PACs in experimental models of intestinal dysfunction and in humans, and to provide insights into the underlying biochemical and molecular mechanisms.

Keywords: gut; permeability; inflammation; metabolic endotoxemia; obesity; IBD; flavonoid; flavan-3-ol; condensed tannin; procyanidin

1. Introduction

The primary function of the intestinal tract is to digest food components and absorb nutrients and water from the lumen to the systemic circulation. The intestine is also a physical barrier that is in contact with the environment. As a result, the intestinal epithelium is constantly exposed to potentially pathogenic microorganisms, toxins,

and harmful components of the diet. When there are disturbances in the barrier function and mucosal immune homeostasis, the influx of intestine luminal content triggers an exaggerated mucosal immune response [1]. Ultimately, chronic exposition to these detrimental environmental stimuli may lead to the development of local and systemic inflammatory conditions [2,3].

Natural products have been recognized as a source of therapeutic agents for many years [4]. Some plant-derived phenolic compounds show promising anti-inflammatory effects and have been associated with the prevention of certain chronic diseases [5]. Proanthocyanidins (PACs), also known as condensed tannins, are oligo- and polymeric end products of the flavonoid biosynthesis pathway in plants [6]. There has been extensive laboratory research into the effects of both pure PAC molecules and PAC-rich extracts on overall health. These phytochemicals show a wide range of physiological activities [7], including anti-inflammatory and barrier protective effects in the intestine [8–10], which may be interesting in the context of diet-induced obesity and inflammatory bowel disease (IBD).

We have reported previously that grape-seed PACs and other flavonoids have beneficial effects on inflammation [11–13] and protect the intestine against alterations associated with diet-induced obesity in rats [8,9,14,15]. In addition, research conducted during the last decade with cell culture and animal models has made significant progress in determining the underlying mechanism of the health promoting properties of PACs in the gastrointestinal tract and peripheral tissues.

2. The Intestinal Barrier

The intestinal epithelium is a single cell-layer responsible for separating underlying mucosal tissues from the environment and is the largest exposed surface area in the body [16]. As there is a prolific commensal microbial community in the intestinal lumen (intestinal microbiota), epithelial integrity plays a pivotal role in maintaining overall health [16,17]. The intestinal epithelium is integrated

by several cell types with specialized functions. The enterocytes are responsible for the absorptive function and constitute the most abundant epithelial cell lineage. The goblet cells are implicated in the synthesis of secretory mucin glycoproteins that form the mucus layer, with mucin 2 (MUC2) being the most prominent [18]. Other cellular types integrating the epithelium, microfold (M) [19], Paneth, and enteroendocrine cells are specialized in antigen sampling and presentation to dendritic cells, synthesis of antimicrobial peptides, and secretion of hormones, respectively.

The first strategy the host tissue has to maintain its homeostatic relationship with the intestinal microbiota is to minimize the physical interaction with microorganisms, thus limiting microbial translocation and physiological inflammation [20,21]. The thick mucus layer secreted by goblet cells represents a primary defense line against environmental insults [18]. In addition, the enterocytes are joined together forming an intricately and well-regulated barrier sustained by intercellular junctions linked to the cell cytoskeleton, such as tight junctions (TJs), desmosomes, and adherent junctions. TJs partially seal the paracellular space and prevent passive transport of large molecules, including microbial components and other potentially proinflammatory agents [1,22]. The main protein components of TJs are claudins, occludins, and junction adhesion molecules (JAMs), which are associated with peripheral proteins such as zonula occludens (ZOs) [22].

3. Intestinal Inflammatory Response: The Critical Role of TLRs and NF-κB

Beneath the intestinal epithelium, a thin mucosal layer of connective tissue known as the lamina propria hosts the gut-associated lymphoid tissue (GALT), which is responsible for the intestinal immunological response to microbial and non-microbial antigens [23]. GALT is distributed along the intestinal tract and includes aggregates of lymphoid cells forming scattered structures such as Peyer's patches, which occur mainly in the ileum [23,24]. In the follicle-associated epithelium (FAE),

M cells actively internalize, process, and present microbial and non-microbial antigens taken from the lumen [19]. M cells also contribute to the induction of antigen-specific immunoglobulin A (IgA), the dominant isotype in mucosal tissues [19].

The constant exposure of the intestine epithelium to the microbiota generates the need for a homeostatic balance between tolerance and the immune response [21]. Thus, intestinal immune cells exert protective immunity against pathogens while they show a limited responsiveness to commensal bacteria and food derived antigens. Failure to maintain this balance may lead to inflammatory conditions [25]. Inflammation is a normal biological response of the immune system triggered by detrimental stimuli or conditions, such as infections and tissue damage. The inflammatory response is the first of several overlapping processes that lead to tissue repair and regeneration [26]. During the inflammatory response, cell signaling pathways are activated to regulate the concentration of proinflammatory mediators in the affected tissue and the recruitment of immune cells from the circulation [27].

The innate immune response is initiated by pattern recognition receptors (PRRs) recognizing microbial antigens known as pathogen-associated molecular patterns (PAMPs). Among others, the PRRs include Toll-like receptors (TLRs) located on the cell membrane and nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) present in the cytoplasm. PRRs are the primary component of innate immunity responsible for preventing systemic dissemination of pathogens by activating proinflammatory intracellular signaling pathways [28]. In addition, TLRs can recognize multiple endogenous molecules derived from damaged tissue (damage-associated molecular patterns or DAMPs) such as peptides, lipids, glycans, and nucleic acids [29]. PRR expression is not uniformly distributed along the proximal/distal axis of the intestinal tract [30,31] or on the crypt/villus axis of the epithelium [30]. In fact, there is a significantly higher expression of TLRs in the

distal segments of the intestine (ileum and colon) where the microbial population is enriched [30,31]. Moreover, a higher PRR expression is found along the crypt/villus axis of the epithelium in both villus and lamina propria [30].

Activation of TLRs (mainly TLR4/myeloid differentiation 2 (MD-2) complex) by bacterial lipopolysaccharides (LPS), induces recruitment and phosphorylation of multiple intracellular protein complexes, leading to the stimulation of the nuclear factor kappa-light-chain-enhancer of activated B-cell (NF-κB) signaling [32]. NF-κB is a family of inducible transcription factors responsible for expression of numerous genes implicated in the innate immune response, including cytokines such as the tumor necrosis factor-alpha (TNF-α), chemokines, adhesion molecules (CAMs), and inducible enzymes such as the nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) [33]. Proteins p50 (NF-κB1), p52 (NF-κB2), RelA (p65), RelB, and c-Rel are present in the cytosol as inactive homo- or heterodimers bound by a family of inhibitory proteins known as inhibitors of κB (IκB), of which IκBα is the most studied member. NF-κB is activated by both canonical signaling, associated with the innate immune response, and non-canonical signaling, implicated in maturation, survival, and homeostasis of B cells [34]. In the canonical signaling pathway, TLR ligands activate the IκB kinase (IKK) complex. IKK induces site-specific phosphorylation of IκB that leads to its ubiquitination and proteasomal degradation, releasing NF-κB dimers, predominantly p50/RelA complex. Subsequent activation of this complex by post-translational modifications promotes its translocation to the nucleus where transcription of target genes is induced [33]. Activation and differentiation of naive T cells are processes of adaptive immunity also influenced by gene programs regulated by NF-κB [35,36]. Depending on the cytokines present during activation, $CD4^+$ T cells differentiate in multiple subsets of T helper cells with different cytokine profiles. Th1 cells play a role in the immune response against intracellular pathogens, essentially through the production of interferon (IFN)-γ. Th2 cells are involved in the response to extracellular pathogens and allergic reactions and are producers of interleukin

(IL)-4, IL-5, IL-6, IL-9, IL-13, and IL-25 [35]. Th17 cells primarily express IL-17 and are also related to the immune response against extracellular pathogens [36].

TLRs are also required for production of IgA, which in turn contributes to determining the microbiota composition and maintenance of the non-inflammatory host-microbial relationship [37]. Furthermore, TLR2 signaling is associated with the regulation of TLR3/4-mediated production of proinflammatory Th1 cytokines through anti-inflammatory IL-10 induction [38]. TLR2 activation also improves the barrier function by means of protein kinase C-mediated sorting and assembly of ZO-1 [39].

4. Cytokine-Induced Regulation of Tight Junctions

TJ protein complexes are not rigid but rather exhibit dynamic elements that ultimately modulate the epithelial paracellular permeability [40]. Phosphorylation of the myosin II regulatory light chain in the perijunctional actomyosin ring by myosin light chain kinase (MLCK), produces a transient TJ opening that enhances paracellular flux [40]. MLCK activation is also implicated in the redistribution of ZO-1 [41] and caveolin-1-dependent occludin endocytosis [42]. TJ protein expression and MLCK activation are both regulated by multiple signaling molecules, including cytokines produced as a result of the activation of NF-κB. These regulatory processes are complex and also involve the activation of multiple kinase enzymes downstream, such as mitogen-activated protein kinases (MAPKs), phosphoinositide-3-kinases (PI3Ks), and monophosphate-activated protein kinase (AMPK) [43]. For example, IFN-γ down-regulates expression of ZO-1 and occludin in an AMPK-dependent pathway [44]. The activation of MLCK by interleukin IL-1β is mediated by p38 MAPK-dependent induction of the transcription factor ATF2, which binds to the MLCK promoter [45]. The activation of MLCK induced by TNF-α is mediated by extracellular signal-regulated kinases (ERK1/2), which phosphorylates and promotes nuclear translocation of the transcription factor ELK-1 [46]. TNF-α also induces expression of the pore-forming claudin-2 through

the upregulation of the transcription factor CDX2 mediated by PI3K/protein kinase B (Akt) signaling [47].

5. Intestinal Dysfunction: Pathophysiological Basis and Environmental Triggers

We define intestinal dysfunction as the state of increased intestinal permeability and pathological inflammation seen in diet-induced obesity and IBD (ulcerative colitis and Crohn's disease) [48]. Evidence suggests that changes in the epithelial barrier function and inflammation are associated with metabolic disorders and alterations in the regulation of body weight [49–51]. This pathological inflammation is characterized by increased levels of proinflammatory mediators and enhanced infiltration of immune cells into the intestinal tissues ([52,53] and **Figure 1**). Regardless of the specific triggering events, intestinal inflammatory states share common immunological processes. However, intestinal dysfunction associated with IBD is significantly more severe than in diet-induced obesity [48]. Diet-induced obesity and Crohn's disease lead to a Th1 response with increased IFN-γ production and reduced Th17 response [53,54], while ulcerate colitis has often been considered a Th2-mediated disease [53].

Multiple environmental factors have been identified as potential triggers of intestinal inflammatory conditions, including Western dietary habits [55]. It has been described that saturated fats play a direct role in inflammatory signaling. Saturated fatty acids (SFA) such as lauric (C12:0) and palmitic (C16:0) directly induce NF-κB activation, acting as non-microbial TLR2 and TLR4 agonists in macrophages [56]. Data suggest that activation of TLRs by SFA is mediated by TLR dimerization and recruitment into lipid rafts [57]. We have reported mild intestinal inflammation and increased permeability in rats feeding on a cafeteria diet consisting of high-saturated fat/high-refined sugar food products [58]. This enhanced permeability has been shown to favor bacterial LPS and other potentially

proinflammatory molecules entering the systemic circulation, which is known as metabolic endotoxemia [15].

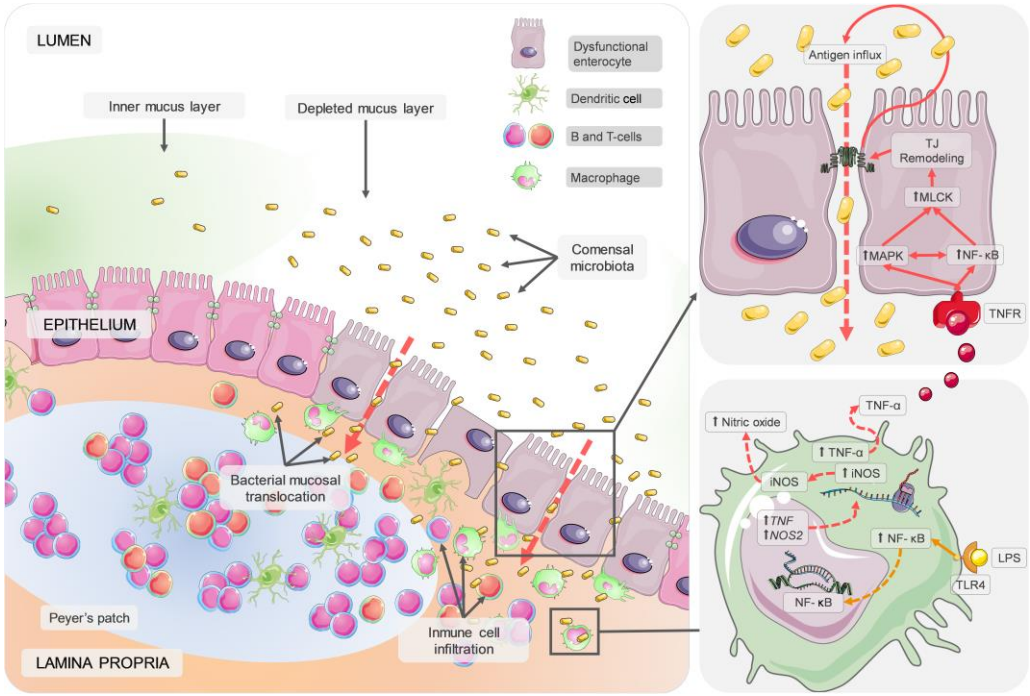


Figure 1. Dysfunctional intestinal mucosal. Chronic exposition to detrimental environmental stimuli, including several food components, may lead to dysbiosis, mucus layer depletion, and breakdown of the epithelial barrier. Constitutive stimulation of NF-κB signaling by bacterial endotoxins induce overproduction of proinflammatory cytokines and reactive species of oxygen and nitrogen, feeding back the epithelial barrier disruption and immune cell infiltration.

Diet plays an important role in the composition of intestine microbiota, promoting or inhibiting growth of microorganisms [59]. Alterations in the composition and metabolism of the intestinal microbiota (dysbiosis) have also been associated with the consumption of high-saturated fat diets in rodents and humans [60,61]. In fact, metagenomic analysis of the intestinal microbiome in Western populations has

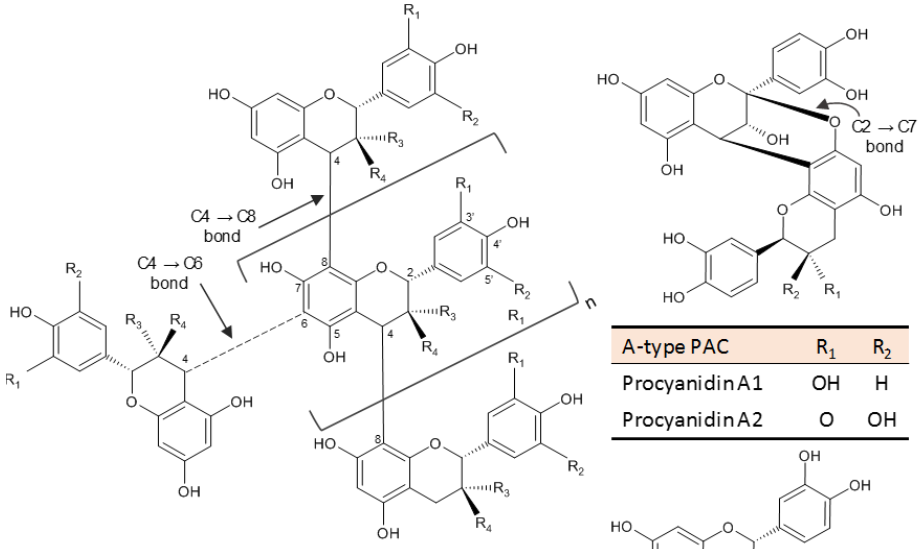
shown a reduction not only of microbial diversity but also of functional potential [62].

Dysbiosis is linked to obesity-associated intestinal inflammation, although the "egg or hen" question related to the cause-effect relationship is not answered yet [63]. High-fat intake in rodents often decreases overall diversity of microbiota and the abundance of *Bacteroidetes*, and increases the relative abundance of *Firmicutes* [64,65]. Several human studies have described similar associations [66,67], but the importance of the ratio *Firmicutes* to *Bacteroidetes* remains controversial [68,69], and some authors state that the experimental results are not sufficiently consistent [70]. Interestingly, the existence of a colitogenic microbiota was demonstrated in T-bet$^{-/-}$ × RAG2$^{-/-}$ deficient mice whose spontaneous ulcerative colitis was horizontally transmissible to wild-type individuals when co-housed [71]. Although mechanisms by which dysbiosis trigger intestinal inflammation are not fully understood, it is known that they involve the loss of immune tolerance due to local immune homeostasis disruption and continuous abnormal activation of TLRs [72]. Overactivation of NF-κB leads to a chronic proinflammatory cytokine release and immune cell infiltration [33]. Furthermore, as stated before, proinflammatory cytokines such as TNF-α and IFN-γ decrease barrier integrity, feeding back the dysfunctional state. The association of NF-κB signaling with intestinal inflammation-related pathologies is supported by the NF-κB-inhibiting properties of drugs used in the treatment of IBD, such as aminosalicylates [73,74]. Given the importance of NF-κB in inflammation-associated pathologies, it is relevant to study dietary components that negatively regulate the NF-κB signaling pathway, such as PACs and other flavonoids [9].

6. PACs: Chemical Structure, Occurrence, and Intake

PACs consist of flavan-3-ol subunits with a degree of polymerization (DP) equal to or greater than 2, mainly linked by (4 → 8) or (4 → 6) carbon-carbon bonds (B-

type PACs) [75]. In some botanical sources an additional (2 → 7) ether-linkage also occurs (A-type PACs) [76] (**Figure 2**).



Flavan-3-ol monomer	R_1	R_2	R_3	R_4
(+)-Catechin	H	OH	H	OH
(−)-Epicatechin	H	OH	OH	H
(+)-Gallocatechin	OH	OH	H	OH
(−)-Epigallocatechin	OH	OH	OH	H
(+)-Afzelechin	H	H	H	OH
(−)-Epiafzelechin	H	H	OH	H

A-type PAC	R_1	R_2
Procyanidin A1	OH	H
Procyanidin A2	O	OH

B-type PAC	R_1	R_2
Procyanidin B1	H	OH
Procyanidin B2	OH	H

Figure 2. Chemical structures of proanthocyanidins (PACs). Flavan-3-ol monomers differ based on the hydroxylation pattern and their cis- or trans- configuration. Dimers A1/A2 and B1/B2 are shown as example of A- and B-type PACs, respectively.

Depending on the type of monomers, PACs can be classified into procyanidins, prodelphinidins, and propelargonidins. The most abundant group, procyanidins, consists exclusively of (+)-catechin and (−)-epicatechin monomers [77]. Prodelphinidins and propelargonidins are composed of (−)-gallocatechin/(−)-epigallocatechin and (+)-afzelechin/(−)-epiafzelechin monomers, respectively [75], and have a more limited distribution.

Dietary assessment studies have shown that PACs, especially procyanidins, are among the most abundant polyphenols in the human diet [6], as they are present in a variety of botanical sources and plant food products such as tea, fruits, nuts, cacao products, legumes, and cereal grains. However, PAC intake varies widely between geographical regions and cultures and is greatly dependent on eating habits, lifestyle behaviors, and socioeconomic status [78]. The daily PAC (dimers to polymers) intakes in adult populations from Korea, the U.S., Mexico, and EU were estimated as 71[79], 73 [78], 103 [80], and 123–180 mg [81,82], respectively, but intakes up to 230 mg d^{-1} have been reported in some regions of Spain and Norway [83].

7. The Fate of PACs after Ingestion

Flavan-3-ols are remarkably stable during gastric transit in humans [84]. Monomers such as (+)-catechin and (−)-epicatechin are readily absorbed in the upper sections of the small intestine [85,86], recognized as xenobiotics and then subjected to an extensive phase II metabolism that generates glucuronidated, sulfated, and methylated conjugates [87]. Flavan-3-ol monomers and their conjugated metabolites reach peak plasma concentration 1–4 h after flavan-3-ol-rich food consumption [88–90]. Studies conducted in cultivated epithelial monolayers [91–93], rats [94,95], and humans [90,96] indicate that PAC absorption is conversely more limited and is highly dependent on DP, and that the permeation of larger oligomers (DP > 5) and polymers is negligible. No PAC transporter has been identified in the enterocyte membrane in the small intestine. Thus, dimers to tetramers are passively transported across the intestinal epithelium essentially by paracellular diffusion. Although transcellular passive diffusion is not likely to occur due to the hydrophilic nature of PACs conferred by the multiple hydroxyl groups, uptake might be possible by endocytic mechanisms [92].

In humans, a study assessed the contribution of the ingested cocoa flavan-3-ols and procyanidins to the systemic pool, and found that the plasma (−)-epicatechin came

from the orally administered cocoa (−)-epicatechin and not from their oligomers or polymers [97]. This is in agreement with evidence obtained with rats that suggests that PACs from different sources do not depolymerize to monomers after ingestion [98,99]. Stalmach et al. [86] conducted a study with ileostomized patients who were administered green tea, and found 70% of the ingested flavan-3-ol in the ileal fluid after 24 h. Altogether, these findings suggest that substantial amounts of ingested flavan-3-ol monomers and PACs remain unabsorbed in the small intestine and reach the colon. There, they are efficiently transformed by the colonic microbiota into low molecular weight phenolic compounds that can be absorbed by colonocytes [87].

In vitro fermentation of purified procyanidin dimers with human fecal microbiota has shown to produce mainly 2-(3′, 4′-dihydroxyphenyl) acetic acid and 5-(3′, 4′-dihydroxyphenyl)-γ-valerolactone [100]. In agreement with this, a randomized cross-over study in healthy humans found that a great portion of the ingested (−)-epicatechin and procyanidin B1 was metabolized by the colonic microbiota to produce phenyl-γ-valerolactones as the major microbial metabolites [90]. In this study, microbial degradation of larger procyanidins was substantially lower, possibly to the inhibition of digesting enzymes or to the antibacterial properties exhibited by these compounds. Other human studies analyzing the bioavailability of flavan-3-ols reported high levels of phenyl-γ-valerolactones in the circulation and urinary excretion after ingestion of a red grape pomace drink [101] and apple juice [102]. In the colonocytes and hepatocytes, these microbial products undergo further metabolism by phase II enzymes to produce conjugated derivatives. Margalef et al. [103] analyzed the tissue distribution of metabolites derived from a grape-seed proanthocyanidin extract (GSPE) 2 h after ingestion by rats. These authors detected a few microbial metabolites (methyl conjugated phenols) at low concentrations in the colon tissue, while most phase II metabolites (glucuronidated and methyl-glucuronidated forms) were found in the kidneys and liver. In humans, the major contributors to the excretion of phenyl-γ-valerolactones after ingestion

of a red grape pomace drink, are sulphated and glucuronidated conjugates of 5-(3′, 4′-dihydroxyphenyl)-γ-valerolactone [101].

8. Studies on the Benefits of PACs for Intestinal Dysfunction

During the last decade, the beneficial properties of PACs for intestinal function have been reported in several studies performed with cell culture models and experimental animals (**Table 1** and **Table 2**). This experimental data indicate that PACs contribute to maintaining the intestinal barrier and improving mucosal inflammation induced by environmental insults. However, there are few studies on the effect of PACs on human intestinal health, although epidemiological studies connect PAC-rich food consumption with a lower risk of colorectal cancer [104].

In vitro models of inflammation have been fundamental in the comprehension of cellular mechanisms driving physiological effects of bioactive molecules. Studies on intestinal dysfunction have employed human colon carcinoma cell lines, with Caco-2 being the most well-established and widely used model of the human intestine barrier ([105] and **Table 1**). Mucus producer [106], macrophages [107], and B cell lines [108] have been employed in co-culture systems to explore the interaction between cell populations.

Although there is a strong trend in the industry towards replacing animal experiments with human cell-culture based models [109,110], there are no *in vitro* models of the human intestine that replicate the complex interplay between cell types and the regulation of the barrier function by the mucosal innate and adaptive immunity. Therefore, most physiologically relevant data on intestinal dysfunction comes from the animal model. Most *in vivo* studies testing the effect of PAC supplementation on intestinal health have been performed in diet-induced obesity models and chemical-induced colitis models. The first resemble intestinal alterations seen in humans with metabolic syndrome [58]. The latter closely mimic histopathological features of human colitis and are frequently used to study the

pathophysiology of IBD and the effectiveness of novel therapeutic drugs [111]. Notably, PAC-rich grape-seed extracts (GSPE) are between the most studied botanical extracts, mainly by *in vivo* approaches in rodents (**Table 2**).

8.1. Studies with Cell Culture Models

The data available on the interaction between PACs and permeability and inflammation markers in cell models of intestinal dysfunction are summarized in **Table 1**. Caco-2-based models have shown to be responsive to proinflammatory stimulation, producing a wide range of inflammatory mediators and increasing the paracellular permeability. Proinflammatory agents such as LPS, phorbol 12-myristate 13-acetate (PMA), and cytokines (TNF-α and IL-1β) have been used in multiple studies testing the effect of PAC molecules and PAC-rich botanical extracts on inflamed Caco-2 cells [10,112–114]. Stimulated-Caco-2 cell monolayers incubated with PACs generally show a reduction in gene expression and secretion of TNFα, IL-6, and IL-8 [10,112,114,115], which is often linked to the downregulation of NF-κB signaling at different levels [10,114,116]. An increased expression of antioxidant enzymes, such as glutathione peroxidase (GPx), superoxidase dismutase (SOD), and hemeoxygenase 1 (HO-1), has also been reported [10].

When permeable support systems such as transwell or Ussing chamber (UCh) are used, alterations in barrier integrity and paracellular permeability of epithelial cell monolayers are evaluated by transepithelial electrical resistance (TEER), an electrophysiological parameter that measures ion conductance across the monolayer, and by the transepithelial transport of molecular markers such as Lucifer yellow (LY) and fluorescently-labelled dextrans (FD) [117,118]. Some *in vitro* studies have associated PACs with increased TEER and decreased transport of permeability markers in the context of barrier dysfunction [113,114,119]. The expression levels of TJ proteins (claudins, occludins, and ZOs) often correlate, but not always [106], with intestinal permeability and are also considered markers of

epithelial integrity. Bitzer et al. [113] found that the dextran sodium sulphate (DSS)-induced loss of barrier function in Caco-2 cells was significantly inhibited by polymeric PACs of cocoa but not by oligomers. Moreover, a higher barrier protective activity was determined in PACs with DP ≥7, which were able to reduce the detrimental effect of DSS in a dose-dependent fashion [113]. Effectiveness of procyanidin B2 ameliorating dextran sodium sulphate (DSS)-induced permeability alterations was examined using a Caco-2/HT29-MTX co-culture model [106]. Although procyanidin B2-incubated cells showed increased levels of the TJ proteins claudin-7, occludin, and ZO-1, these changes did not reduce TEER loss. Altogether, these results suggest that the ability of PACs to strengthen the intestinal barrier integrity depends on the degree of polymerization (DP).

Table 1. Interaction of PACs with permeability and inflammatory markers in cell culture models of intestinal dysfunction.

Extract or Compound	Concentration	Time of Incubation	Cell line(s)	Permeability and/or Inflammatory Inductor	Outcomes		Ref.
					Permeability/ Integrity	Inflammation/ Oxidative Stress	
Apple procyanidins	13–50 μg mL^{-1}	6 h	Caco-2	PMA (300 ng mL^{-1})	ND	↓ IL-8 release	[112]
Apple procyanidin dimer fraction	50–150 μg mL^{-1}	24 h	Caco-2	LPS (50 μg mL^{-1})	↑ Occludin. ↑ ZO-1.	↓ NF-κB and TNF-α gene expression. ↑ GPx, SOD, HO-1.	[10]
Cranberry procyanidins	250 μg mL^{-1}	Preincubation for 24 h	Caco-2/15 cells	Fe/Asc mixture (200 μM/2 mM) or LPS (200 μg mL^{-1}) for 6 h	ND	↓ PGE_2 accretion. ↓ COX-2 protein content. ↓ TNF-α and IL-6 protein content.	[115]
Hexameric procyanidins	20 μM	Preincubation for 30 min	Caco-2	TNF-α (10 ng mL^{-1}) for 60 min	ND	↓ IκBα phosphorylation. ↓ NF-κB p50 and RelA nuclear translocation. ↓ NF-κB-DNA binding. ↓ iNOS mRNA and protein content ↓ ROS.	[116]
Nut polymeric-PAC fraction	4.8–12 (mg cyanidin equivalents mL^{-1})	Preincubation for 1 h followed by co-incubation for 24 h with the inflammation inductor	Caco-2	IL-1β (25 ng mL^{-1})	↑ TEER. ↓ FSA permeation.	↓ IL-6 and IL-8 release. ↓ IκBα phosphorylation. ↓ RelA nuclear translocation.	[114]

Table 1. (Continuation)

Extract or Compound	Concentration	Time of Incubation	Cell line(s)	Permeability and/or Inflammatory Inductor	Outcomes		Ref.
					Permeability/ Integrity	Inflammation/ Oxidative Stress	
Cocoa procyanidin polymers	100 μg mL^{-1}	Preincubation for 24 h	Caco-2	DSS (2% w v^{-1}) for 48 h	ND	↓ IL-8 release.	[113]
			HT-29	TNF-α (5 ng mL^{-1}) for 6 h	↓ FD (4 kD) permeation	ND	
Procyanidin B2	50 μM	Preincubation for 24 h, co-incubation with the inflammation inductor for a further 48h	Caco-2/HT29-MTX co-culture	LPS-activated Raw264.7 medium	≈ TEER. ↑ Claudin-7. ↑ Occludin. ↓ ZO-1.	ND	[106]
Various PAC-rich extracts (apple and avocado peel, cranberry and grape)	12.5–50 μg mL^{-1}	24 h	Caco-2	*p*-Cresol (3.2 mM)	↑ TEER. ↓ FD (4 kD) permeation.	ND	[119]

FSA, fluorescein-5-(and-6)-sulfonic acid trisodium salt. ND, not determined.

Table 2. Interaction of PACs with permeability and inflammatory markers in animal models of intestinal dysfunction.

Extract or Compound	Dose (Way of Administration)	Time of Administration	Animal Model	Permeability and/or Inflammatory Inductor	Outcomes		Ref.
					Permeability/ Integrity	Inflammation/ Oxidative Stress	
GSPE	5, 25 or 50 mg kg^{-1} bw (daily oral gavage)	3 weeks (after 15 weeks of cafeteria diet)	Diet-induced obese Wistar rat	Long-term cafeteria diet (18 weeks)	*Ileum:* ↑ ZO-1 gene expression.	*Ileum:* ↓ IL-1β gene expression. ↓ iNOS gene expression. ↓ MPO activity. ↓ ROS.	[14]
GSPE	500 mg kg^{-1} bw (daily oral gavage)	17 weeks every other week or 10 days (before cafeteria diet).	Diet-induced obese Wistar rat	Long-term cafeteria diet (17 weeks)	↓ Plasma OVA *Duodenum, ileum and colon:* ↑ TEER (*ex vivo*). *Ileum:* ↑ Claudin-1 gene expression.	*Ileum:* ↓ MPO activity.	[8]
GSPE	100 or 500 mg kg^{-1} bw (daily oral gavage)	2 weeks (after 15 weeks of cafeteria diet)	Diet-induced obese Wistar rat	Long-term cafeteria diet (17 weeks)	↓ Plasma OVA *Ileum and colon:* ↑ TEER (*ex vivo*). *Ileum:* ↑ Claudin-1 gene expression.	*Duodenum and colon:* ↓ TNF-α release (ex vivo). Ileum: ↓ MPO activity.	[15]
Pyracantha fortuneana fruit PAC-rich extract	0.4 or 1 g 100 g^{-1} of dry feed weight (orally)	8 weeks (after second week of high-fat diet).	Diet-induced obese Sprague Dawley rat	High-fat diet (10 weeks)	↓ LMR. ↑ Occludin (segment not specified). ↑ ZO-1 (jejunum).	ND	[120]

Table 2. (Continuation)

Extract or Compound	Dose (Way of Administration)	Time of Administration	Animal Model	Permeability and/or Inflammatory Inductor	Outcomes		Ref.
					Permeability/ Integrity	Inflammation/ Oxidative Stress	
GSPE	100, 200 or 400 mg kg^{-1} bw (daily oral gavage)	7 days (after second TNBS-induced colitis)	Wistar rat with TNBS-induced recurrent ulcerative colitis	TNBS (ir. injection of 80 mg kg^{-1}, 30 mg kg^{-1} after 16 days)	ND	*Colon:* ↓ TNF-α. ↓ MPO and iNOS activities. ↓ IKKα/β and IκBα phosphorylation. ↓ NF-κB nuclear translocation. ↓ MDA. ↑ GPx and SOD activities.	[119, 120]
GSPE	100, 200 or 400 mg kg^{-1} bw (daily oral gavage)	7 days (after TNBS-induced colitis)	Wistar rat with TNBS-induced ulcerative colitis	TNBS (ir. injection of 100 mg kg^{-1})	ND	*Colon:* ↓ IL-1β. ↓ MPO activity. ↓ IKK activity. ↓ IκBα phosphorylation. ↓ RelA protein content.	[123]
Procyanidin B2	10, 20 or 40 mg kg^{-1} (daily oral gavage)	11 days	C57BL/6 mouse with DSS-induced colitis	DSS (2.5 g 100 mL^{-1} of drinking water for 9 days)	ND	*Colon:* ↓ MMP9. ↓ Cleaved caspase-1. ↓ RelA phosphorylation. ↓ TNF-α, IL-1β and IL-6 gene expression.	[124]
GSPE	1 g 100 g^{-1} of dry feed weight (orally)	16 weeks	IL10-deficient mouse prone to colitis	None (spontaneous colitis)	ND	*Colon:* ↓ TNF-α, IL-1β, IL-6 and IFN-γ gene expressions. ↓ MPO protein content and gene expression. ↓ RelA phosphorylation.	[125]

Table 2. (Continuation)

Extract or Compound	Dose (Way of Administration)	Time of Administration	Animal Model	Permeability and/or Inflammatory Inductor	Outcomes		Ref.
					Permeability/ Integrity	Inflammation/ Oxidative Stress	
GSPE	0.1 g 100 mL^{-1} of drinking water (orally)	12 weeks	IL10-deficient mouse prone to colitis	None (spontaneous colitis)	ND	*Jejunum:* ↓ TNF-α and IFN-γ. ↑ IκBα protein content. ↑ iNOS gene expression.	[126]
GSPE	75 or 375 mg kg^{-1} bw (daily oral gavage)	15 days (before LPS administration)	Wistar rat with LPS-induced intestinal dysfunction	LPS (ip. injection of 0.3 mg kg^{-1})	↓ Plasma OVA *Duodenum:* ↑ JAM-A gene expression. *Ileum:* ↓ ZO-1, occludin, claudin-2, and JAM-A gene expressions.	*Duodenum:* ↓ COX-2 activity. *Duodenum and ileum:* ↓ MPO activity. *Colon*: ↓ ROS.	[13]

Bw, body weight. LMR, lactulose to mannitol ratio. Ir., intrarectal. Ip., intraperitoneal. ND, not determined.

8.2. In Vivo Studies of Diet-Induced Intestinal Dysfunction

The cafeteria (CAF) diet is a self-selected high-saturated fat/high-refined sugar diet that stimulates hyperphagia and a rapid weight gain in experimental animals [127,128]. In this feeding regime, highly palatable and energy dense foods commercially available, such as muffins, biscuits, bacon, sausages and sugared milk, are offered ad libitum [15,129]. A long-term CAF diet (62% carbohydrate (mostly simple sugar), 23% lipid, and 13% protein in dry matter) has negative effects on intestinal function in rodents, increasing intestinal permeability and inducing mucosal inflammation [58]. We have described the beneficial effects of administering GSPE against the intestinal dysfunction induced by a long-term CAF diet (17–18 weeks) in Wistar rats [8,14,15]. The composition of the GSPE used in these studies has been analyzed in detail and is shown in **Table 3**. Both nutritional (5–50 mg kg^{-1} [14]) and pharmacological (100–500 mg kg^{-1} [8,15]) doses of GSPE administered orally as a preventive [8] or counteractive treatment [14,15] tended to reduce intestinal inflammatory markers such as TNF-α release or myeloperoxidase (MPO) activity (an indicator of neutrophil infiltration in tissues).

The reduction of plasma ovalbumin (OVA), an *in vivo* marker of intestinal permeability, was supported by (1) the increase in TEER in small and large intestine segments. This parameter is determined *ex vivo* by UCh-based protocols [8,15]. And (2) by the upregulation of TJ proteins such as ZO-1 [14] and claudin-1 [8,15]. Notably, the protective effect of GSPE in the intestinal barrier function was linked to the amelioration of metabolic endotoxemia (reduction of plasma LPS) and systemic inflammation (reduction of plasma TNF-α) in obese rats [15,130]. Other authors have also reported the upregulation of ZO-1 and claudin-1 TJ proteins in high-fat fed rats supplemented with other PAC-rich extracts [120].

Table 3. Main compounds of the PAC-rich grape-seed extracts (GSPE) used in the *in vivo* studies on cafeteria (CAF) diet-induced intestinal dysfunction.

Compound	Composition	
	% of Total Flavan-3-ol Content	mg g^{-1} Extract
Flavan-3-ol monomers	21.3	
Catechin		121.32 ± 3.41
Epicatechin		93.44 ± 4.27
Epicatechin gallate		21.24 ± 1.08
PAC dimers	17.4	
Procyanidin B1		88.80 ± 3.46
Procyanidin B2		33.24 ± 1.39
Procyanidin B3		46.09 ± 2.07
Dimer gallate		8.86 ± 0.14
PAC trimers	16.3	4.90 ± 0.47
PAC tetramers	13.3	0.05 ± 0.01
Other PACs (DP > 5)	31.7	n/a

The GSPE (Vitaflavan®) was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the extract has a 75% of procyanidins. HPLC-MS/MS analysis by Margalef et al. Table adapted from [131].

8.3. In Vivo Studies of Chemical-Induced Intestinal Dysfunction

Chemical agents administered orally to induce colitis in rodents include trinitrobenzene sulphonic acid (TNBS) and DSS. These agents erode the colonic mucosal lining and produce the loss of the intestinal barrier function and colonic inflammation. In these models, the severity of outcomes depends on the dose of the chemical agent and the frequency of administration. Li et al. [132] found that intragastric administration of GSPE in rats at pharmacological doses (100–400 mg kg^{-1} d^{-1}) prior to TNBS-induced recurrent colitis reduced weight loss and attenuated macro and microscopic tissue damage scores in the colon. This protective effect was accompanied by a reduction in oxidative stress

(malondialdehyde; MDA), inflammation (IL-1β), and neutrophil infiltration (MPO activity) in colonic tissues. Remarkably, the beneficial effects of low-to-high doses of GSPE were comparable to those of sulfasalazine (200 mg kg^{-1} d^{-1}), a potent inhibitor of NF-κB. Subsequent studies carried out by these authors with the same model confirmed the role of the GSPE down-regulating NF-κB response [122,123]. Amelioration of the redox status due to the increase in GPx and SOD activity was also observed in the colon tissues of GSPE-treated rats [122]. A preventive effect of procyanidin B2 was also evidenced in a mouse model of DSS-induced colitis [124]. Administration of procyanidin B2 (10–40 mg kg^{-1} d^{-1}) attenuated the severity of tissue damage in the colon and reduced the levels of matrix metalloproteinase-9 (MMP-9), a marker of macrophage infiltration. In addition, inhibition of the NF-κB signaling and of NLRP3 inflammasome activation was observed, with a concomitant reduction in the gene expression of proinflammatory cytokines. Overall, the benefits of procyanidin B2 administration, especially at the highest dosage (40 mg kg^{-1}), were comparable to those of mesalazine (200 mg kg^{-1}), a cyclooxygenase (COX) inhibitor. The authors suggest that these effects were largely due to the reduction in activated macrophages infiltrating colonic tissues, probably driven by reactive oxygen species (ROS) clearance.

8.4. Other in Vivo Studies with Animal Models

The IL-10 deficient mouse is a classic knockout model that develops spontaneous colitis under pathogen-free conditions. Some authors have explored the influence of GSPE in this model, supplementing colitic animals with 0.1–1 g 100 g^{-1} of dry feed weight for 12–16 days [125,126]. These studies evidenced a reduction of multiple inflammatory markers in the jejunum and colon, such as TNF-α, IL-1β, IL-6, and IFN-γ gene expressions, as well as MPO activity. This anti-inflammatory effect was associated with the inhibition of the NF-κB signaling. Interestingly, GSPE supplementation also increased the density of goblet cells in the jejunum of treated animals, suggesting that there is an alternative mechanism by which inflammation

is attenuated. Cardoso et al. [13] recently tested both dietary (75 mg kg^{-1}) and pharmacological doses of GSPE (375 mg kg^{-1}) in a rat model of mild intestinal dysfunction induced by intraperitoneal injection of LPS. GSPE was administered daily by oral gavage for 15 days prior to LPS-induced intestinal dysfunction. LPS enhanced intestinal permeability and induced both oxidative stress and inflammation. GSPE-treated animals reduced OVA permeation to the circulation, MPO activity and COX-2 in the small intestine tissues, and reactive oxygen species (ROS) levels in the colon. Furthermore, a gene expression analysis with a low-density microarray technique revealed that unlike the dietary dose of GSPE, the pharmacological dose had a striking effect on the LPS-gene expression profile, showing a strong modulation of multiple genes associated with chemokines and ILs, including upregulation of the anti-inflammatory cytokine IL-13.

8.5. Human ex Vivo Studies

Although the use of animal models is the predominate approximation at preclinical stages for testing novel therapies in intestinal dysfunction, there is a strong trend in the industry towards replacing animal experiments with human cell-culture based models [109,110]. Nevertheless, advantages related to the usefulness of *in vitro* models for screening of bioactives and exploring action mechanisms are offset by limitations regarding the mimicking of the *in vivo* situation and translation to the human [133]. Thus, some human *ex vivo* models have been proposed to test immunomodulatory properties of drug candidates in intestinal explants from IBD patients [134,135]. Intestinal function can also be studied with UCh-based protocols. The UCh system consists of two halves with an opening between them, where mucosal tissue is adapted, thus isolating the apical and basolateral sides of the tissue. This technique has been applied for studying drug absorption [136] and secretion of enterohormones [137] in human endoscopic biopsies. An advantage of UCh models over explant-based models is that UCh models make it possible to measure the electrophysiological parameters, including TEER [136]. All these set-

ups permit analyzing the cytokine profiling of intestinal explants or biopsies retaining their *in situ* conditioning in a polarized fashion [135,138]. We have employed the UCh to determine TEER and cytokine release (TNF-α) in intestinal tissues from CAF diet-induced obese rats treated with GSPE [8,15]. It could also be useful for testing the effect of bioactives on dysfunctional human intestine. A feature of *ex vivo* models is that screening of drug effects does not compromise the patients by exposing them to unknown outcomes.

8.6. Clinical Trials

The use of PACs for intestinal dysfunction is an emerging therapeutic strategy still in preclinical development. There have been no large, well-designed clinical trials that have tested the effectiveness of these phytochemicals. However, current evidence in humans suggests that PAC administration is a promising and safe adjunctive support to current therapies in IBD.

Translation of doses of PAC-rich extracts used in rodent models of intestinal dysfunction to human equivalent doses (HED) indicates that pharmacological doses (up to 5 g d^{-1} for a 60 kg person) could be required to achieve beneficial effects in clinical trials [14,15]. Thus, the first uncertainty involved in assessing the use of PACs as therapy agents in humans is safety. Grape-seed and skin proanthocyanidin-rich extracts have been subjected to toxicological tests in rats to determine their safety for use in functional foods [139–141]. In these studies, the median lethal dose (LD50) was found to be greater than 5000 mg kg^{-1} bw (HED > 50 g) when administered once by oral gavage, and 1400-2000 mg kg^{-1} d^{-1} (HED of ~14–20 g d^{-1}) was found to be the no-observed-adverse-effect level (NOAEL) for systemic toxicity in subchronic administration. A recent study evaluated the safety and tolerability of GSPE intake (up to 2.5 g d^{-1}) in a small number of healthy adults for a four-week period and found a good tolerability without adverse effects on hematological or biochemical parameters [47].

To date, there are few clinical studies that evaluate the influence of PACs on intestinal inflammatory conditions. A pilot study was conducted on paediatric Crohn's disease patients in remission to test the possibility of influencing oxidative stress markers with a procyanidin-rich bark extract from the French maritime pine (Pycnogenol®) [142]. Patients showed reduced antioxidant defenses and increased oxidative stress markers at the beginning of the study compared with healthy controls. After 10 weeks of administration (2 mg kg^{-1} d^{-1}), Pycnogenol had no impact on inflammatory markers (CRP and calprotectin) and the disease activity index score, but it increased the activity of erythrocyte antioxidant enzymes (SOD and GPx), reduced the level of lipid peroxidation markers in the blood (lipoperoxides and 8-isoprostanes), and reduced protein damage (advanced oxidation protein products). Another clinical study revealed that the postprandial increase of plasma LPS associated with the intake of a high-fat meal was significantly reduced in obese subjects who consumed 1 g of GSPE [143]. As translocation of LPS to the circulation is considered a critical factor in the appearance of systemic low-grade inflammation in patients with metabolic syndrome [144], reduction of postprandial endotoxemia could be particularly interesting from a therapeutic perspective. Large double-blind clinical studies need to be conducted to provide more information on PAC clinical efficacy in intestinal dysfunction so that these phytochemicals can be used therapeutically to improve intestinal health in obese and IBD individuals.

9. Biochemical and Molecular Mechanisms Underlying the Barrier Protective and Anti-Inflammatory Properties of PAC in the Intestine

PACs were often considered to be nutritionally undesirable due to their ability to form complexes with macronutrients and reduce the activity of virtually any enzyme implicated in digestion [145,146]. Nevertheless, based on the anti-cancerous, anti-mutagenic, and antimicrobial activities these phytochemicals elicited in laboratory experiments, a role in the modulation of the metabolism and

immune system was suggested [146]. The ability of PACs to form cross-links with biomolecules can be attributed to the hydroxyl groups and aromatic rings in their structure that can establish hydrogen bonds and hydrophobic interactions [147]. PACs have a significant affinity for proline-rich proteins and peptides [148]. In general, binding to proteins seems to increase with the DP as larger PAC molecules have more potential binding sites for the associations with proline residues [148]. This phenomenon, responsible for the "tanning effect" that converts animal skin into leather, accounts for most of their therapeutic properties. The interaction results in effects determined by the biological function of the target protein. Thus, PACs not only alter enzymatic activity, but they may also prevent ligand-receptor interactions and the binding of transcription factors to their specific sites in DNA. In addition, some PAC molecules can be adsorbed non-specifically onto biomembrane surfaces [149], affecting their physical characteristics, such as fluidity and density, and potentially altering membrane-dependent processes, including protein receptor activity [150]. Altogether, these effects lead ultimately to the alteration of cell signaling pathways and the modulation of gene expression (**Figure 3**)

9.1. Antioxidant Activity

For a long time, the significance of PACs for promoting overall health was attributed to the antioxidant and free radical scavenging activity shown in many *in vitro* studies, a hallmark feature of plant phenolic compounds [151]. This property is related to their structure, as the presence of the numerous hydroxyl groups reduces free radicals through electron donation, and the aromatic rings allow the resultant aroxyl radicals to be stabilized by resonance. The antioxidant activity of PACs has been demonstrated in numerous studies performed with PAC-rich foods and derived food products, such as grape, green tea, and cocoa products [152,153]. In particular, GSPEs have shown better free radical scavenging abilities than β-carotene and vitamins C and E [154,155].

New knowledge about the limited bioavailability of PACs meant that the physiological relevance of their antioxidant effects needed to be reconsidered. PACs have been found to have a very limited intestinal absorption [91], and concentrations in the systemic circulation are low with respect to other compounds that display similar antioxidant activity *in vivo* [133]. Thus, PAC antioxidant activity was unlikely to be relevant for the *in vivo* situation in several tissues [133], except for those directly exposed to high concentrations, such as the gastrointestinal tract. *In vitro* studies have suggested that flavan-3-ol and other flavonoids have an inhibitory effect on the NF-κB signaling pathway [156,157]. In addition, it has been demonstrated in HepG2 cells that GSPE improves cell redox status through upregulation of endogenous antioxidant enzymes such as GPx, glutathione S-transferase (GST) and SOD [158]. This is probably mediated by the activation of the nuclear factor-erythroid-2-related factor 2 (NRF2) [159]. These observations support the view of PACs and other polyphenols as versatile bioactives rather than mere antioxidants and encourage the exploration of novel immunomodulatory mechanisms.

9.2. Modulation of Signaling Transduction Pathways

PACs exert direct anti-inflammatory effects modulating kinases activity and transcription factors implicated in the production of cytokines, chemokines, and other inflammatory mediators in epithelial and immune cells. Signaling pathways modulated by PACs include NF-κB, ERK1/2 and p38 MAPKs, and NRF2. Park et al. [157] reported an inhibitory effect of monomeric flavan-3-ol and some oligomeric PACs in NF-κB dependent gene expression of IFN-γ-stimulated RAW 264.7 cells (murine macrophages). However, the first demonstration of flavan-3-ols inhibiting NF-κB by direct interaction with key components of the signaling pathway, came from a study by Mackenzie et al. [160]. In this study, Jurkat (human T lymphocyte) cells incubated with (+)-catechin, (–)-epicatechin or B-type dimeric PACs prior to the PMA (NF-κB activator) stimulation showed a reduction in the nuclear

translocation of NF-κB due to the inhibition of IKKβ and IκBα phosphorylation. In addition, accumulation of these compounds in the nucleus (especially PAC dimers) dose-dependently suppressed the DNA-binding activity of the p50/RelA complex, indicating that inhibition of the NF-κB signaling pathway also occurred downstream. A subsequent study by the same authors [161] evidenced that dimeric procyanidins B1 and B2, but not A1 or A2, inhibited binding of p50/RelA to DNA. Molecular modelling for these interactions indicated that dimeric B-type PACs adopted a folded structure that mimicked the guanine pair in the κB DNA consensus sequence, which is responsible for the binding of arginine residues in the p50/RelA complex [161]. Terra et al. [11] demonstrated that GSPE also down-regulated IκBα mRNA and inhibited RelA nuclear translocation in RAW 264.7 macrophages stimulated with LPS and IFN-γ. Further *in vivo* studies confirmed unequivocally that grape-seed PACs orally administered to rats suppressed canonical activation of NF-κB induced by TNBS and a CAF diet in the intestine [122,123] and liver [130].

ERK1/2 and p38 MAPKs and are a family of serine/threonine kinases that mediate cellular responses to external stress signals and cytokines [162]. Activation of p38 MAPK has been associated with transduction of the proinflammatory cytokine signal within the intestinal epithelial cell [45]. ERK1/2 and p38 are involved in NF-κB transactivation through the phosphorylation and activation of the mitogen- and stress-activated protein kinase-1 (MSK-1), which in turn phosphorylates RelA [163]. They are also implicated in the phosphorylation of the coactivator p300 required for RelA acetylation [164]. PACs have shown to modulate MAPK activity by different mechanisms. Some authors have suggested that hexameric PACs inhibit bile acid-induced activation of ERK1/2 and p38 MAPKs in intestinal epithelial cells by a lipid raft-dependent effect involving inhibition of NADPH oxidase (NOX) [165]. More recently, a molecular docking analysis indicated that procyanidin B1 may bind to the TLR4/MD-2 complex and be able to act as a competitive antagonist of LPS [166]. This effect was associated with the inhibition

of the LPS-induced phosphorylation of p38 MAPK and activation of NF-κB signaling in THP1 (human monocyte) cells. However, PACs may activate ERK1/2 and p38 MAPKs under some oxidative stress situations, which has been associated with translocation of NRF2 to the nucleus. Under oxidative stress, NRF2 promote cell survival by inducing expression of antioxidant enzymes via antioxidant responsive element (ARE) binding [167]. Studies in Caco-2 [159] and HepG2 cells [168] showed that procyanidin B2 and grape-seed PACs induce activation of NRF2, increasing GST [159] and HO-1 [168] activity. More recently, AMPK-induced activation of NRF2 by oral administration of GSPE was associated with a protective effect against lead-induced lung oxidative stress in rats [169].

9.3. Modulation of TJ Integrity

The precise mechanisms underlying the improvement in intestine paracellular permeability due to PACs in inflammation are not yet completely elucidated; however, it is known that they lead ultimately to the upregulation (e.g., ZO-1 and claudin-1 [8,13]) or downregulation (e.g., claudin-2 [125]) of TJ protein expression. Loss of TJ integrity in the proinflammatory state is mediated by the NF-κB signaling pathway and by the activation of protein kinases MAPKs, PI3Ks, AMPK, and MLCK [43]. MLCK is particularly crucial in actomyosin-based cytoskeletal functions and multiple studies highlight its important role in intestinal TJ remodelling [40,41]. PACs reduce the production of proinflammatory mediators (e.g., TNF-α) and reactive oxygen species (i.e., iNOS activity) associated with enhancing intestinal permeability by antagonizing the NF-κB signaling pathway. In addition, PACs are potent inhibitors of kinases including MLCK [43,170]. Contreras et al. [171] suggested that there is an upstream mechanism associated with flavan-3-ols that leads to the prevention of TNF-α-induced intestinal permeability. In this study, TNF-α-stimulated Caco-2 monolayers incubated with (–)-epicatechin showed a reduction of NOX activity, an enzyme that also facilitates activation of TNF-α

signaling. This effect was directly associated with the inhibition of ERK1/2 MAPK activity of IκB phosphorylation and of MLCK activation.

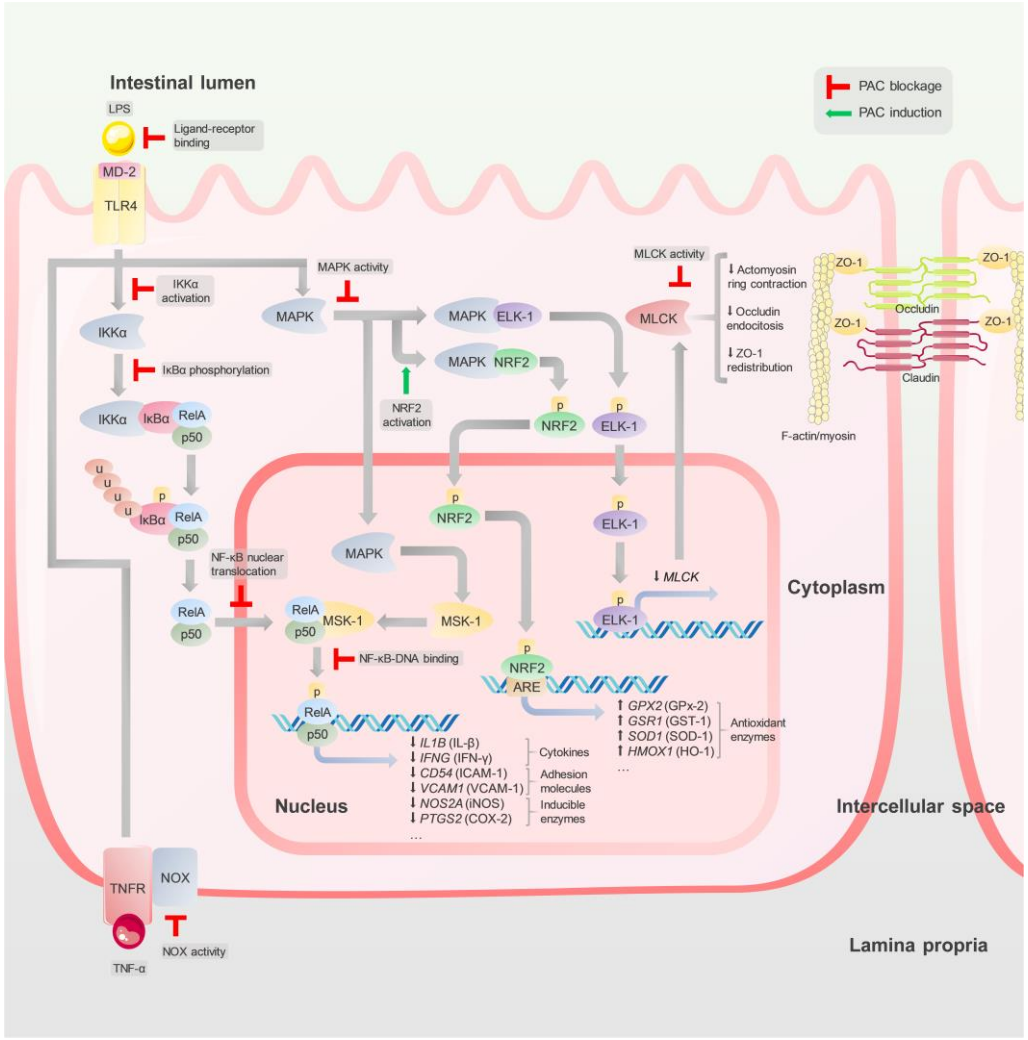


Figure 3. Molecular mechanisms implicated in the physiological effects of PAC in the intestinal mucosa. PACs suppress inflammation interacting with bacterial endotoxins, as well as protein receptors, kinases and transcription factors involved in the proinflammatory signaling (NF-κB and mitogen-activated protein kinases (MAPK) pathways). Oxidative stress is mitigated directly by free-radical scavenging and indirectly by the activation of factor-erythroid-2-related factor 2 (NRF2), leading to antioxidant enzyme production via antioxidant responsive element (ARE) binding.

9.4. Interaction with Bacterial Endotoxins

Delehanty et al. [172] demonstrated that naturally occurring A- and B-type cranberry PACs were able to bind the lipid A moiety of LPS, exhibiting an affinity similar to that of polymyxin B, a potent LPS-binding molecule. In this study, PACs efficiently blocked endocytosis of bacterial LPS in a dose-dependent manner in HEK 293 (human embryonic kidney cells) that expressed receptors TLR4/MD-2 and CD14, thus preventing the induction of the NF-κB signaling pathway without any interaction with cellular components. However, other authors reported that PACs isolated from cocoa beans did not abrogate the binding of LPS to TLR4 in cultivated human dendritic cells [173]. PAC–LPS binding has been linked to the reduction of the post prandial increase in blood LPS associated with the ingestion of a high-fat meal in obese subjects ingesting an oral dose of GSPE [143].

9.5. Modulation of Intestinal Microbiota

Dietary PACs, specifically longer polymers, reach the distal intestine nearly intact, where they become fermentable substrates for the commensal microbiota [174]. PACs have been associated with prebiotic properties, boosting the composition of several kinds of probiotics such as *Bifidobacterium spp.*, *Lactobacillus spp* [175] and the stimulator of MUC2 production *Akkermansia muciniphila* [176,177]. Nevertheless, current evidence is somewhat controversial as effects described in different *in vivo* studies mainly performed with rodents do not always agree. This suggests that interactions between PACs and microbiota depend largely on the botanical source, the types of molecules present in the extracts tested, and the animal model [178].

A recent study by Casanova et al. [179] found that oral administration of GSPE in Wistar rats for eight days resulted in profound changes in the cecal microbiota composition, reducing diversity indices and the ratio of *Firmicutes* to *Bacteroidetes*. Similar results were found in diet-induced obese Sprague Dawley rats

supplemented with a PAC-rich extract of the *Pyracantha fortuneana* fruit, although in this study an increase in microbiota diversity was also reported [120]. GSPE supplementation in IL-10 deficient mice resulted in an increased abundance of *Bacteroides* and *Lactobacilli* [125]. Xing et al. [177] reported that the administration of procyanidin B2 in rabbits feeding a high-fat-cholesterol diet, promoted an increase in the relative abundance of *Akkermansia*. These authors proposed that the reduction of metabolic endotoxemia found in animals treated with procyanidin B2 was attributed to the ability of *Akkermansia* to retain the thickness of the intestinal mucus layer, thus reducing intestinal permeability and the leakage of LPS into the circulation [180].

Cuevas et al. [175] found that *in vitro* fermentation of grape-seed monomers and PACs in human feces resulted in a reduced abundance of *Clostridium histolyticum*. Inhibition of the growth of some infectious microorganisms, such as the mentioned *C. histolyticum* in the intestine and *Helicobacter pylori* in the stomach [181], may be related to the anti-adherence activity that PACs have demonstrated in *in vitro* studies [182], as adherence to the epithelium is a prerequisite for colonization and infection of the intestinal gastrointestinal mucosa.

Finally, phenolic acids and phenyl-γ-valerolactones resulting from the colonic fermentation of PACs also exhibit a significant bioactivity in cell models and experimental animals [183]. They therefore may partially account for the beneficial anti-inflammatory effects reported in intestinal and peripheral tissues *in vivo*. Further research is needed to clarify the importance of these microbial products in health promoting properties associated with the intake of PACs.

10. Conclusions and Future Perspectives

The health promoting properties of PACs in the intestine are attributed not only to the antioxidant activity inherent to phenolic compounds, but also to the capacity of these phytochemicals to interact with multiple biomolecules, including proteins,

biomembrane lipids, and endotoxins. Bioactivity of PACs is highly structure-dependent and enriched botanical extracts composed by a large variety of molecular structures exert a wide range of unrelated physiological effects. In this way, PAC-rich extracts can modulate kinase activity, several signal transduction pathways implicated in the inflammatory response, and the remodelling of TJs. Flavan-3-ol monomers and short PAC oligomers are absorbed by enterocytes and immune cells and exert a direct action on kinases and transcription factors. Bioactivity of larger oligomers and polymeric PACs do not require direct intestinal absorption and are able to bind protein receptors on the enterocyte and immune cell surfaces as well as luminal bacterial endotoxins, thus inhibiting proinflammatory signaling and improving barrier integrity. Due to the negligible absorption of large PAC molecules in the short intestine, phenyl-γ-valerolactones and phenolic acids produced by the microbiota metabolism in the colon are thought to play an important role in these health-promoting effects, and thus need to be further researched.

The barrier-protective and anti-inflammatory properties of PACs are emerging as a potential adjunctive support to current therapies for managing obesity related intestinal dysfunction and IBD. However, there have been no large, well-designed clinical trials establishing the efficacy of these phytochemicals in chronic conditions. At preclinical stages, the use of animal models is the predominate approach for testing novel therapies for intestinal dysfunction, although several strategies for replacing animal experiments have been proposed. As there are still no studies on the impact of PACs on human intestinal health, *ex vivo* models of the human intestine could be a more physiologically reliable alternative to human cell lines and an alternative to animal experimentation in preclinical development.

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II. Hypothesis and Objectives

The integrity of the intestinal barrier has been recognized of relevance for health and disease, and is a topic garnering increasing attention [1]. PACs have been identified as powerful antioxidant and anti-inflammatory bioactive compounds that exert beneficial effects against intestinal inflammation and permeability provoked by nutritional factors [2,3] and colitis-inducing chemicals [4,5] in preclinical studies.

Our research group's development of a successful rat model of CAF diet-induced obesity with intestinal dysfunction [6] allowed us to test the efficacy of the oral administration of GSPE as a therapeutic approach in the context of a Western-style diet (CAF diet) [2,3]. Gil-Cardoso et al. [2] found that nutritional doses sub-chronically administered in these animals at the end of a long-term CAF diet ameliorated the oxidative stress and inflammation produced in the small intestine by this nutritional intervention. This study also suggested the amelioration of the barrier integrity in the ileum, although it was evaluated indirectly by the analysis of changes in the expression of protein genes involved in the maintenance of epithelial junctional complexes. In a further study [3], pharmacological doses of GSPE proved to be effective against intestinal dysfunction and metabolic endotoxemia in CAF-diet fed rats when co-administered or administered preventively. This detrimental effect of a CAF diet on the integrity of the intestinal epithelium has been described as a multifactorial process driven by dysbiosis [7] and by the exposure of intestinal tissues to several proinflammatory nutritional factors, including saturated fats [8]. In this regard, the consumption of large amounts of fructose, a major component of the CAF diet, has also been proposed as a potential inductor of intestinal barrier disruption [9] and chronic diseases in mammals [10], although its effects on human health are still a matter of controversy [11]. Therefore, the detrimental effects of a CAF diet on the intestine could be partially driven by fructose.

Research on PACs in the context of chronic disease is still at the preclinical stage, and most of the data available has been provided by studies with experimental animals and cell lines. Therefore, one of the most important findings to emerge from our literature review (**Manuscript 1** [12]) was the limited availability of data regarding the influence of PACs on intestinal health in humans. It has been reported that an oral dose of GSPE can prevent the acute increase in plasma endotoxin levels associated with a high-fat meal in obese adults [13] and this could indicate the reinforcement of the intestinal barrier.

Based on the above, we hypothesized that **the administration of pharmacological doses of GSPE as a counteractive or preventive treatment can improve the intestinal dysfunction and metabolic endotoxemia provoked by nutritional factors associated with the Western diet and can be beneficial to human intestinal health in the context of acute dysfunction.**

To assess the validity of this hypothesis, as the overall objective of this thesis we proposed **to evaluate the impact of GSPE on intestinal barrier function alterations, local inflammation and metabolic endotoxemia in preclinical models of intestinal dysfunction and to investigate its effectiveness in humans.**

To meet this overarching objective, we established a set of specific objectives:

1. To test the efficacy of orally administered pharmacological doses of GSPE in reversing intestinal dysfunction and metabolic endotoxemia induced by a long-term CAF diet in obese rats and to perform a comprehensive analysis of changes in the intestinal barrier function and inflammatory response through the implementation of *in vivo* and *ex vivo* protocols.

2. To explore the impact of high concentrations of fructose on the barrier function and inflammatory response in a cell culture-based model of human

intestinal epithelium and to assess the influence of GSPE on its putative detrimental effects.

3. To gain insight into the influence of GSPE on human intestinal health, evaluating its effectiveness in the prevention of intestinal mucosa alterations using an *ex vivo* model of acute colonic dysfunction produced by a colitis-inducing chemical.

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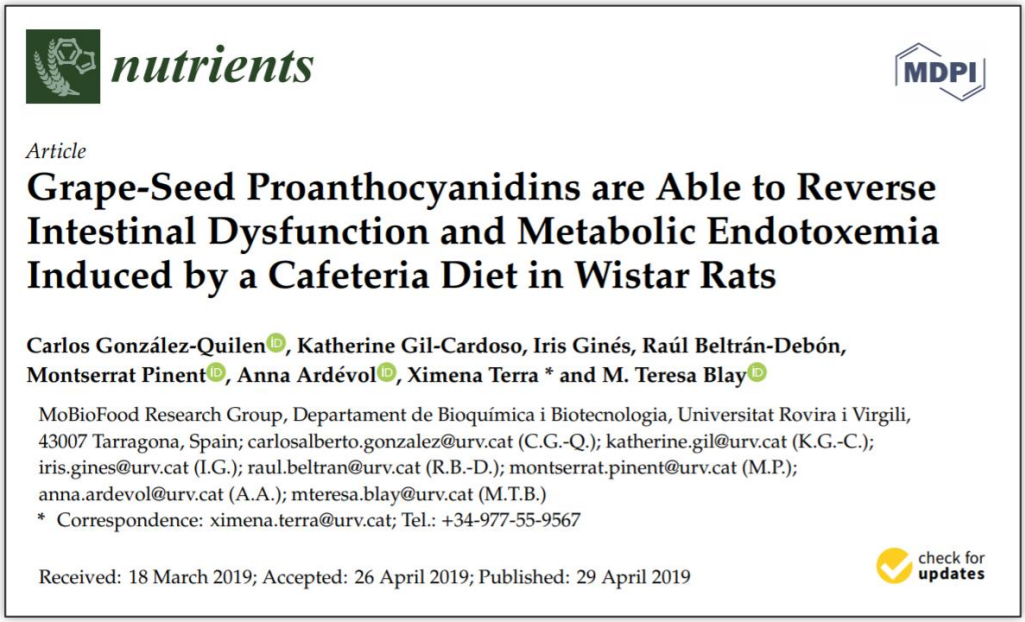
III. Results

1. *In Vivo* and *in Vitro* Studies of Intestinal Dysfunction Induced by Nutritional Factors

In the first part of this Results section, we present two studies that tested the therapeutic properties of GSPE in the context of the intestinal dysfunction associated with the Western diet:

- In the study described in **Manuscript 2**, pharmacological doses of GSPE were co-administered to CAF-diet fed Wistar rats for 15 days at the end of a 17-week diet intervention. This study aimed to explore the impact of GSPE on intestinal permeability and inflammatory markers by means of both *in vivo* and *ex vivo* techniques and its effectiveness in reversing metabolic endotoxemia.
- In the study described in **Manuscript 3**, the effect of high concentrations of fructose accessible in the context of the CAF diet were evaluated in the barrier function and inflammatory response of human colon carcinoma Caco-2 cell monolayers. The influence of GSPE on fructose-induced detrimental effects was also assessed.

1.1. Manuscript No. 2



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Grape-Seed Proanthocyanidins are Able to Reverse Intestinal Dysfunction and Metabolic Endotoxemia Induced by a Cafeteria Diet in Wistar Rats

Carlos González-Quilen, Katherine Gil-Cardoso, Iris Ginés, Raúl Beltrán-Debón, Montserrat Pinent, Anna Ardévol, Ximena Terra * and M. Teresa Blay

MoBioFood Research Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, 43007 Tarragona, Spain; carlosalberto.gonzalez@urv.cat (C.G.-Q.); katherine.gil@urv.cat (K.G.-C.); iris.gines@urv.cat (I.G.); raul.beltran@urv.cat (R.B.-D.); montserrat.pinent@urv.cat (M.P.); anna.ardevol@urv.cat (A.A.); mteresa.blay@urv.cat (M.T.B.)

* Correspondence: ximena.terra@urv.cat; Tel.: +34-977-55-9567

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Grape-Seed Proanthocyanidins are Able to Reverse Intestinal Dysfunction and Metabolic Endotoxemia Induced by a Cafeteria Diet in Wistar Rats

By Carlos González-Quilen, Katherine Gil-Cardoso, Iris Ginés, Raúl Beltrán-Debón, Montserrat Pinent, Anna Ardévol, Ximena Terra and M. Teresa Blay

MoBioFood Research Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, 43007 Tarragona, Spain

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Abstract: We evaluated the effectiveness of pharmacological doses of grape-seed proanthocyanidin extract (GSPE) in reversing intestinal barrier alterations and local inflammation in female Wistar rats fed a long-term obesogenic diet. Animals were fed a 17-week cafeteria diet (CAF diet), supplemented with daily GSPE doses (100 or 500 mg kg^{-1} body weight) during the final two weeks. CAF diet enhanced the intestinal permeation of an orally administered marker (ovalbumin, OVA) and increased the plasma levels of tumor necrosis factor-α (TNF-α) and lipopolysaccharides (LPS) in 2–3-fold. *Ex vivo* Ussing chamber assays showed a 55–70% reduction in transepithelial electrical resistance (TEER) and increased the TNF-α secretions in both small and large intestinal sections with a 25-fold increment in the ileum. Ileal tissues also presented a 4-fold increase of myeloperoxidase (MPO) activity. Both GSPE-treatments were able to restitute TEER values in the ileum and colon and to reduce plasma LPS to basal levels without a dose-dependent effect. However, effects on the OVA permeation and TNF-α secretion were dose and section-specific. GSPE also reduced ileal MPO activity and upregulated claudin 1 gene expression. This study provides evidence of the efficacy of GSPE-supplementation ameliorating diet-induced intestinal

dysfunction and metabolic endotoxemia when administered at the end of a long-term obesogenic diet.

Keywords: flavan-3-ol; gut; inflammation; obesity; tight junction; Ussing chamber; transepithelial electrical resistance

1. Introduction

It has been reported that there is a causal relationship between pathophysiological changes in the intestinal epithelium and obesity in animal models and humans [1–3]. Studies aiming to identify natural compounds that modulate intestinal alterations in obesity are therefore promising future therapeutic strategies [2,4].

The rising prevalence of obesity in Western societies and developing countries is a serious public health concern that, in part, results from the consumption of unbalanced hypercaloric diets [5,6]. High-saturated fat/high refined-carbohydrate diets, that are low in fiber and plant flavonoids, induce not only weight gain in humans and laboratory animals, but also alterations in gut microbiota (dysbiosis) [7–9] and intestinal dysfunction (increased permeability and local inflammation) [10].

Increased intestinal permeability leads to a higher flow of intestinal endotoxins, e.g., lipopolysaccharides (LPS) deriving from luminal gram-negative bacteria, to the inner intestinal layers [11–13]. This causes a local inflammatory response in the intestine that aggravates barrier function deficiency in a vicious cycle. When LPS reaches the general circulation due to the inability of the intestinal immune response to neutralize it, it can spread systemically causing metabolic endotoxemia and obesity-related complications such as adipose tissue dysfunction as well as systemic low-grade inflammation [14].

Intestinal permeability and inflammatory response are site-specific processes, i.e., the small and large intestines behave in different ways in terms of permeability increase and susceptibility to inflammation [15,16]. The duration of the dietary feeding period has a strong influence on the initiation of intestinal dysfunction and obesity in animals consuming a high-fat/high-sugar diet. Rats fed an obesogenic diet show evidence of intestinal dysfunction at 8–12 weeks [3,8,17,18]. By this time, an increment of permeability (most commonly measured as plasma concentration of an orally administered marker), proinflammatory cytokine production (TNF-α, IL-6, etc.) and myeloperoxidase (MPO) activity are evidenced in the intestinal mucosa. The downregulation of tight-junction (TJ)-related genes and proteins (zonula occludens 1, occludin and claudin 1) also occurs.

Anti-obesity treatments have proven to be of limited effectiveness. Complementary dietary strategies such as bioactive compounds with anti-obesity effects could, therefore, be an adjunctive support to current therapies and reinforce obesity treatments. Indeed, the supplementation of hypercaloric diets with flavonoid sources could become a reliable strategy for correcting the intestinal dysfunction associated with the obesogenic process [18,19]. Notably, in European countries, the average habitual intake of flavonoids is considerably below the amounts used in most dietary intervention studies [20].

Multiple studies demonstrate the beneficial effects of a grape-seed proanthocyanidin extract (GSPE) on obesity-related outcomes, including the amelioration of intestinal dysfunction [19,21–23]. Proanthocyanidins are oligomeric and polymeric flavan-3-ols, mainly constituted of catechin and epicatechin subunits. They are an important component of plant-based diets and are abundant in green tea, certain fruits and beverages and in spices such as cinnamon [24].

We have described a combination of GSPE doses and frequencies of administration that can effectively prevent some of the metabolic and intestinal alterations

associated with diet-induced obesity in rats [18,23]. In a previous study, we also tested the effect of nutritional doses of the GSPE (5-50 mg kg^{-1} d^{-1}) after 15 weeks of cafeteria (CAF) diet administration. We evidenced a reduction of oxidative stress (reactive oxygen species and iNOS mRNA expression) and inflammatory markers (MPO activity and IL-1β mRNA expression) in ileal tissues, as well as the upregulation of zonula occludens 1 gene expression. Nevertheless, we found no differences in plasma LPS levels between experimental groups at these doses. Our hypothesis is that, in rats fed a CAF diet for a period of 17 weeks leading to obesity, supplementation with the GSPE at pharmacological doses during the final two weeks can reverse the diet-associated intestinal dysfunction and metabolic endotoxemia.

In this study, we determined the effectiveness of two pharmacological doses of GSPE (100 and 500 mg kg^{-1} body weight), administered daily for two weeks at the end of a 17-week obesogenic diet. The doses and conditions of administration of the GSPE were evaluated by *in vivo* and *ex vivo* measurements in both small (duodenum and ileum) and large (colon) intestines. We focused on intestinal permeability, local inflammatory status, metabolic endotoxemia and systemic inflammation. Here, we corroborate the relationship between CAF diet consumption, metabolic endotoxemia and the associated intestinal dysfunction. Additionally, we provide evidence of the counteractive effect of GSPE on the alterations induced by a long-term CAF diet (obese animals) at the intestinal and systemic levels.

2. Materials and Methods

2.1. Grape-Seed Proanthocyanidin Extract

The grape-seed proanthocyanidin extract (GSPE) was provided by Les Dérivés Résiniques et Terpéniques (Dax, France). The GSPE administered in this study (batch number 124029) has the following composition: monomers of flavan-3-ols

(21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5–13 units; 31.7%) of proanthocyanidins. A detailed analysis of the monomeric, dimeric, and trimeric structures can be found in the work by Margalef et al. [25].

2.2. Experimental Animals

Forty-seven-week-old female Wistar rats (240–270 g) purchased from Charles River Laboratories (Barcelona, Spain) were individually caged in animal quarters at 22 °C with a 12-h light/12-h dark cycle and fed a standard chow diet (STD diet; Panlab 04, Barcelona, Spain) and tap water *ad libitum*. After an acclimation period, animals were randomly distributed into four experimental groups (n=10). The control group (STD group) received only the STD diet. The other groups were fed a CAF diet as a model of high saturated-fat/high refined-carbohydrate diet until the end of the animal experiment. The CAF diet was offered ad libitum and replenished every day with a quantity that was enough for 17 weeks. CAF-fed animals also had free access to standard chow. The composition of the diets supplied is shown in **Table 1**. During the final two weeks of the CAF intervention, two CAF-diet groups received daily GSPE doses of 100 and 500 mg kg^{-1} bw as a corrective treatment (groups CORR100 and CORR500, respectively). The GSPE was dissolved in water and orally gavaged every day to each animal at 18:00 h in a final volume of 0.5 mL. Non-supplemented animals received water as a vehicle. The experimental design is shown in **Figure 1**. All procedures involving the care and use of animals in this work were reviewed and approved by The Animals Ethics Committee of the Universitat Rovira i Virgili (code: 0152S/4655/2015).

Table 1. The composition and energy content of diets administered.

	STD	CAF
Content (g 100 g^{-1} dry matter)		
Available carbohydrate	48.0	62.6
Sugar	~0.0	46.0
Protein	14.3	15.1
Fat	4.0	17.2
Saturated	0.6	8.1
Fiber	4.1	1.7
Energy contribution		
kJ g^{-1} dry matter	12.1	20.7
Carbohydrate (%)	67.2	52.0
Protein (%)	20.2	14.1
Fat (%)	12.6	33.9

STD diet, standard chow diet; CAF diet, cafeteria diet. The CAF diet consisted of bacon, sausages, biscuits with paté, carrots, muffins, and sugared milk, which induces voluntary hyperphagia. ~, approximately.

2.3. Blood and Tissue Collection

At the end of the study, the animals were fasted for 14 h, anesthetized with sodic pentobarbital (70 mg kg^{-1} bw; Fagron Iberica, Barcelona, Spain) and exsanguinated from the abdominal aorta. The total blood was collected using heparin (Deltalab, Barcelona, Spain) as an anticoagulant. Plasma was obtained by centrifugation (1500 × g for 15 min at 4 °C) and stored at −80 °C until analysis. White adipose tissue depots were rapidly removed and weighed. The small intestine, defined as the portion of the gastrointestinal tract between the pylorus and the ileocecal valve, was dissected. Colon tissues were also removed. Five-centimeter segments of the

duodenum, ileum and colon were taken for Ussing chamber assays. Leftover segments of small and large intestines were stored at −80 °C until analysis.

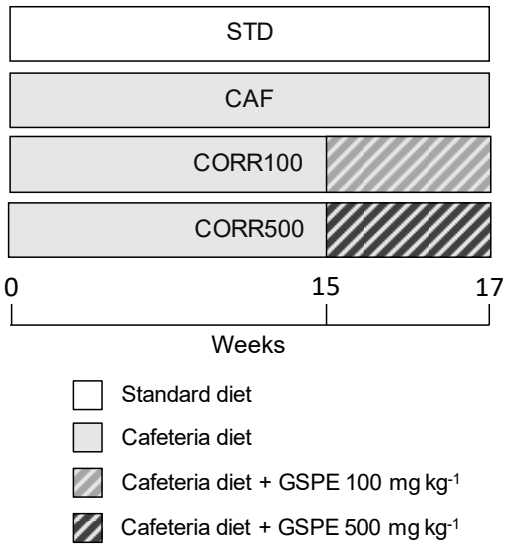

Figure 1. The schematic diagram of the experimental design. STD group, rats fed standard chow diet; CAF group, rats fed cafeteria (CAF) diet; CORR100 group, CAF-fed rats supplemented with 100 mg kg^{-1} body weight of grape-seed proanthocyanidin extract (GSPE); CORR500 group, CAF-fed rats supplemented with 500 mg kg^{-1} bw of GSPE. GSPE doses were administered daily by oral gavage during the final two weeks of the animal experiment. The number of animals included in this study was n = 10 for each group.

2.4. Morphometric and Biochemical Parameters

Body weight was monitored weekly until the end of the experiment. Body weight gain was calculated by subtracting the initial body weight from the final body weight. Adiposity was expressed as an adiposity index, which was based on total fat pad measurements. This was computed for each rat as previously described [26]. These variables were evaluated as physiological indicators of the degree of obesity in the experimental animals. Enzymatic colorimetric kits were used to

measure the plasma levels of glucose (QCA, Amposta, Spain) and triacylglycerols (TAG) (QCA, Amposta, Spain).

2.5. LPS and TNF-α Plasma Determinations

Plasma LPS levels were determined using a Pyrochrome lysate mix diluted in Glucashield buffer (Associates of Cape Cod, E. Falmouth, MA, USA), which inhibits cross-reactivity with (1→3)-β-d-glucans. Plasma concentrations of tumor necrosis factor-α (TNF-α) were measured by ELISA (Merck Millipore, Madrid, Spain) with a sensitivity of 4.2 pg mL^{-1}.

2.6. Oral Intestinal Permeability Test

In vivo intestinal permeability was assessed using the ovalbumin (OVA) test at 17 weeks, for which the animals were previously fasted for four hours. OVA (Sigma-Aldrich, Madrid, Spain) was administered by oral gavage at 250 mg kg^{-1} bw in a final volume of 0.5 mL of phosphate buffer solution. One hour later, blood was collected from the saphenous vein, then heparinized and centrifuged (12,000 × g for 10 min at 4 °C). Plasma OVA levels were determined by ELISA (MyBioSource, Madrid, Spain) with a detection range of 16–10,000 pg mL^{-1}.

2.7. Ex Vivo Assessment of Intestinal Dysfunction: Intestinal Barrier Integrity and Local Inflammation

Ex vivo intestinal permeability and local inflammation were evaluated in an Ussing chamber system (Dipl.-Ing. Muβler Scientific Instruments, Aachen, Germany) by measuring transepithelial electrical resistance (TEER) and TNF-α secretions in the basolateral medium. At the end of the experiment, fresh intestinal tissues (duodenum, ileum and colon) were immediately placed in a cold oxygenated Krebs-Ringer bicarbonate buffer (KRB buffer), dissected to remove serosal and muscular layers, and placed on 0.237 cm^2 aperture Ussing chambers. Mucosal preparations were mounted within 10 min following euthanasia and apically and basolaterally

bathed with 1.5 mL of the KRB buffer. The basolateral bathing solution, which contained 10 mM of glucose (Panreac, Barcelona, Spain), was osmotically balanced on the apical compartment with 10 mM of mannitol (Sigma, Madrid, Spain). Bathing solutions were oxygenated and circulated in water-jacketed reservoirs maintained at 37 °C. The transepithelial potential difference (PD) was short-circuited through Ag-AgCl electrodes with a voltage clamp that corrected for fluid resistance. TEER (ohm.cm^2) was calculated from the transepithelial PD and short-circuit current in accordance with Ohm's law.

After a 20-min equilibration period, the KRB buffer in the apical and basolateral compartments was replaced by a fresh KRB buffer containing 10 mM of glucose and protease inhibitors (10 μM amastatin (Enzo Life Sciences, Madrid, Spain), 500 KIU aprotinin (Sigma, Madrid, Spain) and 0.1 % bovine serum albumin (BSA) fatty acid free). Mucosal preparations were incubated for an additional 30 min, after which basolateral media were collected for determining TNF-α secretions by ELISA (Merck Millipore, Madrid, Spain).

2.8. Quantification of MPO Activity in the Ileum

MPO activity was used as an indicator of neutrophil accumulation in the rat ileum. Tissue samples were homogenized with a TissueLyser LT system (Qiagen, Hilden, Germany) in 50 mM of potassium phosphate buffer (Panreac, Barcelona, Spain). The homogenate was centrifuged at 15,000 × g for 15 min at 4 °C, and the resulting supernatant was discarded. The pellet was then homogenized with hexadecyltrimethylammonium bromide (HTBA) (Sigma-Aldrich, Madrid, Spain) and 50 mM of potassium phosphate buffer. The homogenate was sonicated (20 s), subjected to three freeze-thaw cycles, and centrifuged at 15,000 × g for 10 min at 4 °C. To determine MPO activity we used an adaptation of the Lenoir method [27]. The supernatant was mixed into a solution of a 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**.** MPO activity was expressed as U per mg of protein in the final fraction.

2.9. Tissue RNA Extraction and Gene Expression Analysis by RT-qPCR

The total RNA was extracted from 50 mg of ileum using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's instructions. Complementary DNA (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 polymerase chain reaction (qPCR) amplification and detection were performed in a qPCR system (Applied Biosystems, Madrid, Spain) using the TaqMan Universal PCR Master Mix (Applied Biosystems, Madrid, Spain) and the respective specific TaqMan probes (Applied Biosystems, Madrid, Spain): Rn02116071_s1 for zonula occludens 1 (*Tjp1*), Rn00587389_m1 for junctional adhesion molecule 1 (*F11r*), Rn00581740_m1 for claudin 1 (*Cldn1*), Rn02063575_s1 for claudin 2 (*Cldn2*). The results were normalized with respect to the cyclophilin A gene (*Ppia*) (Rn00690933_m1). Reactions were performed using the following thermal profile: 2 min at 50 °C, 2 min at 95 °C, and 40 cycles of 15 s at 95 °C and 2 min at 60 °C. The relative mRNA expression levels were calculated following the $2^{-\Delta\Delta Ct}$ method [28], where $\Delta Ct = Ct\ gene\ of\ interest - Ct\ cyclophilin$ and $\Delta\Delta Ct = \Delta Ct\ treated\ samples - the\ mean\ of\ \Delta Ct\ control\ samples$.

2.10. Statistical Analysis

Results are expressed as the mean value ± the standard error of the mean (SEM). Statistical comparisons between groups were assessed by a two-sided Student's *t*-test or ANOVA followed by Tukey's HSD test when the variances were equal and Dunnett's T3 test when they were not. *P*-values < 0.05 were considered statistically significant. Analyses were performed with IBM SPSS statistics 22 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Morphometric and Biochemical Parameters

Here, we tested the GSPE supplementation (100 and 500 mg kg^{-1} bw per day) in Wistar rats during the final two weeks of a 17-week CAF diet. Morphometric and biochemical parameters are presented in **Table 2**. To assess the influence of the GSPE treatment on the CAF-diet detrimental effects, we first tested its effectiveness in controlling body weight and adiposity. GSPE-supplementation did not bring about any change in the final body weight or adiposity index compared to the CAF group, even though the body weight gain of the CORR500 group showed a statistically significant reduction.

Table 2. The morphometric and biochemical parameters in experimental groups at 17 weeks.

	STD	CAF	CORR100	CORR500
Morphometric parameters				
Initial body weight (g)	220.7 ± 4.5	216.6 ± 3.5	221.4 ± 5.3	219.6 ± 3.3
Final body weight (g)	273.7 ± 7.8 ^{a}	346.2 ± 12.0 ^{b}	358.7 ± 13.3 ^{b}	332.7 ± 14.0 ^{b}
Body weight gain (g)	48.4 ± 3.4 ^{a}	131.3 ± 12.2 ^{c}	106.7 ± 12.4 ^{b,c}	89.6 ± 6.7 ^{a,b}
Adiposity (%)	5.6 ± 0.5 ^{a}	11.8 ± 0.8 ^{b}	12.0 ± 1.0 ^{b}	11.3 ± 4.9 ^{b}
Biochemical parameters				
Glucose (mM)	8.9 ± 0.7	10.2 ± 0.1	11.5 ± 1.0	10.4 ± 0.4
Triacylglycerols (mM)	0.41 ± 0.1	0.57 ± 0.1	0.45 ± 0.1	0.46 ± 0.1

STD group, rats fed standard chow diet; CAF group, rats fed cafeteria (CAF) diet; CORR100 group, CAF-fed rats supplemented with a 100 mg kg^{-1} body weight of grape-seed proanthocyanidin extract (GSPE); CORR500 group, CAF-fed rats supplemented with a 500 mg kg^{-1} bw of GSPE. The number of animals included in this study was n = 10 for each group. Values indicate mean ± standard error (SEM). Different letters indicate statistically

significant differences between groups ($p < 0.05$); ANOVA test followed by Tukey's HSD test.

Regarding biochemical parameters, GSPE-supplementation did not produce any significant changes in the glucose or triacylglycerol levels. Non-statistically significant differences were found between the CORR100 and CORR500 groups for the parameters already mentioned.

3.2. In Vivo Intestinal Permeability

The effects of the CAF diet and GSPE on intestinal permeability were determined *in vivo* by the measurement of OVA concentrations in plasma, 1 h after oral administration. After 17 weeks, the CAF group showed a 3-fold increase of plasma OVA levels compared to the STD group (24.2 ± 2.2 and 8.2 ± 1.7 ng mL^{-1} respectively; **Figure 2A**). Interestingly, GSPE supplementation decreased the OVA permeation by 30–60% with respect to the CAF group, but only the CORR100 group showed a significant reduction in this parameter (9.8 ± 2.3 ng mL^{-1}).

3.3. Metabolic Endotoxemia and Systemic Inflammation

The expected increased influx of the endotoxin through the intestinal epithelium of obese animals and the potential ameliorative effect of GSPE on metabolic endotoxemia were assessed by determining plasma LPS levels. The CAF group showed significantly higher LPS levels in plasma with respect to the STD group (136.9 ± 17.1 and 56.2 ± 7.7 EU mL^{-1} respectively; **Figure 2B**). Plasma LPS levels estimated in GSPE-treated groups were comparable to the STD group (64.7 ± 10.8 and 64.1 ± 14.8 EU mL^{-1} for CORR100 and CORR500 respectively), which represents a significant reduction with respect to the CAF group without a dose-dependent effect.

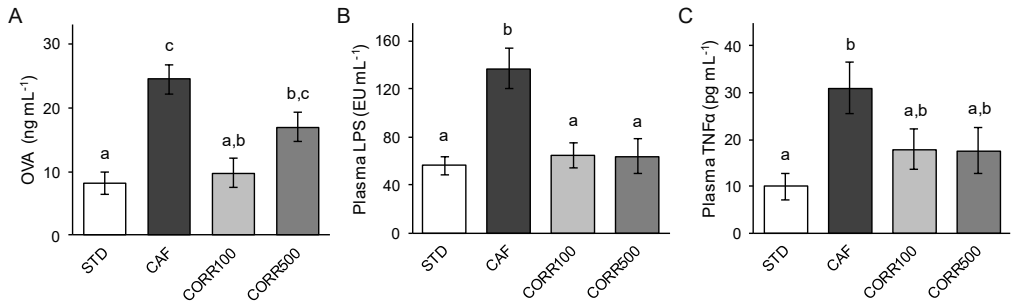

Figure 2. The effect of the grape-seed proanthocyanidin extract (GSPE) treatment on intestinal permeability, metabolic endotoxemia and systemic inflammation in diet-induced obese rats. Values represent mean ± SEM; *n*=8–10. Different letters indicate the statistically significant differences between groups ($p<0.05$); ANOVA test followed by Tukey's HSD test when variances were equal and Dunnett's T3 test when they were not.

TNF-α was measured in plasma as a systemic inflammation marker. The CAF group showed a 3-fold increase in plasma TNF-α with respect to the STD group (30.9 ± 7.7 and 10 ± 2.8 pg mL^{-1}). A non-statistically significant reduction in this marker was found in the CORR100 and CORR500 groups compared to the CAF group (17.9 ± 4.4 and 17.6 ± 4.9 pg mL^{-1} respectively; **Figure 2C**).

3.4. Ex Vivo Intestinal Barrier Integrity

Barrier integrity in small and large intestine sections was evaluated in an Ussing chamber system by TEER measurements of mucosal preparations from all experimental groups. Similar TEER values were found throughout the intestine of the STD group (24.9 ± 1.8, 25.0 ± 1.3 and 29.4 ± 2.3 ohm.cm^2 for duodenum, ileum and colon respectively; **Figure 3**). TEER values in all the intestinal sections from animals fed the CAF diet were significantly lower than those from the STD group (55-70% TEER reduction).

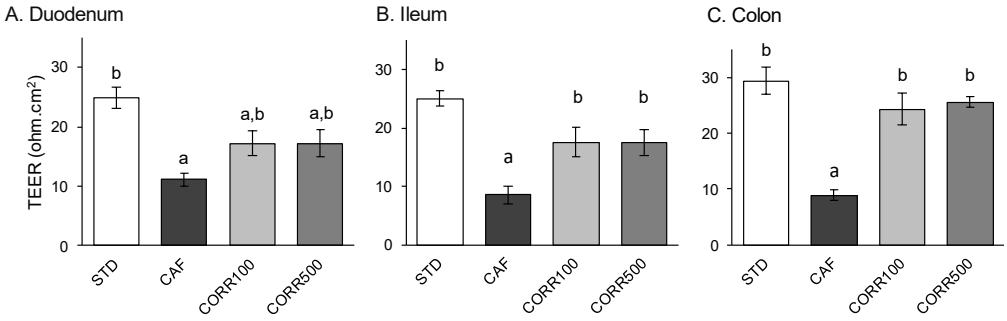

Figure 3. The effect of the GSPE treatment on the intestinal barrier integrity of small and large intestines in diet-induced obese rats. TEER (transepithelial electric resistance) values were estimated from fresh duodenal, ileal and colonic mucosal preparations mounted in an Ussing chamber system. Values represent mean ± SEM; *n*=8–10. Different letters indicate statistically significant differences between groups ($p<0.05$); ANOVA test followed by Tukey's HSD test when variances were equal and Dunnett's T3 test when they were not.

Notably, TEER values from the GSPE-treated groups showed a tendency to increase in the duodenum (approx. 54%; **Figure 3A**) but exhibited statistically significant increases in the ileum (approx. 107%; **Figure 3B**) and colon (approx. 180%; **Figure 3C**). Non-statistically significant differences in TEER values were found between the CORR100 and CORR500 groups.

3.5. The Expression of Tight Juntion Protein Genes

To evaluate the influence of GSPE-supplementation on intestinal tight-junction integrity, we analyzed the expression of the genes encoding tight junction-associated proteins in the ileum. The CORR100 and CORR500 groups showed a two-fold increase in the expression of the claudin 1 gene in comparison to the CAF group, without a dose-dependent effect (**Figure 4C**). The expression of the zonula occludens 1 (**Figure 4A**), junctional adhesion molecule 1 (**Figure 4B**) and claudin 2 (**Figure 4D**) genes did not differ significantly between experimental groups.

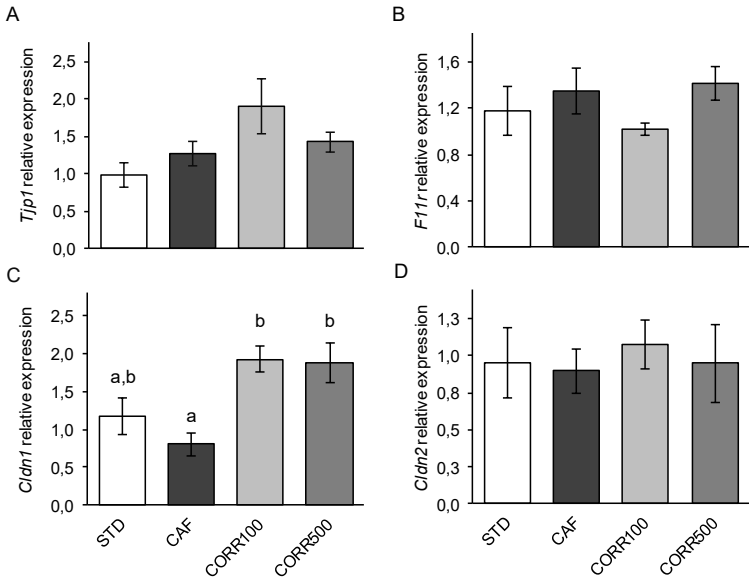

Figure 4. The effect of the GSPE treatment on tight juntion-related gene expression in the ileum of diet-induced obese rats. The expression of target genes was normalized to cyclophilin A gene expression (*Ppia*). Values represent mean ± SEM; n=8–10. Different letters indicate statistically significant differences between groups (p<0.05); ANOVA test followed by Tukey's HSD test.

3.6. Ex Vivo Intestinal Inflammation

To evaluate intestinal local inflammation, we determined the level of TNF-α secreted by the intestinal mucosal preparations subjected to Ussing chamber assays in the basolateral media. TNF-α secreted from intestinal tissues in STD diet-fed animals varied between 0.2–13.4 pg mL^{-1}, with the highest levels released by the colon (**Figure 5**). The CAF diet promotes a significant increase in TNF-α secretion from all intestinal tissues in comparison to the STD group (5–20.2 pg mL^{-1}), with the ileum being the intestinal section most susceptible to CAF intervention with a 25-fold increase in this marker (**Figure 5B**). The GSPE-treated group presented lower TNF-α secretions from the duodenum and colon compared

to the CAF group. Only the CORR500 treatment was able to reduce the TNF-α secretion to basal levels in the colon (**Figure 5C**), although both treatments were equally effective in the duodenum (**Figure 5A**). No GSPE-treatment was effective in reducing the TNF-α secretion from the ileum.

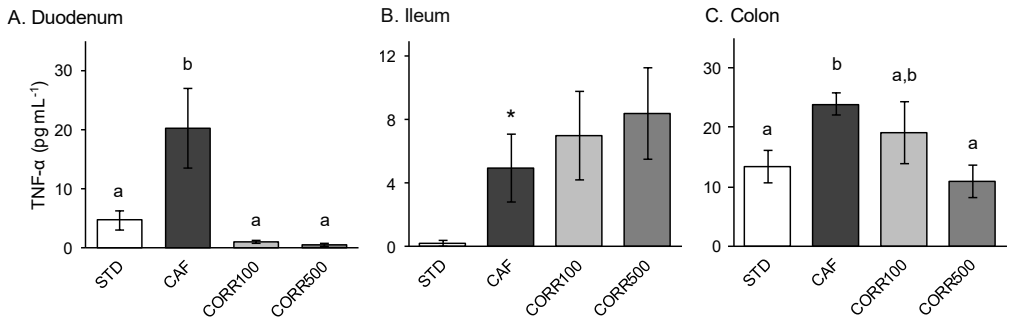

Figure 5. The effect of the GSPE treatment on the local inflammation of small and large intestines in diet-induced obese rats. TNF-α levels were determined from the basolateral media from the Ussing chamber after 30 min of incubation. Values represent mean ± SEM; *n*=8–10. Different letters indicate statistically significant differences between groups ($p<0.05$); ANOVA test followed by Tukey's HSD test when variances were equal and Dunnett's T3 test when they were not. *Significant statistical differences with respect to the STD group; two-sided Student's *t*-test.

3.7. Ileal MPO Activity

MPO activity was determined on ileum tissues as a marker of neutrophil accumulation. The CAF diet induced a 4-fold increase in MPO activity (7.52 and 2.0 U mg^{-1} for the CAF and STD groups respectively). Furthermore, the GSPE treatment reduced MPO activity to levels comparable to the control STD (3.04 and 3.35 U mg^{-1} for the CORR100 and CORR500 groups respectively) without a dose-dependent effect.

3.8. Association between Metabolic Endotoxemia and Intestinal Barrier Integrity

To check the association between endotoxemia and integrity of the intestinal barrier we performed Pearson correlation tests between plasma LPS and TEER values determined *ex vivo* for each intestinal section (**Figure 6**). We found negative and statistically significant associations for these variables in all the intestinal sections studied. As shown in Figure 6, the strength of the linear relationship increased in the distal intestine.

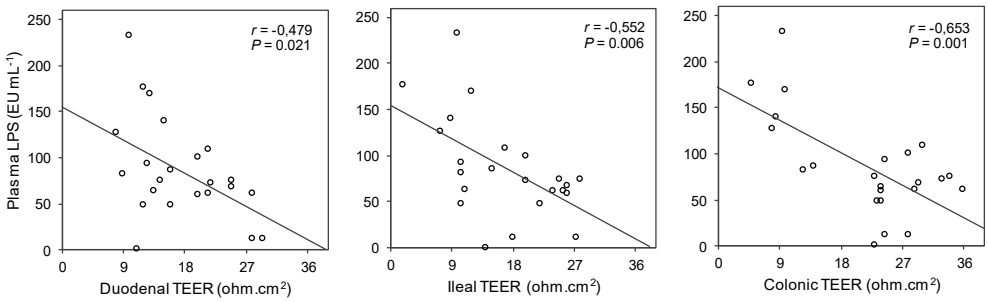

Figure 6. The linear relationship between endotoxemia and TEER values in small and large intestines of rats from the STD, CAF, CORR100 and CORR500 experimental groups. The inset shows the Pearson's *r* correlation and the corresponding *p*-value; n=23. p<0.05 was considered statistically significant.

4. Discussion

The intestinal barrier plays a major role in protecting the organism against foreign agents. Its function can be compromised by chronic exposure to diet-related components such as saturated fats and refined carbohydrates [10]. These alterations promote the influx of bacterial endotoxins (i.e., LPS) and exacerbate the immune response. The subsequent systemic inflammation has been associated with obesity-related pathologies [11,13].

Phytochemicals such as flavonoids have been shown to reduce intestinal alterations induced by high saturated-fat/high refined-carbohydrate diets. In this regard, we have found that proanthocyanidins are able to attenuate or even prevent diet-induced alterations to the intestinal barrier and local inflammation [18]. We hypothesized that pharmacological doses of GSPE (100 and 500 mg kg^{-1} bw) may reverse some of the detrimental effects of a long-term obesogenic diet, ameliorating intestinal dysfunction.

We designed an experiment in which diet-induced obese rats received pharmacological doses of GSPE daily for two weeks. Initially, the animals were fed a typical CAF diet, a highly palatable and hypercaloric diet that resembles the Western human diet [29]. The CAF diet is considered a robust inductor of obesity because it is rich in refined carbohydrates as well as saturated fats. In this study, the CAF diet-induced voluntary hyperphagia, resulting in the rapid weight gain and an increased adiposity index compared to animals fed the control STD diet. Similar results have previously been reported in this animal model [3,18,19].

To assess the intestinal barrier permeability and local inflammation we analyzed multiple markers at the end of the experiment. Our results showed lower TEER values in both the small and large intestines and higher levels of plasma OVA in CAF-fed rats after 17 weeks, suggesting a decrease in intestinal epithelial integrity and enhanced paracellular transport. Interestingly, GSPE supplemented animals evidenced the restitution of the intestinal epithelium's integrity, particularly in the ileum and colon. We also found a significant reduction of the paracellular transportation as a result of CORR100 administration and a tendency toward the reduction in response to CORR500. Thus, GSPE-treatments exerted a clear beneficial effect in the permeability of distal sections of the intestine. Accordingly, a favorable effect of GSPE on the intestinal barrier function was found in a study performed with IL-10-deficient mice that develop spontaneous colitis [30]. In this study, the supplementation with GSPE 0.1% (w v^{-1}) in drinking water for 10 weeks

reduced significantly *in vivo* permeation of fluorescently-labeled dextran to the blood after an oral load.

Ex vivo assays also showed that intestinal tissues from animals fed the CAF diet released higher levels of TNF-α. This proinflammatory cytokine is a key mediator of intestinal inflammation that induces TJ opening via myosin light-chain kinase phosphorylation to enhance paracellular flux [31]. Clinical and experimental studies have demonstrated that defects in the intestinal TJs and increased permeability are present in various intestinal and systemic diseases [32]. In the present work, both GSPE-treatments exerted an anti-inflammatory effect on duodenal tissues, reducing TNF-α secretions to basal levels. Nevertheless, only in colon tissues, CORR500 was able to reverse proinflammatory cytokine production. Previous studies have revealed the critical role of the ileum in the pathogenesis of chronic intestinal diseases associated with intestinal permeability alterations and local inflammation [33]. GSPE supplementation did not significantly alter TNF-α secretion from the ileum, but it did promote a decrease in CAF diet-induced neutrophil infiltration as indicated by the reduction in MPO activity. MPO is a key component of oxygen-dependent microbial activity by neutrophils, but it has also been linked to tissue damage in acute or chronic inflammation [34]. In agreement with these findings, a study performed on a rat model of spontaneous inflammatory bowel disease (HLA-B27) found that diet supplementation with 7.6% (w w^{-1}) of lyophilized apples rich in flavan-3-ol monomers and proanthocyanidins (2.8 and 26.1 mg g^{-1} respectively), reduced MPO activity along with cyclooxygenase-2 and inducible nitric oxide synthase gene expression in intestinal tissues [35].

Regarding inflammatory parameters, plasma LPS were markedly elevated in the CAF-group at the end of the experiment. Under normal conditions, the presence of LPS in the intestinal lumen does not cause adverse health effects. Nevertheless, the loss of intestinal barrier integrity associated with the consumption of saturated fat/high-sugar diets may favor LPS penetration through the intestinal epithelium

to the blood [11]. Two- to three-fold increases in levels of LPS in plasma trigger metabolic endotoxemia, and this process is closely linked to systemic inflammation [12,13]. In the present study, a clear linear relationship between CAF diet-induced TEER loss and LPS levels in plasma was found. This association was stronger in the ileum and colon, suggesting a higher contribution of the distal intestine to metabolic endotoxemia. This observation can be explained by the abundant bacterial population present in distal sections of the intestinal tract [36]. Once it has entered the circulation, LPS activates a series of proinflammatory receptors, i.e., toll-like receptors 2 and 4 and the CD14 receptor, leading to cytokine release [13,37]. Accordingly, in this study, the CAF-diet-fed rats also showed higher levels of TNF-α in plasma, indicating a state of systemic inflammation in this group of animals. Remarkably, both GSPE doses were also effective in decreasing plasma LPS to basal levels and tended to reduce the TNF-α release to circulation. These findings are consistent with the observations discussed above regarding GSPE effects on intestinal permeability.

The mechanisms underlying the anti-inflammatory and barrier-protective effects of flavonoids on the intestine were reviewed by Gil-Cardoso et al. [2]. Flavonoids, including proanthocyanidins, reduce intestinal inflammatory processes driven by NF-κB activation, a key factor in proinflammatory gene expression. *In vitro* studies have demonstrated that procyanidin-rich red wine extract down-regulates IκB kinase complex signal transduction, inhibiting the IκB degradation and NF-κB translocation to the nucleus [38]. Another interesting point to consider is the modulation of TJ protein genes by flavonoids. In a recent study, it was observed that Granny Smith apple procyanidin extract increases the expression of TJ protein genes, including occludin and zonula occludens 1 in LPS-treated Caco-2 cells [39]. We demonstrated that CAF diet-induced obese rats supplemented with GSPE (5–500 mg kg^{-1} d^{-1}), show an increased expression of zonula occludens 1 [19] and claudin 1 [18] genes in the ileum. A significant increase of claudin 1 protein content has also been estimated in ileal tissues of IL-10 deficient mice supplemented with

GSPE 1% (w w^{-1}) for 16 weeks [40]. Claudin proteins are considered key components and the structural backbone of TJs [41], with claudin 1 being involved in TJ tightening. Thus, the upregulation of claudin 1 gene expression found in the present work might partially explain the restitution of TEER and intestinal permeability at the ileum level in GSPE-supplemented animals.

Most studies in rodents describing a beneficial effect of polyphenol-rich extracts on diet-induced intestinal dysfunction and metabolic endotoxemia involve preventive or long-term treatment approaches [18,42–44]. To our knowledge, there is no other study in the literature describing an ameliorative effect of a polyphenol-rich extract in these alterations as a result of short-term chronic treatment. Data show that pharmacological doses of grape-seed proanthocyanins can normalize intestinal permeability and local inflammation markers altered by a long-term obesogenic diet, as well as the associated metabolic endotoxemia. The effects of both pharmacological doses of GSPE on CAF-diet-induced intestinal dysfunction were similar and, therefore, we found no dose-response relationship. Both doses proved to ameliorate intestinal alterations, but the 100 mg kg^{-1} bw dose seems to be the most effective in correcting intestinal permeability, whereas the 500 mg kg^{-1} bw dose corrected local inflammation more effectively.

GSPE doses used in this study are high but feasible in a translational perspective. We applied a rat-to-human correction factor that relates the body weight and body surface to estimate the theoretical human equivalent doses (HEB) [45]. Considering the 100 mg kg^{-1} bw dose of GSPE, the estimated HED is approximately 0.97 g d^{-1} for a 60 kg person. A clinical study was carried out with 31 volunteers with different body mass index to determine the effect of GSPE administration in postprandial concentrations of plasma LPS after a high-fat breakfast (63 g of fat and 990 kcal) [46]. These authors found that 1 g of GSPE effectively prevented the postprandial increase of plasma LPS after 4 h compared with the placebo. This effect was statistically significant in the obese group. Another recent clinical study assessed

the safety and tolerability of GSPE intake in a small number of healthy adults [47]. This study tested the oral administration of the extract up to 2.5 g d^{-1} for a 4-week period, finding a good tolerability without adverse effects on hematological and biochemical parameters.

5. Conclusions

In summary, the consumption of a CAF diet leads to an increase in intestinal permeability and metabolic endotoxemia, which together contribute to the systemic inflammation state present in obese animals. We found that the administration of grape-seed proanthocyanidins not only improves the intestinal health in diet-induced obese rats, effectively ameliorating permeability alterations and local inflammation at the end of a long-term obesogenic diet, but also reduces metabolic endotoxemia. Thus, supplementation with proanthocyanidin-rich natural extracts is outlined as a promising nutritional and therapeutic treatment to ameliorate the diet-induced intestinal dysfunction associated with obesity.

Author Contributions: Conceptualization, X.T. and M.T.B.; Data curation, C.G.-Q. and K.G.-C.; Formal analysis, C.G.-Q. and K.G.-C.; Funding acquisition, M.P., A.A., X.T. and M.T.B.; Investigation, C.G.-Q., K.G.-C., I.G., R.B.-D., M.P., A.A., X.T. and M.T.B.; Methodology, R.B.-D., M.P., A.A., X.T. and M.T.B.; Project administration, A.A. and M.T.B.; Resources, M.P. and A.A.; Supervision, X.T. and M.T.B.; Validation, X.T. and M.T.B.; Visualization, C.G.-Q.; Writing – original draft, C.G.-Q.; Writing – review & editing, X.T. and M.T.B.

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Conflicts of Interest: The authors declare that there is no conflict of interests regarding the publication of this paper.

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1.2. Manuscript No. 3

Detrimental Effects of High Fructose Concentrations in Monolayers of Human Colon Carcinoma Caco-2 Cells: The Role of Proanthocyanidins in Permeability Alterations and Proinflammatory Changes

Carlos González-Quilen, Raúl Beltrán-Debón, Montserrat Pinent, Anna Ardévol, Ximena Terra, Maria Teresa Blay

MoBioFood Research Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, 43007 Tarragona, Spain.

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MoBioFood Research Group, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, 43007 Tarragona, Spain.

Abstract:

Scope**:* Fructose is a ubiquitous component of the Western diet that has been associated with metabolic disorders when consumed in large quantities. However, its contribution to the toll of chronic illness is highly controversial. It has been hypothesized that a high fructose intake affects the intestinal barrier function, thus favoring the influx of bacterial endotoxins and inducing systemic inflammation and alterations in the metabolism. Proanthocyanidins are naturally occurring flavonoids that have been shown to ameliorate diet-induced intestinal dysfunction in rodents. Here, we investigated the effect of fructose at high concentrations (50, 150 and 300 mmol L^{-1}) achievable in the small intestine on the functionality of human Caco-2 monolayers and the effectiveness of a grape-seed proanthocyanidin extract (GSPE) (25, 50 and 100 μg mL^{-1}) in preventing fructose-induced changes. ***Methods and results: A 5-h incubation period with high fructose concentrations (≥150 mmol L^{-1}) drastically reduced transepithelial electrical resistance (38–69%) and promoted the permeation of fluorescent markers across cellular monolayers. These changes were associated with increases in both the activity of LDH released

into the culture medium (two-fold) and the levels of IL-8 secreted basolaterally (up to 106-fold). A 20-h incubation period with GSPE prior to the high-fructose treatment was incapable of preventing detrimental changes. ***Conclusions:*** High fructose concentrations achievable in the human small intestine provoked cytotoxic and proinflammatory responses in Caco-2 monolayers, leading to barrier integrity loss and permeability alterations that were not prevented by GSPE in this *in vitro* model.

Keywords: intestine, permeability; inflammation; Western diet, sugar; transepithelial electrical resistance

1. Introduction

The Western diet is characterized by the high consumption of energy-dense ultra-processed foods, rich in saturated fats and simple carbohydrates. In this dietary pattern, more than 14% of the calorie intake comes from sugars, with sweet products and sweetened beverages the main contributors to sugar consumption [1,2]. As a result, concerns have been raised regarding the impact of these food products on the global epidemic of obesity [3]. The World Health Organization recommends limiting added sugars to one-tenth of the daily calorie intake, namely 25 g per day for a 2500-calorie diet [4]. Nevertheless, a typical 330-mL can of a regular soft drink contains up to 40 g of free sugars (>600 mmol L^{-1}). In addition, a large portion of sugar is hidden in foods marketed as healthy choices (e.g. breakfast cereals, yogurt and other sugar-sweetened dairy products) and daily intake per capita in some developed countries can reach up to 145 g [1]. Nearly half of the sugar content in the Western diet is fructose derived from sucrose and corn syrup [5]. Epidemiological studies associate the consumption of fructose with metabolic syndrome [6]. However, the claim that fructose and other sugars directly contribute to the toll of chronic illness independently of body weight and fat gain

remains controversial, although some plausible mechanisms have been proposed [3,7,8].

Studies in mammals have reported that high fructose intake increases intestinal permeability and the translocation of bacterial endotoxins [9–11], which may be a major driver of metabolic diseases [12]. Rats fed a Western-style cafeteria diet (rich in fructose and saturated fats) not only gain body weight but also manifest mild intestinal inflammation and enhanced permeability (intestinal dysfunction) [13,14]. Intestinal permeability might be the result of increased paracellular transport, which in turn is regulated by tight junction (TJ) proteins located between adjacent epithelial cells. The overactivation of proinflammatory signaling pathways alters the integrity of TJs [15]. As the intestinal epithelial barrier prevents proinflammatory agents such as bacterial endotoxins from entering the systemic circulation, loss of barrier integrity may trigger a vicious circle of inflammation and permeability [16].

Proanthocyanidins are a group of flavonoids present in a variety of botanical sources such as cocoa, nuts, fruit and spices, which have demonstrated antioxidant, anti-inflammatory and immunomodulatory effects *in vivo* [17,18], and could offer a safe adjunctive support to current therapies in the treatment of various chronic diseases, including obesity [19–21]. Oral administration of grape-seed proanthocyanidins extract (GSPE) in rats has demonstrated beneficial effects against the increased intestinal permeability and inflammation (intestinal dysfunction) developed as a consequence of a long-term cafeteria diet [14,22] or colitis-inducing chemicals [23].

The amount of sugars in ultra-processed foods and sweetened beverages should result in high postprandial concentrations of free sugars in the lumen of the upper small intestine (up to 100 mmol L^{-1} in rodents [24]). Higher concentrations of free sugars would be expected near the brush borders following the hydrolysis of sucrose and other disaccharides by disaccharidases [25]. In this study, we aimed to

investigate whether high concentrations of fructose achievable in the human small intestine in the context of the typical Western diet promotes alterations in the functionality of human colon adenocarcinoma Caco-2 cell monolayers. We also tested the efficacy of GSPE in preventing these fructose-induced changes.

2. Materials and Methods

2.1. Chemicals

DMEM (with 4.5 g L^{-1} glucose), L-glutamine solution (200 mmol L^{-1} in 0.85% NaCl), penicillin-streptomycin mixture (10,000 U mL^{-1}) and trypsin–EDTA solution (500 mg L^{-1} trypsin and 200 mg L^{-1} EDTA in Hank's Balanced Salt Solution) were purchased from Lonza Verviers SPRL (Verviers, Belgium). Fetal bovine serum (FBS), EGTA (≥97%), and D-(−)-fructose (≥99%) were provided by Sigma–Aldrich Chemie (Steinheim, Germany). Hank's Balanced Salt Solution (HBSS with calcium and magnesium) and HEPES buffer solution (1 mol L^{-1}) were from GIBCO (Grand Island, NY, USA). Lucifer yellow (LY) potassium salt (522 g mol^{-1}) was purchased from Biotium (Hayward, CA, USA). Fluorescein isothiocyanate-dextran 4 kDa (FD4) came from TdB Consultancy AB (Uppsala, Sweden).

2.2. Proanthocyanidin extract

The grape-seed proanthocyanidin extract (GSPE) was provided by Les Dérivés Résiniques et Terpéniques (batch number 124029; Dax, France). According to the manufacturer, the composition of the GSPE is as follows: monomers of flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5–13 units; 31.7%) of proanthocyanidins. A detailed analysis of the monomeric, dimeric, and trimeric structures of the GSPE can be found in the work by Margalef et al. [26]. The GSPE was dissolved in ethanol absolute to prepare a stock solution of 50 mg mL^{-1}.

2.3. Cell culture

Caco-2 cells (HTB-37) were obtained from the ATCC (American Tissue Culture Collection) and used in experiments between passages 18 and 30. The cells were maintained in 75-cm^2 culture flasks (Greiner Bio-One, Frickenhausen, Germany) at 37 °C in an atmosphere of 5% CO_2 with the medium being changed every 2–3 days. The growth medium consisted of DMEM supplemented with 10% (v v^{-1}) heat-inactivated FBS, 2 mmol L^{-1} L-glutamine, 1 mol L^{-1} HEPES and 100 U mL^{-1} penicillin-streptomycin mixture. When confluence reached ~80%, the cells were harvested by treatment with trypsin–EDTA solution for 10 min, and then split and sub-cultured in fresh growth medium.

2.4. Measurement of transepithelial electrical resistance

Permeable transwell inserts with a 1.1-cm^2 surface area and 1-μm pore size were placed in 12-well plates (Merck Millipore, Darmstadt, Germany) and 0.4 mL of cell suspension (1.5×10^4 cells mL^{-1}) was added. Wells were added with 1 mL of growth medium and both apical and basolateral media were changed every other day. After 21 days of cell differentiation, transepithelial electrical resistance (TEER) was measured (ohm cm^2) using a Millicell-ERS 2 voltohmmeter (EDM Millipore, Billerica, MA, USA) in triplicate per insert and corrected by subtracting the mean value from a cell-free insert. D-Fructose was dissolved in experimental medium consisting of supplemented DMEM with 1% (v v^{-1}) heat-inactivated FBS for preparation of the high-fructose treatments: Fruc50 (50 mmol L^{-1}), Fruc150 (150 mmol L^{-1}) and Fruc300 (300 mmol L^{-1}). EGTA, which is known to disrupt epithelial TJs, was dissolved in experimental medium (2 mmol L^{-1}) and was included as a positive control of permeability. All media were sterilized by filtration prior to the experiments using a 0.22-μm syringe-driven filter (Jet Bio-Filtration Co., Guangzhou, China). Both the inserts and wells were rinsed with a sterile, prewarmed PBS solution and the treatments were added apically, except for EGTA, which was also added basolaterally. The plates were then incubated for 5 h at 37 °C.

To evaluate the effectiveness of GSPE in preventing fructose-induced changes in TEER, the GSPE solutions (10, 25 and 50 μg mL^{-1}) were prepared in experimental medium, added apically and incubated for 20 h at 37 °C prior to the high-fructose treatments. The concentration of ethanol was kept at ≤0.1% (v v^{-1}) in the apical medium. All experiments were performed at least three times with biological duplicates or triplicates per condition. Changes in TEER were calculated for every experimental group as the percentage of the initial value recorded before treatments were added.

2.5. Assessment of molecular permeability using fluorescent markers

Stock solutions of LY (100 mmol L^{-1}) and FD4 (50 mmol L^{-1}) were prepared in HBSS. Inserts and wells were rinsed twice with sterile, prewarmed PBS and fluorescent markers were then added apically (0.4 mL) at a final concentration of 100 μmol L^{-1}. HBSS (1.2 mL) was added basolaterally and the plates were incubated at 37 °C for 60 min. The amount of fluorescent marker that crossed to the well was measured with a PerkinElmer LS-30 fluorimeter (Beaconsfield, U.K.) at λ_{exc}=430 nm and λ_{em}=540 nm and compared with a standard curve. The transepithelial transport of LY and FD4 was calculated as apparent permeabilities (*Papp*) using the following equation:

$$Papp\ (mL/cm^2 \times s) = (V/A \times t) \times (C_{ba}/C_{ap}),$$

where *V* is the basolateral volume, A is the exposed surface area, *t* is the incubation time and C_{ba} and C_{ap} are the concentrations of the fluorescent marker in the basolateral (well) and apical (transwell insert) media, respectively.

2.6. Quantification of secreted IL-8

Human IL-8 was determined using a commercially available ELISA kit according to the manufacturer's instructions (Sigma–Aldrich Chemie, Steinheim, Germany). IL-8 levels corresponded to the total amount secreted into the basolateral media at the

end of the 5-hour treatment. Absorbances were measured with a BioTek Eon microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) at 450 nm. IL-8 levels were expressed as picograms per milliliter with an analytical sensitivity of 1 pg mL^{-1}.

2.7. Quantification of LDH activity

The damage induced by fructose on Caco-2 cells was assessed by means of lactate dehydrogenase (LDH) activity assays. In brief, Caco-2 cells (1.5×10^4 cells mL^{-1}) were seeded in 24-well culture plates (0.5 mL per well) (Merck Millipore, Darmstadt, Germany) in growth medium. The cells were incubated for 21 days until full differentiation, and the experiments were performed as described above. LDH activity was measured from plate media using the SFBC method with a commercially available kit (QCA S.A., Amposta, Spain) and normalized to the protein content quantified from the whole-cell lysates by means of a Pierce BCA assay (Thermo Fisher Scientific, Rockford, IL). Enzymatic activity was expressed as mU mg^{-1} protein.

2.8. RNA extraction and gene expression analysis by RT-qPCR

The total RNA was isolated from the cells using the Qiagen RNeasy Mini Kit (Qiagen, Barcelona, Spain) following the manufacturer's instructions, and it was quantified with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany). cDNA was obtained using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain). cDNA (2.5 ng mL^{-1}) was subjected to qPCR amplification using iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain). Genes encoding the zonula occludens 1 and claudin 1 (*TJP1* and *CLDN1*, respectively) were analyzed because they are key components of TJs. Myosin light chain kinase (encoded by *MYLK*) is involved in TJ opening and enhancement of paracellular permeability. The forward (Fw) and reverse (Rv) primer sequences of the target genes are shown in **Table 1**. Amplifications were

performed using a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Barcelona, Spain) with thermal profile settings as follows: 95 °C for 30 seconds and then 40 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds. The relative mRNA expression levels were calculated following the $2^{-\Delta\Delta Ct}$ method [27] and the results were normalized in relation to the human peptidylprolyl isomerase A gene (*PPIA*) as follows:

$$\Delta Ct = Ct\ gene\ of\ interest - Ct\ peptidylprolyl\ isomerase\ A,\ \text{and}$$

$$\Delta\Delta Ct = \Delta Ct\ treated\ samples - the\ mean\ of\ \Delta Ct\ control\ samples.$$

Table 1. Primer sequences for target genes.

Gene name	Symbol	Accession number	Sequences	Product size (bp)
Tight junction protein 1	*TJP1*	NM_003257.5	F: 5'-ccagcctgctaaacctactaa-3' R: 5'-ccatctcttgctgccaaact-3'	126
Claudin 1	*CLDN1*	NM_021101.5	F: 5'-agtcttctgccaggaatccc-3' R: 5'-gtggtgagtagaagtcccgt-3'	137
Myosin light chain kinase	*MYLK*	NM_053025.4	F: 5'-agatgacgtgggagtgtaca-3' R: 5'-cctgacatctgaattggtggc-3'	142
Peptidylprolyl isomerase A	*PPIA*	NM_001300981.2	F: 5'-tgctggacccaacacaaatg-3' R: 5'-ggcctccacaatattcatgcc-3'	118

F, forward. R, reverse

2.9. Statistical Analysis

Results were expressed as the mean ± SEM. Statistical comparisons between groups were assessed by ANOVA followed by Tukey's HSD test when the variances were equal or the Games-Howell test when they were not. P-values <0.05 were considered statistically significant. Analyses were performed with the XLSTAT 2019.1.3 software (Addinsoft, NY, USA).

3. Results

3.1. High fructose concentrations induced loss of Caco-2 monolayer integrity and enhanced permeability

We first evaluated the influence of high fructose concentrations on the integrity of Caco-2 monolayers by means of a TEER measurement. The initial TEER of the cell monolayers ranged from 231.0 to 291.9 ohm cm^2 with an average value of 260.9 ± 3.5 ohm cm^2 (*n*=26). TEER was markedly modulated by fructose (≥150 mmol L^{-1}) with a pronounced dose-dependent increment during the first 60 min of incubation (106–135% compared to baseline), followed by a sustained reduction in the subsequent hours (**Figure 1A**).

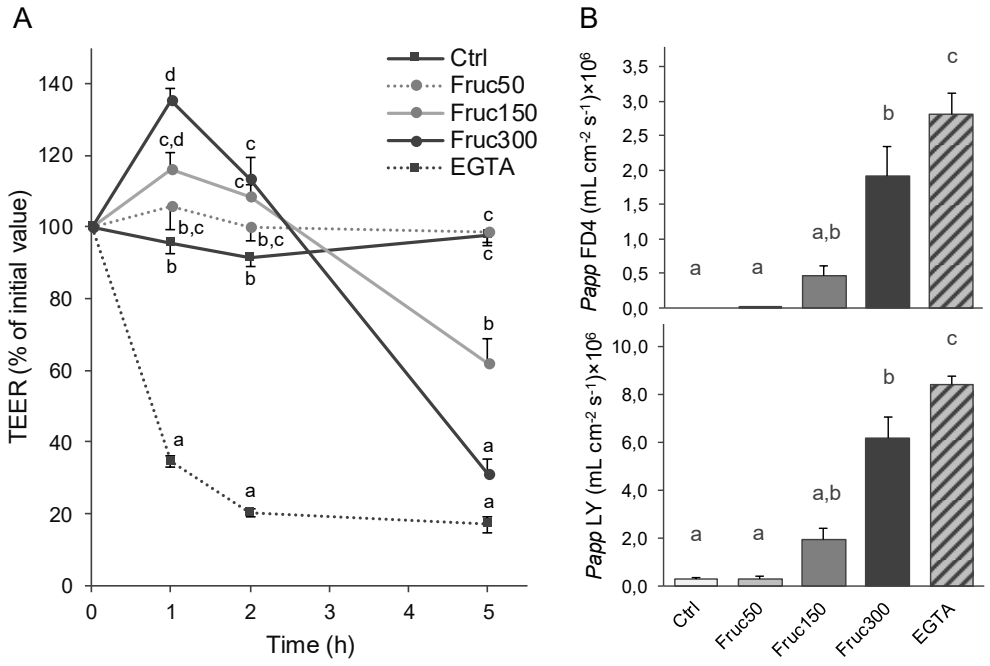

Figure 1. Effect of high fructose concentrations on the integrity and permeability of Caco-2 monolayers. (A) Transepithelial electrical resistance (TEER) time course. (B) Apparent permeabilities (*Papp*) of Lucifer yellow (LY) and fluorescein isothiocyanate-dextran 4 kDa

(FD4). Fructose concentrations of 50, 150 and 300 mmol L-1 were tested. EGTA (2 mmol L^{-1}) was used as positive control of permeability. Values presented are mean ± SEM of four to six independent experiments. The different letters indicate statistically significant differences, $p<0.05$.

Changes associated with Fruc150 and Fruc300 were statistically significant during the time-course experiment when compared to Ctrl ($p<0.05$). The TEER drop at 5 h was moderate in Fruc150 and severe in Fruc300 (loss of 38 and 69% in relation to the baseline, respectively) with the latter comparable to that produced by the positive control (2 mM EGTA).

The permeation profiles were similar for both molecular weight markers (**Figure 1B**). In Ctrl, FD4 was not detected in the basolateral medium, while very low quantities of LY permeated across monolayers ($0.26 \pm 0.07 \times 10^{-6}$ mL cm^{-2} s^{-1}). This is consistent with FD4 being a high-weight molecule and therefore a marker of paracellular transport, while LY could also be transported transcellularly. The reduction of monolayer integrity at 5 h was associated with an increase in the transepithelial transport of molecular markers in a dose-dependent manner. Changes in Fruc150 were not statistically significant and agreed with a mild loss of monolayer integrity ($p>0.05$). In Fruc300, permeations of FD4 ($0.63 \pm 0.14 \times 10^{-6}$ mL cm^{-2} s^{-1}) and LY ($2.1 \pm 0.2 \times 10^{-6}$ mL cm^{-2} s^{-1}) were significantly higher ($p<0.05$ vs. Ctrl) and in accordance with a more severe alteration, suggesting the opening of paracellular pathway and/or an enhanced molecular flux due to cell detachment.

In order to determine whether the effects described above were associated with the paracellular pathway opening driven by the disruption of TJs, we quantified the mRNA levels of the key genes involved in the integrity and regulation of TJs (**Table 2**). No statistically significant differences were found between the experimental conditions for the expression of TJ protein genes (*TJP1* and *CLDN1)* or myosin light chain kinase genes *(MYLK*) ($p>0.05$), suggesting that the integrity of TJs was not compromised at the transcriptional level. To determine whether the

effects described above were associated with the partial detachment of cell monolayers, we measured the protein content of the cells still attached to the culture plates after the 5-hour incubation period (**Figure 2A**). Slight but significant reductions in the cell protein content were quantified in Fruc150 and Fruc300 (loss of 6.3% and 9.0%, respectively) when compared to Ctrl (p<0.05), indicating a mild loss of cell mass.

Table 2. Fold change in TJ-related gene expression of Caco-2 cells incubated with high fructose concentrations.

Gene	Ctrl	Fruc50	Fruc150	Fruc300
TJP1	100.0 ± 4.6	101.6 ± 14.1	100.6 ± 8.9	102.0 ± 14.4
CLDN1	100.0 ± 15.0	97.8 ± 5.4	98.1 ± 7.9	98.2 ± 8.9
MYLK	100.0 ± 21.9	97.4 ± 13.9	96.2 ± 8.9	99.7 ± 19.6

Data represent the normalized target mRNA levels quantified by RT-qPCR using *PPIA* as reference gen. Values are relative to Ctrl which is considered 100% and are presented as mean ± SEM of three independent experiments.

3.2. High fructose concentrations induced cell damage and proinflammatory changes

As the detachment of Caco-2 monolayers could be caused by cytotoxicity, we measured the activity of LDH released into the medium as a marker of cell injury and death. A dose-dependent increase in LDH activity was found in the media of treated cells (**Figure 3B**), with statistically significant changes in Fruc150 (1.6-fold) and Fruc300 (2.2-fold) compared to Ctrl (p<0.05).

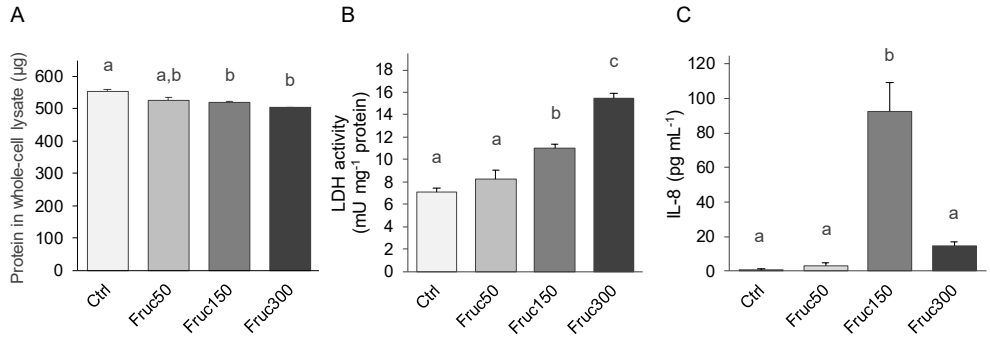

Figure 2. Cell injury and IL-8 secretion induced by high fructose concentrations in Caco-2. (A) Protein content, (B) lactate dehydrogenase (LDH) specific activity and (C) basolateral IL-8 secretion were measured after 5 h of incubation with high fructose concentrations (50, 150 and 300 mmol L^{-1}). Values presented are mean ± SEM of three independent experiments. The different letters indicate statistically significant differences, $p<0.05$.

Cell injury in the fructose-treated cells did not seem to be linked to oxidative damage, as we did not find a significant increase in the production of reactive oxygen species in the culture media (results not shown). However, Fruc150 did promote a striking 106-fold increase in IL-8 secreted basolaterally ($p<0.05$, **Figure 3C**), suggesting a proinflammatory state. The effect on IL-8 was not statistically significant in Fruc300 when compared to Ctrl ($p<0.05$), possibly due the reduction in IL-8 synthesis capacity resulting from cell death.

3.3. GSPE was not able to prevent fructose-induced detrimental effects

GSPE has demonstrated barrier-protective and anti-inflammatory properties in the intestinal mucosa of animal models of diet-induced obesity [28]. Here, we exposed Caco-2 monolayers to GSPE for 20 h (10, 25 and 50 µg mL^{-1}) prior to a high fructose treatment in order to test its effectiveness in preventing the alterations described above (**Figure 3**). For these experiments, we chose Fruc150 as it was the lowest fructose concentration tested that promoted significant changes in Caco-2

monolayers. TEER did not vary significantly at the end of GSPE incubation at the concentrations tested (p>0.05; results not shown).

We next evaluated whether GSPE was able to exert preventive effects against the monolayer integrity loss and proinflammatory state promoted by Fruc150. GSPE did not prevent a fructose-induced TEER drop at 10 or 25 μg mL^{-1}, but a tendency (p<0.1) was found at 50 μg mL^{-1} (**Figure 3A**). However, this slight effect was not associated with changes in the permeation of FD4 (**Figure 3B**) or in the expression of TJ-related genes (results not shown). Finally, the increase in IL-8 secretion promoted by Fruc150 was not effectively attenuated at any concentration of GSPE (p>0.05; **Figure 3C**).

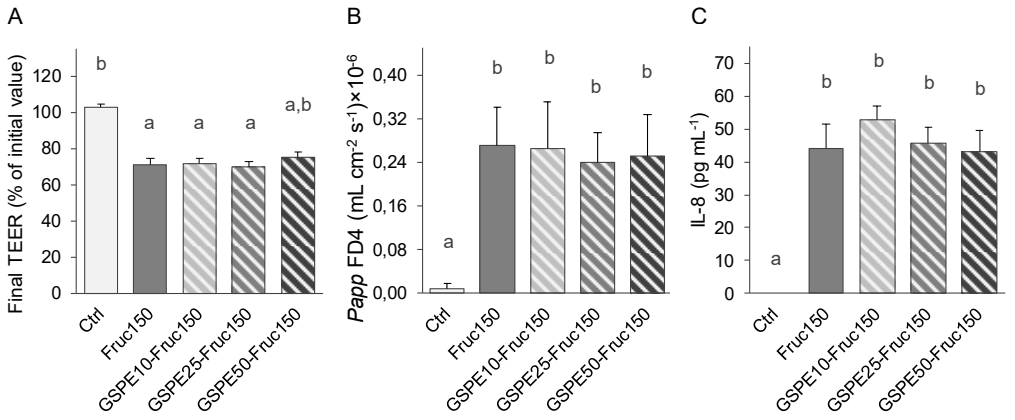

Figure 3. Influence of preventive GSPE treatment on permeability and proinflammatory changes induced by high fructose concentrations in Caco-2 monolayers. (A) Transepithelial electrical resistance (TEER), (B) apparent permeability (*Papp*) of fluorescein isothiocyanate-dextran 4 kDa (FD4) and (C) basolateral IL-8 secretion were measured after cell monolayers were exposed to GSPE (10, 25 and 50 μg mL^{-1}) for 20 h followed by a 5-h incubation period with fructose at high concentration (150 mmol L^{-1}). Values presented are mean ± SEM of three independent experiments. The different letters indicate statistically significant differences, p<0.05.

4. Discussion

The intake of fructose in the diet has been on the rise over the past few decades, and the consumption of large quantities seems to be related to metabolic alterations and illness [6], although the link is not fully understood and remains controversial [8]. Our findings are in agreement with several lines of evidence suggesting that high fructose intake causes intestinal permeability [11,29], which in turn seems to play a pivotal role in metabolic disorders [30].

The oral administration of high doses of fructose to experimental animals was reported to induce metabolic endotoxemia and systemic inflammation, which is consistent with an enhanced influx of luminal content across the intestinal epithelium [9,31,32]. In the current study, high concentrations of fructose (≥150 mmol L^{-1}) promoted drastic but transient increases in TEER of Caco-2 monolayers. This most likely indicated the collapse of lateral intercellular spaces due to a sudden increase in apical medium osmolarity and not an actual integrity reinforcement [33,34]. After the first hour of incubation, the increase in TEER was followed by a gradual drop accompanied by enhanced permeation of molecular markers. Increases in permeability were also recently reported in the monolayers of human HT-29 [29] and T84 [32] colon cells exposed to lower concentrations of fructose than those tested in this study (0.1–10 mmol L^{-1}), but incubated for longer periods of time (up to 24 h). In both studies, the enhanced permeability was associated with the disruption of TJ due to the downregulation of TJ proteins such as zonula occludens 1, occludin, claudin 1 and claudin 4. Although we did not find any changes in the expressions of the TJ protein genes examined or the myosin light chain kinase gene at the fructose concentrations and incubation times tested, we cannot rule out the occurrence of changes in protein synthesis affecting the integrity of junctional complexes. This is a possibility that should be further explored.

In accordance with other *in vitro* studies performed with cell lines [29,35,36], fructose promoted injury and a proinflammatory state in Caco-2 cells. Increases in proinflammatory cytokine production, including IL-8, have also been reported under apical hyperosmolarity in Caco-2 [34,37], although in these studies the hyperosmotic media had no effect on cell viability (LDH release) up to 24 h. This suggests that the cell injury found in this study is directly linked to the presence of fructose itself and not to osmotic stress [38]. Fructose is a unique monosaccharide that bypasses the regulatory phosphofructokinase step of glycolysis, entering directly into the glycolytic pathway as fructose-1-phosphate after phosphorylation by fructokinase (KHK), predominantly by KHK C. Unlike phosphofructokinase, KHK C is not regulated by negative feedback, and high intracellular levels of fructose can result in a transient phosphate and ATP depletion process that might arrest protein synthesis and produce a proinflammatory environment [35,39,40]. This process has been associated with hepatotoxicity in experimental animals [41] and it might also occur in the proximal segment of the small intestine where KHK C is highly expressed [38,42].

As mentioned earlier, subchronic oral administration of GSPE effectively prevented/ameliorated intestinal dysfunction and metabolic endotoxemia in Wistar rats fed a cafeteria diet [14,22]. Recently, a loquat extract enriched with polyphenols was also reported to attenuate the intestinal permeability and increased hepatic endotoxin levels induced by a high-fructose diet in mice when co-administered orally [43]. In the present study, a 20-h incubation period with GSPE was not able to prevent the proinflammatory changes and permeability alterations promoted by high fructose concentrations in Caco-2 monolayers. We speculate that the adverse metabolic effects of fructose at the concentrations/incubation time tested were too severe for the associated detrimental changes in inflammation and permeability to be effectively counteracted by GSPE under these experimental conditions. In addition, it is possible that a substantial part of the health-promoting properties attributed to GSPE *in vivo* are related to the microbial degradation of

parental compounds during intestinal transit [44,45]. Thus, the use of a GSPE-digested phenolic extract could be an interesting approach to test this assumption.

In summary, high concentrations of fructose achievably in the small intestine in the context of the typical Western diet promoted inflammation, injury and death of cultivated Caco-2 cells within a short period of time, thus negatively affecting the integrity of cell monolayers and enhancing molecular permeability. The detrimental changes induced by fructose could not be prevented by GSPE under the experimental conditions established in this study.

Author contributions: C.G.-Q, X.T. and M.T.B. conceived the ideas and the experimental design. C.G.-Q. performed the experiments and data collection, C.G.-Q. and M.T.B performed the statistical analysis and drafted the manuscript. R.B.-D., M.P., A.A. and X.T. contributed to the revision. All authors read and approved the final manuscript.

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Conflict of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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2. Human *ex Vivo* Study of Intestinal Dysfunction Induced by a Chemical Agent

The limitations inherent to cell culture-based models of intestinal dysfunction gave rise to the need for adopting an experimental model closer to the *in vivo* situation in humans that also share the physiological complexity of *in vivo* models.

In **Manuscript 4**, we describe a standardized human *ex vivo* model of acute colonic dysfunction induced by a colitis-inducing chemical in order to test the efficacy of GSPE administered as a preventive treatment.

Human colon samples were collected from adult oncological patients who underwent surgery at University Hospital Joan XXIII, Tarragona, Spain, and who provided prior informed consent as donor patients.

2.1. Manuscript No. 4

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ORIGINAL CONTRIBUTION

Protective properties of grape-seed proanthocyanidins in human ex vivo acute colonic dysfunction induced by dextran sodium sulfate

Carlos González-Quilen[1] · Carme Grau-Bové[1] · Rosa Jorba-Martín[2] · Aleidis Caro-Tarragó[2] · Montserrat Pinent[1] · Anna Ardévol[1] · Raúl Beltrán-Debón[1] · Ximena Terra[1] · M. Teresa Blay[1]

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Ximena Terra and M. Teresa Blay contributed equally to this work.

✉ Raúl Beltrán-Debón
raul.beltran@urv.cat

[1] Departament de Bioquímica i Biotecnologia, MoBioFood Research Group, Universitat Rovira I Virgili, 43007 Tarragona, Spain

[2] Servei de Cirurgia General i de l'Aparell Digestiu, Hospital Universitari Joan XXIII, Tarragona, Spain

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Protective properties of grape-seed proanthocyanidins in human *ex vivo* acute colonic dysfunction induced by dextran sodium sulphate.

By Carlos González-Quilen[1], Carme Grau-Bové[1], Rosa Jorba-Martín[2], Aleidis Caro-Tarragó[2], Montserrat Pinent[1], Anna Ardévol[1], Raúl Beltrán-Debón[1], Ximena Terra[1] and M Teresa Blay[1]

[1] *Universitat Rovira i Virgili, Departament de Bioquímica i Biotecnologia, MoBioFood Research Group, 43007 Tarragona, Spain*

[2] *Servei de Cirurgia General i de l'Aparell Digestiu, Hospital Universitari Joan XXIII, Tarragona, Spain*

Abstract

Purpose: Anti-inflammatory and barrier protective properties have been attributed to proanthocyanidins in the context of intestinal dysfunction, however little information is available about the impact of these phytochemicals on intestinal barrier integrity and immune response in the human. Here we assessed the putative protective properties of a grape-seed proanthocyanidin extract (GSPE) against dextran sodium sulphate (DSS)-induced acute dysfunction of the human colon in an Ussing chamber system. ***Methods***: Human proximal and distal colon tissues from colectomized patients were submitted *ex vivo* to a 30-minute preventive GSPE treatment (50 or 200 μg mL^{-1}) followed by 1-hour incubation with DSS (12% w v^{-1}). Transepithelial electrical resistance (TEER), permeation of a fluorescently-labelled dextran (FD4) and proinflammatory cytokine release (tumor necrosis factor (TNF)-α and interleukin (IL)-1β) of colonic tissues were determined. ***Results***: DSS reduced TEER (45-52%) in both the proximal and distal

colon; however, significant increments in FD4 permeation (4-fold) and TNF-α release (61%) were observed only in the proximal colon. The preventive GSPE treatment decreased DSS-induced TEER loss (20-32%), FD4 permeation (66-73%) and TNF-α release (22-33%) of the proximal colon dose-dependently. The distal colon was not responsive to the preventive treatment but showed a reduction in IL-1β release below basal levels with the highest GSPE concentration. ***Conclusions***: Our results demonstrate potential preventive effects of GSPE on human colon dysfunction. Further studies are required to test whether administering GSPE could be a complementary therapeutic approach in colonic dysfunction associated with metabolic disorders and inflammatory bowel disease.

Keywords: Human colon; Ussing chamber; inflammation; permeability; flavonoid; procyanidin

1. Introduction

Intestinal dysfunction, characterized by an increase in epithelial permeability and mucosal inflammation, is a manifestation commonly observed in obesity and inflammatory bowel disease (ulcerative colitis and Crohn's disease; IBD) [1]. Intestinal barrier integrity is a key feature in human health as it is essential for maintaining normal intestinal permeability. When the barrier function is compromised by detrimental agents (e.g. diet components and chemicals) the permeability is altered, which can lead to the translocation and dissemination of luminal content into the underlying tissue, including bacterial lipopolysaccharides (LPS) [1,2]. Chronic exposition to these elements triggers the ubiquitous expression of inflammatory transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which leads to the production of proinflammatory cytokines like tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, with a concomitant decrease in the anti-inflammatory cytokines and

proteins [3,4]. This disruption of the intestinal epithelial-cell barrier is closely associated with the onset of metabolic disorders [1,5,6].

The link between alterations of the intestinal barrier function and inflammation has been studied in animals exposed to various chemicals [7]. In recent years, many *in vivo* experiments have employed dextran sodium sulphate (DSS) as a chemical inductor of colonic dysfunction because of its simplicity and reproducible effect that closely resembles human ulcerative colitis [8]. DSS is a water-soluble sulphated polysaccharide of negative charge and highly variable molecular weight. The administration in murines of 40-50 kDa DSS added to drinking water (up to 10% w v^{-1}) results in the erosion of the colon epithelium, which compromises barrier integrity and increases colonic epithelial permeability [8]. The DSS-induced damage is almost completely restricted to the colon mucosa, although the reason for this is not known. It has been suggested that DSS forms nano-lipocomplexes with medium-chain-length fatty acids in the colon, which disrupts the epithelial barrier [9].

Interventions for improving intestinal barrier integrity have shown promising results in the context of inflammatory bowel disease and metabolic disorders [1]. Multiple studies using experimental animals demonstrate the beneficial effects of proanthocyanidin, which can ameliorate intestinal dysfunction derived from the diet [10,11], spontaneously-induced or produced by chemical agents [12]. Proanthocyanidins are oligomeric and polymeric flavan-3-ols, mainly composed of (+)-catechin and (–)-epicatechin monomers. They are present in a wide variety of plant derived foods and beverages and constitute one of the most abundant groups of phenolic compounds in the human diet [13]. Proanthocyanidins from grape seeds have been shown to improve colonic permeability alterations and local inflammation induced by DSS and other chemicals in experimental rats, often exerting an effect comparable to sulfasalazine, an anti-inflammatory drug [14–16]. The metabolic effects of grape-seed proanthocyanidins on intestinal mucosa

described in these *in vivo* studies include increasing antioxidant enzyme activity and reducing proinflammatory mediators, such as TNF-α and IL-1β, as well as the associated immune cell infiltration. It has been proposed that the reduction in mucosal inflammation is mediated by the inhibition of the NF-κB signal transduction pathway [17,18].

Although there is much evidence supporting the gut-protective properties of grape-seed proanthocyanidins, there are few studies that analyze the efficacy of these phytochemicals in humans and thus efforts should focus on this. The Ussing chamber technique is a valuable tool for studying human intestinal function [19,20]. The set-up consists of two half chambers separating the apical and basolateral domains of the mucosal preparation, thus mimicking the *in vivo* situation of the epithelium and permitting an accurate prediction of intestinal function. In this set-up, tissue integrity can be monitored by electrophysiological parameters such as the transepithelial electrical resistance (TEER), that reflects the ionic conductance of the paracellular pathway. Furthermore, preserving the tissue architecture maintains the interplay between the different cell types, which not only makes it possible to study site-specific transport of molecules across the epithelium but also the metabolic effects of bioactives administered in a donor compartment that simulates the intestinal lumen [19].

In the present study, healthy/normal colon tissues from donor oncology patients who underwent colectomy were used to perform Ussing chamber-based experiments. In these *ex vivo* assays, human proximal and distal colon tissues were exposed to DSS as an inductor of acute dysfunction to evaluate the putative barrier-protective and anti-inflammatory properties of a grape-seed proanthocyanidin extract (GSPE).

2. Materials and methods

2.1. Proanthocyanidin extract

The grape-seed proanthocyanidin extract (GSPE) was provided by Les Dérivés Résiniques et Terpéniques (batch number 124029; Dax, France). According to the manufacturer the GSPE has the following composition: monomers of flavan-3-ols (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%) and oligomers (5–13 units; 31.7%) of proanthocyanidins. A detailed analysis of the monomeric, dimeric, and trimeric structures of the GSPE can be found in the work by Margalef et al. [21]. The GSPE was dissolved in DMSO 50% to prepare a stock solution of 100 mg mL^{-1}.

2.2. Collection of human tissues

Human colon tissues were collected from 62 consecutive donor patients with pathologically confirmed colorectal carcinoma and a median age of 65 years (range: 28–82 years), who underwent colon surgery between 2016 and 2019 in the University Hospital Joan XXIII (Tarragona, Spain). Exclusion criteria included the consumption of anti-inflammatory drugs, alcohol abuse and the presence of IBD or celiac disease as these would alter intestinal functioning. All donor patients gave informed consent and the study was approved by the ethics committee of the University Hospital Joan XXIII (ref. CEIm 101/2017). The characteristics of patients included in this study are summarized in **Table 1**.

Non-diseased tissues that were not strictly needed for diagnosis purposes were excised from the proximal colon (cecum, ascending, hepatic flexure, and transverse colon; *n*=27) and distal colon (splenic flexure, descending and sigmoid colon; *n*=34). After resection, these colon tissues were transferred from the hospital within 30 min in ice-cold Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) saturated with 95% oxygen and 5% CO_2. After rinsing, tissues were mounted in a plastic tube to facilitate the removal of the serosal and outer muscular layers with a scalpel

(stripping). The stripped preparations were placed apical side up on Parafilm M (Heathrow Scientific, Vernon Hills, IL, USA) and segments of approximately 1.5 × 1.0 cm were cut for the Ussing chamber experiments.

Table 1. Descriptive statistic and biochemical parameters of the donor patients.

	All (*n*=61)	**Proximal colon donors (*n*=27)**	**Distal colon donors (*n*=34)**
Age (years)			
<50	14 (22.5%)	5 (18.5%)	9 (25.7%)
50-60	30 (48.4%)	12 (44.5%)	18 (51.4%)
>60	18 (29.0%)	10 (37.0%)	8 (22.9%)
Gender			
Male	34 (54.8%)	13 (48.1%)	21 (60.0%)
Female	28 (45.2%)	14 (51.9%)	14 (40.0%)
Tobacco consumption			
Never	50 (80.6%)	20 (74.0%)	30 (85.7%)
<20 cigarettes per day	9 (14.5%)	4 (14.8%)	5 (14.3%)
>20 cigarettes per day	3 (4.9%)	3 (11.2%)	0 (0.0%)
Alcohol consumption			
Never	35 (56.4%)	14 (52.4%)	20 (58.1%)
Mild-Moderate	27 (43.6%)	13 (47.6%)	15 (41.9%)
BMI (kg m^{-2})	27.6 ± 0.7	28.1 ± 1.1	27.3 ± 0.8
Glucose (mM)	5.8 ± 0.2	5.7 ± 0.4	5.9 ± 0.3
Cholesterol (mg dL^{-1})			
Total	187.4 ± 5.6	183.0 ± 9.6	190.0 ± 6.9
HDL	55.4 ± 3.7	53.0 ± 6.5	57.0 ± 4.3
LDL	108.1 ± 7.8	100.8 ± 10.3	114.1 ± 11.4
Triglycerides (mg dL^{-1})	114.2 ± 7.7	105.3 ± 10.2	120.0 ± 10.1

Data is presented as number of patients (percentage) or mean ± SEM.

2.3. Ussing chamber experiments

Depending on tissue availability, up to four stripped proximal or distal colon segments of one patient were placed in 0.237 cm^2 aperture Ussing chambers (Dipl.-Ing. Mussler Scientific Instruments, Aachen, Germany) for each experiment. Ussing chambers were bathed apically and basolaterally with 1.5 mL of fresh KRB buffer (pH 7.4). The basolateral bathing solutions contained 10 mM of glucose (Panreac, Barcelona, Spain) and were osmotically balanced in the apical compartments with 10 mM of mannitol (Sigma, Madrid, Spain). Bathing solutions were continuously bubbled with a O_2/CO_2 (95%/5%) gas mixture and circulated in water-jacketed reservoirs kept at 37 °C.

After a 20-30-min equilibration period, the colon segments were randomly assigned to one of four experimental conditions (**Figure 1**). The bathing solution of the apical compartments was replaced by KRB buffer containing GSPE at 50 μg mL^{-1} (GSPE50-DSS condition) or 200 μg mL^{-1} (GSPE200-DSS condition) or plain KRB buffer (DSS condition). The DMSO concentration was kept at ≤0.1% in the apical media. A KRB buffer with protease inhibitors (10 μM amastatin (Enzo Life Sciences, Madrid, Spain), 500 KIU aprotinin (Sigma, Madrid, Spain) and 0.1% bovine serum albumin fatty acid free) was added to the basolateral compartments. Tissues were incubated for 30 min, after which the apical media were replaced by KRB buffer containing 12% w v^{-1} of dextran sodium sulphate (DSS, MW: 36,000–50,000; MP Biomedicals, Solon, OH, USA). After one additional hour, the basolateral media were stored at −80 °C for further analysis. A control (Ctrl condition) with plain KRB buffer was also included to assess the effect of the DSS.

2.4. Histology

Stripped and non-stripped colon segments were haematoxilin-eosin stained to evaluate the tissue structure after stripping. Tissues were fixed in 4% diluted formaldehyde. After 24 h of fixation, the tissues were successively dehydrated

(alcohol/ethanol 70%, 96% and 100%; plus xylol/dimethylbenzene) and paraffin infiltration-immersed at 52 °C. Then, sections 2 µm thick (Microm HM 355S, Thermo Scientific) were obtained, deposited on slides (JP Selecta Paraffin Bath), and subjected to automated hematoxylin-eosin staining (Shandon Varistain Gemini, Thermo Scientific).

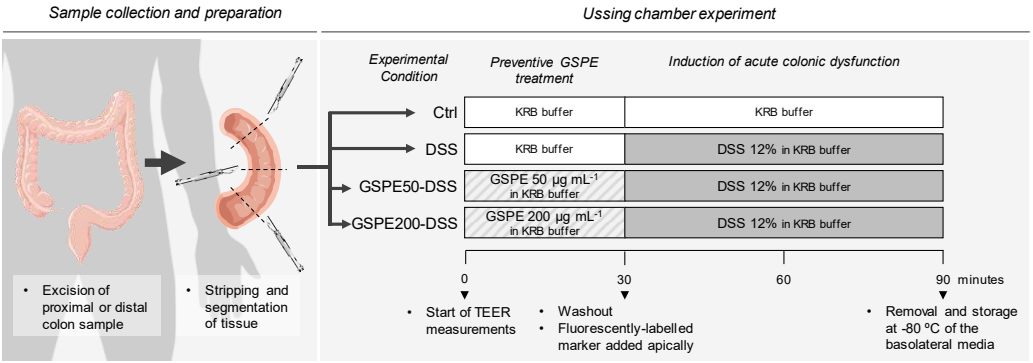

Figure 1. Schematic diagram of the experimental protocol. For each experiment carried out, four stripped proximal or distal colon segments of one patient were randomly assigned to one of four experimental conditions. Ctrl (control), only Krebs-Ringer bicarbonate (KRB) buffer; DSS, acute colonic dysfunction induced by 12% of dextran sodium sulphate in KRB buffer; GSPE-DSS, incubation with the grape-seed proanthocyanidin extract (GSPE) followed by DSS-induced acute colonic dysfunction. Specified media were added apically. KRB-glucose buffer with protease inhibitors was used in the basolateral compartment (see text for details).

2.5. Electrophysiological parameters

A four-electrode system coupled to an external 6-channel voltage/current clamp electronic unit (Dipl.-Ing. Mussler Scientific Instruments, Aachen, Germany) was used for monitoring the electrophysiological parameters in each Ussing chamber. One pair of Ag/Cl electrodes was used for measuring the potential difference (PD) and another pair for the current passage. The spontaneous transepithelial PD was measured under open-circuit conditions after appropriate correction for fluid

resistance. TEER (ohm cm^2) was calculated every 30 minutes from the transepithelial PD and the short-circuit current in accordance with Ohm's law.

2.6. Paracellular transport of fluorescently labeled dextran

A stock solution of 110 mg mL^{-1} of 4-kDa fluorescein isothiocyanate-dextran (FD4; TdB Consultancy AB, Uppsala, Sweden) was prepared in phosphate-buffered saline. FD4 was added apically in each Ussing chamber at a final concentration of 5.6 mg mL^{-1} and incubated for 1 h during the induction of acute colonic dysfunction by DSS. The amount of FD4 that crossed to the basolateral compartment was measured in a PerkinElmer LS-30 fluorimeter (Beaconsfield, U.K.) at λ_{exc}=430 nm and λ_{em}=540 nm and compared with a FD4 standard curve. FD4 transport across the colon mucosa was calculated as apparent permeability (*Papp*) using the following equation:

$$Papp\ (mL/cm^2 \times s) = (V/A \times t) \times (C_{ba}/C_{ap}),$$

where *V* is the basolateral volume, A is the exposed surface area, *t* is the incubation time and C_{ba} and C_{ap} are the concentrations of FD4 in the basolateral and apical media, respectively. To compare the permeation of FD4 between the DSS and GSPE-DSS experimental conditions, values were taken relative to the Ctrl levels.

2.7. Proinflammatory cytokine release

Human TNF-α and IL-1β levels were determined using commercially available ELISA kits according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Cytokine levels corresponded to the total amount released from colon tissues to the basolateral media of each Ussing chamber at the end of the experiments. The absorbances were measured with a BioTek Eon microplate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) at 450 nm and cytokine levels were expressed as picograms per milliliter. The analytical sensitivities of the assays were 0.13 and 0.3 pg mL^{-1}, respectively.

2.8. Statistical analysis

Unless otherwise indicated, results are expressed as the mean ± standard error of the mean (SEM). The mean represents the average value of determinations performed in n patients. The sample size (*n*) for each variable measured is indicated in the corresponding figure caption. Descriptive statistics and comparisons between groups were assessed with unpaired one-sided Student's *t*-tests, and *p*-values <0.05 were considered statistically significant. Analyses were performed with the XLSTAT 2019.1.3 software (Addinsoft, NY, USA). Linear regressions were fitted in Microsoft Excel 2016 software (Microsoft Corporation, Redmond, WA, USA).

3. Results

3.1. Structural evaluation of colonic tissues after removal of the external muscular layer

We evaluated the protective properties of the GSPE in the human colon in DSS-induced dysfunctional mucosa. For this purpose, we mounted stripped preparations from human proximal and distal colon tissues in an Ussing chamber system. The stripped preparations were structurally conserved and consisted of the epithelial cell layer, the lamina propria, the muscularis mucosae and the submucosal layer (**Figure. 2b**).

3.2. Electrophysiological parameters of colonic tissues

The basal electrophysiological parameters of all colon segments were measured. The spontaneous transepithelial PD and initial TEER values were −0.66 ± 0.13 mV and 41.6 ± 1.7 ohm cm^2 for the proximal colon (*n*=27) and −0.42 ± 0.13 mV and 31.8 ± 1.4 ohm cm^2 (*n*=34) for the distal colon. TEER differences between the proximal and distal colon were statistically significant (*p*<0.01). Barrier integrity is malleable and depends on multiple factors. Therefore, potential differences in the

initial TEER due to gender were evaluated in the proximal colon (males (n=17): 39.6 ± 2.0 ohm cm^2 vs. females (n=10): 45.0 ± 3.1 ohm cm^2; p>0.05) and distal colon (males (n=21): 32.5±1.7 ohm cm^2 vs. females (n=13): 30.7 ± 2.2 ohm cm^2; p>0.05).

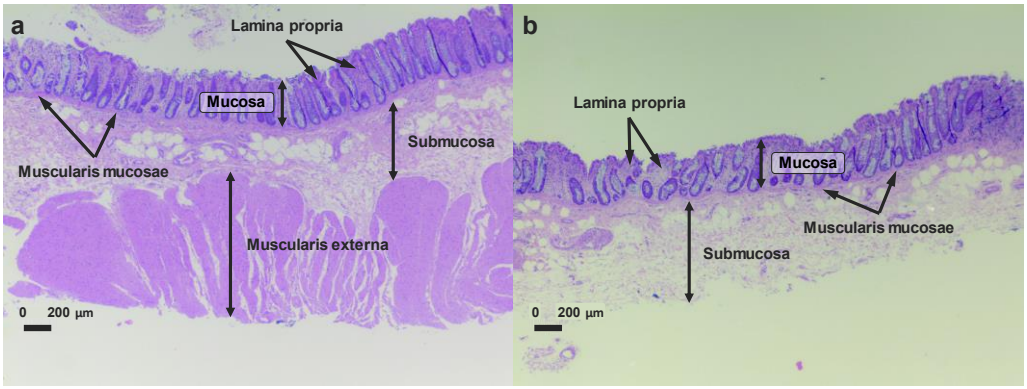

Figure 2. Haematoxilin-eosin staining of transversal sections from the human colon before (a) and after (b) the serosal and outer muscular layers were removed. The muscularis externa is completely absent after stripping.

We also performed correlations between BMI, age and initial TEER values in order to discard a potential influence of the patient's body weight and/or age on differences in the inter-variability of tissue integrity (**Figure 3**). There were no statistically significant correlations between these variables in any colon region. TEER values of the control conditions did not show significant changes during the 90-minute experiments in either kind of tissue (**Figure 4a**).

3.3. Basal macromolecular permeability of the proximal and distal colon

We found detectable FD4 permeations in the control conditions of the proximal (6.3 ± 1.4 ×10^{-6} mL cm^{-2} s^{-1}, n=15) and distal colon (2.5 ± 0.5 ×10^{-6} mL cm^{-2} s^{-1}, n=15) at the end of the experiments. Differences were statistically significant (p<0.05) and in agreement with those found in TEER.

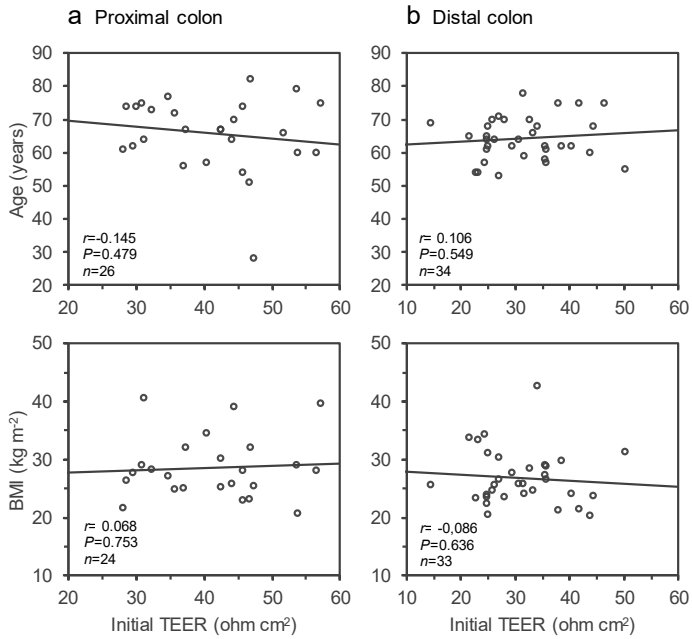

Figure 3. Linear relationships between age, BMI and TEER values in the proximal (a) and distal (b) colon. The inset shows the Pearson's r correlation and the corresponding *p*-value

3.4. A preventive GSPE treatment attenuates DSS-induced permeability in the proximal colon.

The presence of DSS had a detrimental effect on tissue integrity. This effect was more severe in the proximal colon with a 44% reduction in TEER at 90 min compared to the control ($p<0.01$; **Figure 4a**). In the distal colon a reduction of 37% at 90 min was estimated ($p<0.01$). The loss of tissue integrity induced by DSS came with a 2–4-fold increase in FD4 permeation to the basolateral media. However, the effect of DSS on FD4 permeation was only statistically significant in the proximal colon (DSS: $8.8 \pm 2.5 \times 10^{-6}$ mL cm^{-2} s^{-1}, $n=15$ vs. Ctrl: $2.5 \pm 0.5 \times 10^{-6}$ mL cm^{-2} s^{-1}, $n=16$; $p<0.05$). Even though the presence of the GSPE in the apical medium did not significantly change the TEER of the proximal or distal colon during the initial 30-minute incubation at any concentration ($p>0.05$; **Figure 4a**), interestingly GSPE

attenuated the DSS-induced decreased integrity in the proximal colon at 60 and 90 min (p<0.05). This effect was dose dependent and therefore more pronounced in the GSPE200-DSS condition, with a 32% reduction in TEER loss at 90 min (p<0.01; **Figure 4a**). FD4 permeation was also reduced in the GSPE-DSS conditions by 66-73% (p<0.05; **Figure 4b**). We did not find any significant effect of the preventive GSPE treatments on TEER or FD4 permeation in the distal colon.

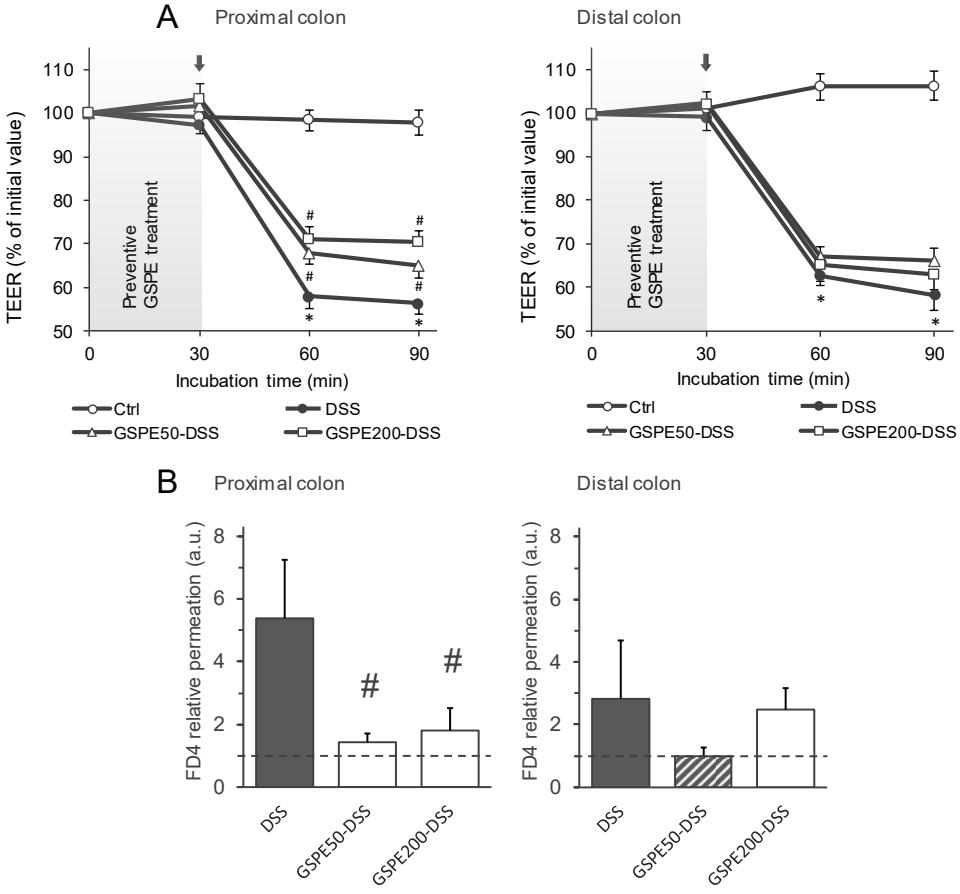

Figure 4. Effect of the preventive GSPE treatment on TEER (a) and FD4 relative permeation (b) in DSS-induced dysfunctional human colon. GSPE (50 and 200 μg mL^{-1}) was incubated apically during the first 30 min at 37 ºC. After a washout, DSS at 12% was added apically (black arrows) and maintained until the end of the experiment. Ctrl (control), only Krebs-

Ringer bicarbonate buffer. FD4 was added apically at 30 min and determined from the basolateral media at 90 min. The dashed lines indicate the basal FD4 permeation (Ctrl condition). Values are presented as mean ± SEM. The sample size for each variable was: TEER (proximal, distal colon), Ctrl *n*=26, *n*=21; DSS *n*=27, *n*=23; GSPE50-DSS *n*=23, *n*=19; GSPE200-DSS *n*=22, *n*=17. FD4 permeation (proximal, distal colon), Ctrl *n*=15, *n*=14; DSS *n*=15, *n*=13; GSPE50-DSS *n*=13, n=7; GSPE200-DSS *n*=10, n=10. **p*<0.05 versus control; #*p*<0.05 versus DSS.

3.5. Basal release of pro-inflammatory cytokines

We found detectable concentrations of the proinflammatory cytokines TNF-α and IL-1β in the basolateral media of the Ussing chamber at 90 min of incubation. While similar basal levels of TNF-α were found in the proximal and distal colon (*p*>0.05; **Figure 5a**), the basal release of IL-1β was approximately 3-fold higher in the latter (*p*<0.05; **Figure 5b**).

3.6. A preventive GSPE treatment reduces DSS-induced TNF-α release in the proximal colon and modulates IL-1β release in the distal colon.

DSS increased (61%) TNF-α release in the proximal colon at 90 min (*p*<0.01; **Figure 5a**); however, we did not observe significant changes in the distal colon (**Figure 5b**). In addition, DSS did not change IL-1β release significantly in the proximal or distal colon. Lastly, the preventive GSPE treatment reduced the DSS-induced TNF-α release of the proximal colon by 22–33% (**Figure 5a**), although only GSPE200-DSS exerted a significant effect (*p*<0.05). We did not find any significant effect of GSPE on the TNF-α release of the distal colon; however, GSPE200-DSS modulated IL-1β secretion in this tissue with a 56% reduction with respect to the control (*p*<0.05; **Figure 5b**).

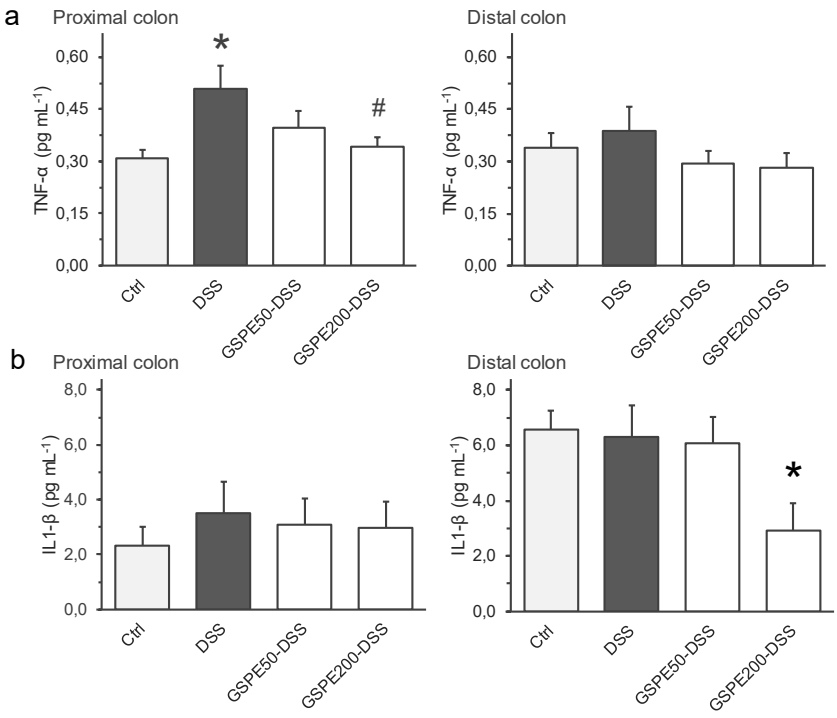

Figure 5. Effect of the preventive GSPE treatment on the secretion levels of TNF-α (a) and IL-1β (b) in DSS-induced dysfunctional human colon. Ctrl (control), only Krebs-Ringer bicarbonate (KRB) buffer; DSS, acute dysfunction induced by 12% of dextran sodium sulphate in KRB buffer; GSPE-DSS, incubation with the GSPE followed by DSS-induced acute dysfunction. Values are presented as mean ± SEM. The sample size for each variable was: TNF-α (proximal, distal colon), Ctrl *n*=20, *n*=19; DSS *n*=20, *n*=19; GSPE50-DSS *n*=17, *n*=15; GSPE200-DSS *n*=16, *n*=14. IL-1β (proximal, distal colon), Ctrl *n*=14, n=19; DSS *n*=14, *n*=19; GSPE50-DSS *n*=12, *n*=14; GSPE200-DSS *n*=12, *n*=14. **p*<0.05 versus control; #*p*<0.05 versus DSS.

4. Discussion

We developed a feasible *ex vivo* Ussing chamber-based model to analyze the therapeutic potential of GSPE in human colon tissues exposed to a detrimental chemical agent (DSS), thus avoiding some of the challenges and limitations of *in vivo* studies in humans [22].

In our model, TEER of the control conditions of both proximal and distal colon tissues was very stable during the Ussing chamber experiments. In previous studies, TEER values of 29-39 ohm cm^2 have been found in distal colon tissues taken from endoscopy biopsies [23] and approximately 109-120 ohm cm^2 in tissues from different colonic locations obtained from surgical procedures [20,24]. Basal electrophysiological parameters usually vary greatly even within segments of the same tissue. This variability has been described in other *ex vivo* studies performed with the human intestine [25]; however, regional variations in colonic integrity have not been studied extensively. It is noteworthy that we observed a higher TEER and lower macromolecular permeation in the proximal colon compared to the distal colon, which suggests a decline in tissue integrity along the large intestine. These findings replicate regional variations of colonic integrity observed in rats [26,27] and are in agreement with results obtained in a recent study performed with colon biopsies of healthy donors [27].

The effect of DSS on TEER was particularly severe in the proximal colon and consistent with the enhanced macromolecular permeability also found in this tissue. Our results are in agreement with *in vitro* studies performed in human epithelial colorectal adenocarcinoma Caco-2 cells, where DSS added apically rapidly decreased monolayer TEER [28,29] and led to an enhanced FD4 permeation to the basolateral medium [29,30]. These similarities validate our *ex vivo* model with the added value of preserving the tissue architecture and cell diversity, features not achieved with *in vitro* models.

We also examined the immune system response to DSS to get a more in-depth characterization of the *ex vivo* model. The activation of the intestinal immune system due to the epithelial barrier alterations leads to the production of inflammatory mediators [31,32]. Our results show that the detrimental stimuli of DSS produces slight but significant increases in TNF-α release in the proximal colon after a short period of time. TNF-α is a key immunoregulatory cytokine mainly secreted by the monocytic lineage that amplifies the inflammatory response to recruit other immune cells [33]. Histochemical analyses of uptake and tissue distribution of DSS in mice indicate that DSS rapidly penetrates the colon mucosa and small amounts are found in resident macrophages as early as the day after administration [34]. Since most of the resident macrophages of the lamina propria are hypo-responsive to proinflammatory elements as an adaptation to the antigen-rich microenvironment [35], the DSS-induced release of TNF-α seen here probably reflects the small number of $CD14^+$ macrophages of normal mucosa that are involved in sensing bacterial LPS [36]. It cannot be ruled out that other cell types contribute to this because intestinal epithelial cells may also secrete TNF-α in an injury context [37].

Once a robust human *ex vivo* model of intestinal dysfunction had been defined, we assessed the potential gut-protective effects of grape-seed proanthocyanidins. Incubation with the GSPE prior to the DSS treatment attenuated the integrity loss and the concomitant increase in macromolecular permeation induced by DSS in the proximal colon. Indeed, both dietary and pharmacological doses of GSPE administered subchronically in rats prevented intestinal permeation after intraperitoneal injection of LPS [38]. In addition, we have previously reported the upregulation of genes involved in the reinforcement of tight-junction (TJ) in the intestine of diet-induced obese rats by dietary and pharmacological GSPE doses with different frequencies of oral administration [11,39,40]. The effect of proanthocyanidins on the TJ protein gene expression was recently described in LPS-induced Caco-2 cells co-treated with a procyanidin-rich apple extract [41].

The GSPE (200 μg mL^{-1}) also attenuated the increase in TNF-α release induced by DSS in the proximal colon. This effect is in line with (1) the reduction of macromolecular permeation across the mucosa and (2) the anti-inflammatory action of the GSPE suppressing the NF-κB inflammatory signal pathway described elsewhere [17,18,42]. Remarkably, the barrier protective and anti-inflammatory effects of GSPE in the proximal colon were long-lasting because they were still exhibited after the media were washed out completely. Thus, beneficial long-lasting effects of a subchronic oral GSPE treatment on cafeteria diet-induced alterations (including intestinal inflammation) have been described in rats, but the associated mechanisms need to be studied further [11,43]. The reduction of IL-1β release estimated in the distal colon is also notable as this cytokine appears to be key in the onset of diarrhea, the main symptom of severe intestinal inflammation [44].

Finally, some considerations should be taken into account. First, the colorectal tumor is not an isolated entity but rather may alter both the macromolecular permeability [26] and the gene expression of non-tumor adjacent mucosa [45]. Thus, although colon segments used in this work were anatomopathologically tested, it cannot be ruled out that the integrity and metabolism of healthy tissues could be influenced by their proximity to cancerous lesions. Second, proanthocyanidins are partially degraded *in vivo* in low molecular weight phenolics by the intestinal microbiota, thus altering the bioavailability and bioactivity of the parent compounds [46,47]. Some microbial products of proanthocyanidin degradation exhibit anti-inflammatory properties and likely account for part of the beneficial effects associated with proanthocyanidin consumption in the intestinal mucosa and a large proportion of their effects at systemic level [48]. Here we exposed human colon tissues to the parent compounds of the GSPE for a short time window, which is feasible *in vivo* in the proximal colon [49,50]. However, the presence of these compounds is less likely in distal regions. In this scenario, it is also important to take into consideration the presence of intestinal microbiota in our samples, which can metabolize the GSPE. In this study the preparation of the

biological samples did not undergo a thorough cleansing to avoid undesirable changes in the integrity of tissues. Then, in our *ex vivo* model, the presence of remnants of microbiota was likely and some degree of microbial degradation of the parent compounds would be expected. Third, it is very difficult to translate the concentrations tested here (50–200 μg mL^{-1}) into oral doses intended for therapy; however, the gut-protective effects of GSPE found in this work would probably only be achieved in humans with pharmacological doses. A recent study conducted in healthy humans found that the daily ingestion of oral pharmacological doses of GSPE (1000–2500 mg) in healthy adults is safe and doses are well tolerated during a 4-week period [51]. Therefore, effective GSPE doses in humans need to be established by further clinical trials.

Taken together, our results indicate that the detrimental effect of DSS on tissue integrity, extent of paracellular pathway opening and local inflammatory response were more prominent in the proximal colon. We found that GSPE administered as a treatment prior to damage induction dose-dependently attenuated the epithelial barrier disruption and the local inflammatory response of the proximal colon. Furthermore, these effects were long-lasting and endured even though proanthocyanidins were not present. The distal colon was not responsive to the preventive treatment; however, basal IL-1β release decreased with high concentrations of GSPE. Therefore, our results demonstrate the potential preventive effects of GSPE on the acute dysfunction of the human colon. Controlled trials are necessary to test the administration of GSPE as a complementary therapeutic approach for the colonic dysfunction associated with metabolic disorders and IBD.

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Conflict of interest: The authors declare that they have no conflict of interest.

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IV. General Discussion

The intestinal tract is an interface between the host and the environment where complex interactions between host cells, microbiota and external factors take place. Thus, diverse regulatory mechanisms cooperate to maintain tissue homeostasis, and a breakdown in these processes can result in dysfunction and the development of pathological conditions [1]. Additionally, the intestine serves as a target organ for naturally occurring bioactive compounds such as plant flavonoids, where they can modulate the activity of its intrinsic machinery [2]. In this scenario, PACs have emerged as potential adjunctive therapeutic agents for the treatment of intestinal alterations prevalent in diet-induced obesity and IBD [3]. In this doctoral thesis we explored the influence of grape-seed PACs in various models of intestinal dysfunction induced by nutritional or chemical agents.

1. The CAF Diet Induces Detrimental Effects in the Intestine That Can Impact Overall Health

In **Manuscript 2** [4]**,** we showed that a 17-week CAF diet greatly increased body weight and adiposity in Wistar rats. These changes in body mass and composition came with substantial alterations in the barrier function and inflammatory status of the intestinal mucosa.

The OVA assay revealed augmented intestinal permeability in CAF-diet fed rats after 17 weeks of dietary intervention. A direct consequence of this increased permeability was a substantial rise in LPS plasma levels (metabolic endotoxemia). Increased plasma LPS has been reported in other diet-induced obesity models, and it has been linked to adipose dysfunction and systemic inflammation in human obesity [5]. Our findings were consistent with this, as we found increased levels of plasma TNF-α, suggesting a systemic inflammatory state. The effect of diet composition on metabolic endotoxemia was also evidenced in several human

studies which found a positive correlation between dietary fat content and plasma LPS [6,7]. The data also suggests that the occurrence of this effect can be either chronic [7] or acute [6,8].

Ex vivo UCh assays showed the increased secretion of TNF-α from duodenum, ileum and colon tissues, which is in agreement with previous studies reporting intestinal inflammation in rodents fed a Western-style diet [9,10]. One of the characteristics of chronic inflammation is immune-cell infiltration. In this regard, the increased MPO activity estimated in the ileum showed clear indications of neutrophil accumulation in inflamed tissues [11]. Although neutrophil infiltration is crucial to limit microbial invasion and essential in the resolution of inflammation, it can contribute to oxidative stress in pathological conditions [12]. In this process, MPO is a potential source of reactive species such as hypochlorous acid/hypochlorite, tyrosyl radicals, chloramines, and nitrogen dioxide [13] that can react with proteins and DNA in host cells and contribute to tissue damage [11]. Thus, elevated levels of MPO may potentiate mucosal injury.

Another observation in the ileal tissues was the decreased expression of the claudin 1 gene (*Cldn1*), suggesting alterations in the integrity of TJs. The downregulation of TJ proteins in rodents fed a Western-style diet has been extensively reported in the literature [10,14–17]. The UCh system allowed us to get a direct measurement of barrier integrity (TEER values) and to discern site-specific changes in this parameter along the intestinal tract. With this *ex vivo* approach, we were able to confirm the disruption of barrier integrity in all the tissues studied (duodenum, ileum and colon). Additionally, we were able to determine that the distal segments, especially the colon, contributed to metabolic endotoxemia to a greater extent than the proximal intestine. This observation can be explained by the continuous increase in the number of commensal bacterial cells along the mammalian gastrointestinal tract [18], as the microbiota is a natural reservoir of endotoxins.

The disruption of barrier integrity in the context of the obesogenic diet has been attributed to changes in the composition and functionality of intestinal microbiota caused by SFA [19]. These dietary components also seem to affect the inflammatory status and barrier integrity by activating TLR2/4-mediated proinflammatory signal transduction [20,21], although more recent evidence argues against previous research and postulates an alternate mechanism involving the reprogramming of macrophage metabolism [22]. Fructose, another major component of most obesogenic diets, has also been implicated in detrimental effects to intestinal health [23–25]. This component represents nearly 20% of food dry weight in the CAF diet (see **Table 1** in **Manuscript 2** [4]). Interestingly, high fructose intake has been associated with metabolic endotoxemia in animals [26] and increases in plasma endotoxin levels in humans [27] even in the context of isocaloric diets. Considering that commercial sugar-sweetened beverages typically have up to 11 g of free sugars per 100 mL, a single 330-mL can of soft drink can contain ~20 g of fructose (>100 mmol) [28]. Therefore, it is reasonable to think that fructose concentration at the very proximal part of the small intestine can reach hundreds of millimoles per liter after a particularly sugary meal. Few studies have quantified luminal sugar concentrations in the intestine of experimental animals in the postprandial state (up to 100 mmol L^{-1}) [29], and we are not aware of studies in humans.

In **Manuscript 3**, we evaluated the effect of high concentrations of fructose (50–300 mmol L^{-1}) achievable in the context of the CAF diet, on the functionality of human colon carcinoma Caco-2 cells. We showed that cell monolayers exposed to fructose (≥150 mmol L^{-1}) on the apical side for 5 h underwent alterations in the barrier integrity (TEER drop) by means of a combination of osmotic stress and cell injury (increase in LDH activity in the culture media), resulting in increased molecular permeability (increased *Papp* of FD4 and LY). Fructose has also been associated with increased permeability [24,25] and injury [24] in other intestinal cell lines and with hepatic cytotoxicity in animal models and humans [30,31]. Some authors have reported the downregulation of TJ protein genes in intestinal cells

incubated with fructose [24,25]. In our study, we did not observe a change in the expression of the TJ protein genes analyzed, but we cannot rule out the occurrence of alterations in protein synthesis or of processes directly affecting the integrity of TJs. In this regard, recent research using animal and cell line models has found that metabolic alterations produced by high intracellular levels of fructose in the enterocyte or hepatic cells can increase the activity of ethanol-inducible cytochrome P450-2E1 (CYP2E1) and iNOS to promote the nitration of TJ proteins [25]. However, in our *in vitro* model we did not find any significant increase in ROS production and therefore there was no indication of oxidative stress. Lastly, the mechanisms underlying cell injury are not clear yet, but a growing body of evidence suggests that it could involve the depletion of ATP driven by uncontrolled fructose assimilation, which promotes a proinflammatory state [23,32]. In keeping with this, we quantified high levels of IL-8 in the basolateral side of monolayers apically treated with 150 mmol L^{-1} of fructose.

Taken together, our results indicate that the consumption of a CAF diet compromises intestinal health. In this process, fructose may play a pivotal role in inducing a proinflammatory environment in epithelial cells which may lead to cell injury and integrity disruption. The enhanced permeability would favor the entrance of bacterial LPS into the systemic circulation, thereby promoting metabolic endotoxemia and systemic inflammation.

2. Preclinical Research: Unveiling the Influence of GSPE on Intestinal Dysfunction Induced by Nutritional Factors

The evidence provided in this thesis confirms the experimental data yielded in previous studies on the beneficial effect of GSPE against dysfunction promoted by detrimental factors in the intestinal mucosa.

In the study presented in **Manuscript 2** [4], we tested the effectiveness of pharmacological doses of GSPE in reversing the intestinal permeability and inflammation induced by the CAF diet when co-administered for two weeks at the end of a 16-week diet intervention. Gil-Cardoso et al. [16] evaluated nutritional doses (5–50 mg kg^{-1} bw) administered orally in the same model and found that they were able to partially reverse the detrimental effects of the CAF diet by lowering ileal markers of oxidative stress (ROS) and inflammation (MPO). In the same study, changes in the expression of TJ protein genes were quantified as indirect markers of permeability alteration. Here we contributed to expanding the body of knowledge on the effectiveness of this therapeutic approach by testing pharmacological doses of GSPE (100–500 mg kg^{-1} bw) that have been proven elsewhere [33] to exert beneficial effects in CAF-diet fed rats when co-administered or administered as preventive treatments. We consider that these pharmacological doses, although quite high, are reliable when translated from rats to humans (HEB of ~1–5 g d^{-1} for a 60 kg person). As a matter of fact, the safety of the oral intake of GSPE of up to 2.5 g d^{-1} for four weeks was demonstrated in a small group of healthy adults [34] and the NOAEL for systemic toxicity subchronically administered in rats has been estimated at 1400–2000 mg kg^{-1} d^{-1} (equivalent to 14–20 g d^{-1} in humans) [35,36].

We found that the administering GSPE to the animals ameliorated CAF-diet induced intestinal permeability (measured as plasma OVA) when compared to CAF-diet fed non-treated animals. The normalization of intestinal permeability was consistent with the upregulation of the claudin 1 gene (*Cldn1*) in the ileum and the restitution of TEER in the ileum and colon. Gil-Cardoso et al. [16] found an increased expression of the ZO-1 gene (*Tjp1*) in the ileum of CAF-diet fed rats treated with nutritional doses of GSPE. Recently, other authors have also reported the upregulation of the ZO-1 and occludin (*Ocldn*) genes in the colon of Wistar rats fed a high fat diet co-administered with a pharmacological dose of GSPE (200 mg kg^{-1} bw).

GSPE administration produced a beneficial effect at the systemic level, ameliorating metabolic endotoxemia, although contrary to other strategies of administration described elsewhere [33], the counteractive approach was not effective in reducing systemic inflammation (measured as plasma TNF-α). The reduction in plasma endotoxin concentrations was most probably driven by the restitution of the barrier function, although a direct interaction between PACs and luminal LPS provoking endotoxin neutralization cannot be ruled out [37].

GSPE administration was associated with a reduction in the basolateral production of TNF-α in the duodenum and colon mucosa. A reduction in the synthesis of proinflammatory mediators has also been reported in numerous *in vivo* and *in vitro* studies testing the anti-inflammatory properties of PACs (extensively reviewed in **Manuscript 1** [38]) and their microbial metabolites [39]. In this regard, accumulated evidence indicates that grape-seed PACs interfere with the NF-κB and MAPK-mediated signaling pathways involved in the synthesis of cytokines and chemokines [40,41]. A direct effect of the inhibition of proinflammatory mediators was the reduction of neutrophil infiltration in the ileum (MPO activity) and, therefore, a presumable improvement in oxidative stress [16].

It is known that the grape-seed PACs exert antioxidant effects by means of radical scavenging [42] and through the upregulation of antioxidant enzymes [43]. Several *in vitro* studies performed with cell lines have demonstrated the antioxidant properties of the GSPE [44]. For instance, Pinent et al. [45] found that a 20 h incubation period with GSPE was able to prevent oxidative stress in Caco-2 cells treated with tert-butyl hydroperoxide. In our *in vivo* study (**Manuscript 3**) GSPE did not prevent detrimental changes induced by fructose under similar experimental conditions. As cell injury induced by high fructose concentrations was not associated with an increase in ROS generation, but it was with IL-8 secretion, we infer that a proinflammatory environment rather than oxidative stress was the root cause of cell injury in this model.

The microbial metabolism of GSPE during intestinal transit may yield low-weight phenolic compounds with comparable or even higher anti-inflammatory bioactivities than those exhibited by parental compounds [39]. Thus, we believe that the lack of effect of GSPE in this study can be attributed to the inherent limitations of the *in vitro* model. It is possible that, due to the severity of the proinflammatory changes provoked by fructose at the concentrations tested, the parental compounds were not able to attenuate the detrimental effects on monolayer functionality. Thus, although useful for the exploration of molecular mechanisms, most cell culture-based *in vitro* models lack physiological complexity and fail to mimic the *in vivo* situation [46].

Taken together, the evidence provided by *in vivo* studies with experimental animals is promising. The reduction in plasma LPS concentrations seen herein is particularly remarkable, as metabolic endotoxemia has been associated with the onset of obesity and comorbid conditions such as insulin resistance [47] and cardiovascular disease [48]. However, it should be noted that animal models cannot accurately reflect human pathophysiology and therefore are not necessarily good predictors of human responses [49]. Furthermore, current clinical data is limited

and shows a very preliminary correlation between PAC consumption and health benefits. *In vivo* human experimentation is challenging and has obvious ethical restrictions with regard to exposing patients to unknown outcomes. In the next study (**Manuscript 4** [50]), we standardized an *ex vivo* model of dysfunctional colon with human samples, thus overcoming the limitations of animal- and cell culture-based models and, at the same time, the challenges related to clinical trials.

3. Bridging the Gap Between Preclinical Research and Clinical Trials: A Human *ex Vivo* Model of Acute Colonic Dysfunction

In the study described in **Manuscript 4** [50]**,** we tested the influence of GSPE on the human intestine in the context of acute dysfunction. Fresh mucosal preparations from human proximal and distal colon were mounted in an UCh system and incubated on the apical side for 60 min with DSS (12% w v^{-1}), a chemical agent used to induce IBD in rodents [51]. The rationale for using DSS rather than fructose or another more physiological proinflammatory/permeability inductor is based on the fact that fresh samples drastically reduce viability as UCh experiments are conducted over an extended period of time [52]. Therefore, dysfunction had to be induced within a short time span. Furthermore, for these experiments we collected human colon samples, as this segment of the intestinal tract was identified as the most important contributor to metabolic endotoxemia *in vivo* (**Manuscript 2** [4]). An obvious advantage of this model in contrast to the cell culture-based model described in **Manuscript 3** was the preservation of the tissue structure as it would be in an *in vivo* situation, thus maintaining the complex interplay between the different cell types in the intestinal mucosa.

DSS produced some features of acute colitis, i.e. loss of barrier integrity (TEER drop), increased permeability (increased *Papp* of FD4) and a proinflammatory state (increased basolateral secretion of TNF-α) similar to that observed with high-fructose exposure *in vitro* (**Manuscript 3**), but in a comparatively short time period (60 min). The increase in TNF-α secretion was only significant in the proximal colon, indicating that this tissue was more prone to proinflammatory stimulation than the distal colon. This is probably due to the natural adaptation (immune tolerance) of the distal colon to the particularly abundant microbiota inhabiting this segment of the intestinal tract [53,54].

We tested the effectiveness of GSPE (50 and 200 mg mL^{-1}) in preventing acute intestinal dysfunction by incubating intestinal tissues for 30 min prior to washout and the following treatment with DSS. Given that sample processing did not involve a thorough cleansing of tissue samples and remnants of microbiota were surely present, we speculate that the possible limitations related to the microbial metabolism of PACs in the *in vitro* model were partially surpassed *ex vivo*. However, supplementary studies are needed to evaluate the production of microbial-derived phenolic metabolites under the experimental conditions assayed. In any event, we found clear indications that GSPE bioactivity (1) reduced TEER drop and *Papp* of FD4 and (2) reduced the basolateral secretion of TNF-α in agreement with that found *in vivo* (**Manuscript 2** [4]). The effects on integrity, permeability and TNF-α secretion were dose-dependent and only observed in the proximal colon. Moreover, these changes were evident after complete washout and, therefore, in the absence of GSPE in the apical media. This suggests the rapid modulation of cell signal transduction, either by highly polymerized PACs though protein receptors in the epithelial cell membrane or by low-weight microbial phenolics, monomeric- and/or dimeric- flavan-3-ol intracellularly. This long-lasting protection could also relate to observations in CAF-diet-fed Wistar rats experiencing a memory effect 12 weeks after a subchronic 15-day treatment with GSPE [33], although the underlying mechanisms need to be studied in more detail.

In summary, the administration of GSPE as a counteractive or preventive treatment resulted in an overall improvement in intestinal health in the context of intestinal dysfunction induced by nutritional or chemical agents. The therapeutic effect of GSPE appears to be effective in the human colon and comprises the reinforcement of barrier integrity and the reduction in local inflammation. These effects may also have an impact at a systemic level by reducing the entrance of LPS into circulation and therefore metabolic endotoxemia. Lastly, the translation of the GSPE concentrations tested here to oral human doses is not straightforward. However, evidence derived from this thesis, several animal studies and a limited number of clinical trials suggest that the doses therapeutically effective for intestinal dysfunction might be pharmacological rather than nutritional.

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V. Conclusions

The main conclusions of this doctoral thesis are listed below:

1. **Nutritional factors associated with the Western diet compromised intestinal barrier integrity and promoted a proinflammatory environment in animal and cell culture-based models.**
    - A long-term CAF diet in Wistar rats increased the influx of intestinal luminal content, thus producing a sustained local inflammatory response, as well as metabolic endotoxemia and systemic inflammation. Integrity disruption in distal segments of the intestine (mainly colon) greatly contributed to metabolic endotoxemia in CAF diet-induced intestinal dysfunction.
    - High concentrations of fructose produced cell injury and a pro-inflammatory state in human colon Caco-2 cells, and were associated with increased monolayer permeability.
2. **The subchronic oral administration of pharmacological doses of GSPE in rats after a long-term CAF diet was able to reverse the detrimental effects of the diet intervention locally and systemically.**
    - The amelioration of intestinal permeability alterations and local inflammation by GSPE was site-specific with a remarkable restitution of barrier integrity in distal segments.
    - The therapeutic effects of GSPE on the barrier function had a systemic impact through the reduction in metabolic endotoxemia.
3. **The GSPE lessened chemically-induced dysfunction in mucosal tissues of human colon *ex vivo.***
    - A short preventive treatment with GSPE was able to attenuate the loss of integrity and increased permeability caused by a colitis-inducing chemical in the human proximal colon.
    - This therapeutic approach also contributed to ameliorating the proinflammatory environment in both the proximal and distal colon

List of Publications

Manuscripts Included in this Thesis

- **González-Quilen, C.**; Beltrán-Debón, R; Pinent, M.; Ardévol, A.; Terra, X.; Blay, M. Teresa. Detrimental Effects of High Fructose Concentrations in Monolayers of Human Colon Carcinoma Caco-2 Cells: Role of Proanthocyanidins in Permeability Alterations and Proinflammatory Changes. **2020**, [manuscript in preparation].
- **González-Quilen, C.**; Grau-Bové, C.; Jorba-Martín, R.; Caro-Tarragó, A.; Pinent, M.; Ardévol, A.; Beltrán-Debón, R.; Terra, X.; Blay, M. Teresa. Protective properties of grape-seed proanthocyanidins in human *ex vivo* acute colonic dysfunction induced by dextran sodium sulfate. *European Journal of Nutrition* **2020**, [published online ahead of print]. DOI: 10.1007/s00394-020-02222-3.
- **González-Quilen, C.**; Rodríguez-Gallego E.; Beltrán-Debón, R.; Pinent, M.; Ardévol, A.; Blay, M. Teresa; Terra, X. Health-Promoting Properties of Proanthocyanidins for Intestinal Dysfunction. *Nutrients* **2020**, 12(1), 130. DOI: 10.3390/nu12010130.
- **González-Quilen, C**., Gil-Cardoso, K; Ginés, I; Beltrán-Debón, R.; Pinent, M.; Ardévol, A.; Terra X.; Blay, M. Teresa. Grape-Seed Proanthocyanidins are Able to Reverse Intestinal Dysfunction and Metabolic Endotoxemia Induced by a Cafeteria Diet in Wistar Rats. *Nutrients* **2019**, 11(5), 979. DOI: 10.3390/nu11050979.

Other Manuscripts

- Grau-Bové, C.; **González-Quilen, C.**; Terra, X.; Blay, M Teresa; Beltrán-Debón, R; Jorba-Martín, R.; Espina, B; Pinent, M.; Ardévol, A. Effects of flavanols on enteroendocrine secretion. *Biomolecules* **2020**, 10(6), 844. DOI: 10.3390/biom10060844.
- **González-Quilen, C.**; Rodríguez-Gallego E.; Beltrán-Debón, R.; Pinent, M.; Ardévol, A.; Blay, M. Teresa; Terra, X. Beneficial Effects of Proanthocyanidins on Intestinal Permeability and Its Relationship with Inflammation. *IntechOpen* **2020**, [published online ahead of print]. DOI: 10.5772/intechopen.91212.

Abstracts

- **Gonzalez-Quilen, C**.; Gil-Cardoso, K.; Terra, X; Blay, M. Teresa. Grape-seed proanthocyanidins reduce intestinal permeabilization and systemic endotoxemia induced by cafeteria diet in Wistar rats. Poster presented at 15th NuGOweek: Mitochondria, Nutrition and Health 2018, Sep 3–6 Newcastle, UK.
- Robles-Bolivar, P.; **Gonzalez-Quilen, C.**; Ginés, I.; Blay, M Teresa; Terra, X.; Pinent, M.; Ardévol, A. Identification of pure bioactive molecules on enteroendocrine secretions in different pig intestine segments. Poster presented at XL SEBBM Congress 2017, Oct 23–26. Barcelona, Spain.
- **Gonzalez-Quilen C**., Robles-Bolivar, P., Ginés I., Pinent M, Ardévol A Blay M Teresa, Terra X. *In vitro* and *ex vivo* models of intestinal mucosal alteration: Grape seed proanthocyanidin extract ameliorates transepithelial electrical resistance loss under different detrimental stimulus. Poster presented at XL SEBBM Congress 2017, Oct 23–26. Barcelona, Spain.
- **González-Quilen, C.**; Robles-Bolívar, P.; Gil-Cardoso, K.; Ardévol, A.; Pinent, M.; Blay, M. Teresa; Terra, X. Modelo *ex vivo* de alteración de la mucosa

intestinal: Evaluación del efecto de las proantocianidinas de pepita de uva sobre la resistencia eléctrica transepitelial en colon ascendente de cerdo. Poster presented at IX Seminario sobre Alimentación y estilos de vida saludables 2019, Jul 20–21. Tarragona, Spain.

The intestinal tract is a site of interaction with microorganisms and potentially detrimental environmental factors. The high intake of fructose and saturated fats typical of the Western diet has been associated with intestinal dysfunction (disruption of barrier function and inflammation) and an increased influx of proinflammatory bacterial endotoxins into the systemic circulation. In turn, high concentrations of plasma endotoxins (metabolic endotoxemia) are a precursor to the onset of metabolic syndrome. In view of the above, the intestine is emerging as a target for disease prevention and therapy. Proanthocyanins (PACs) are naturally occurring phenolic compounds with remarkable anti-inflammatory properties in the intestinal mucosa, according to preclinical studies. Thus, PAC administration is a promising adjunctive therapeutic strategy for the treatment of intestinal dysfunction, but its efficacy in humans is yet to be confirmed.

The main objective of this doctoral thesis was to evaluate the impact of a grape-seed PAC extract (GSPE) on preclinical models of intestinal dysfunction and to investigate its effectiveness in humans. We found that a long-term Western-style diet (cafeteria diet) induces intestinal dysfunction in rats, and that alterations in the permeability of the colon largely contribute to metabolic endotoxemia. These effects are partially driven by high luminal concentrations of fructose and could be effectively reversed *in vivo* by pharmacological doses of GSPE. Lastly, we compared these findings with evidence derived from an *ex vivo* human model of chemically-induced colonic dysfunction in which we were able to replicate the reduction of intestinal permeability and the amelioration of inflammatory status by means of GSPE found *in vivo*.

In conclusion, the administration of GSPE results in the overall improvement of intestinal dysfunction and associated metabolic endotoxemia. Effective doses in humans are probably pharmacological and will have to be determined in clinical trials.

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