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Perspective

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Polyphenols and intestinal permeability: rationale and future perspectives

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Abstract

Increasing evidence links intestinal permeability (IP), a feature of the intestinal barrier (IB), to several pathological or dysfunctional conditions. Several host and environmental factors, including dietary factors, can affect the maintenance of normal IP. In this regard, food bioactives such as polyphenols have been proposed as potential IP modulators even if the mechanisms involved are not fully elucidated yet. The aim of the present paper is to provide a short overview of the main evidence from *in vitro* and *in vivo* studies supporting the role of polyphenols in modulating IP and briefly discuss future perspectives in this research area.

Keywords: polyphenols, intestinal permeability; in vitro studies, animal studies, human studies

Abbreviations

IP, intestinal permeability; IB, intestinal barrier; IME, intestinal microbial ecosystem; TJ, tight junction; GJ, gap junction; AJ, adherent junction; JAM, junctional adhesion molecules; ZO, zonula occludens; MLCK, myosin light chain kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; TLR4, toll-like receptor 4; NAFLD, non-alcoholic fatty liver disease; MS, multiple sclerosis; CNS, central nervous system; TNF, tumor necrosis factor; MD, mediterranean diet; SCFAs, short chain fatty acids; PPs, polyphenols; NF- kB, nuclear factor-kB; Nrf-2, nuclear factor erythroid 2–related factor 2; IL, interleukine; HO, oxigenase enzyme; SOD, superoxide dismutase; GPx, glutathione peroxidase; DNA, deoxyribonucleic acid; IKK, ikB-kinase; PI3K, phosphoinositide-3-kinases; AMPK, AMP-activated protein kinase; TEER, transepithelial electrical resistance; INF-γ, interferon-γ; ERK, extracellular regulated kinase; MaPLE, Microbiome mAnipulation through Polyphenols for managing gut Leakiness in the Elderly.

Introduction

Over the last ten years there has been significant research effort to investigate the central role of gut function and properties in the promotion of human health and/or the development of several pathological conditions.

The intestine is the main organ involved in the absorption of nutrients and water and it is the largest area of contact with environmental factors. It contains a large number of specialized immune cells that can coordinate with defensive responses that prevent or counteract exposure of the host and its immune system to luminal antigens of different origins (e.g. microbial and dietary origin) [1].

The definition and specific ontology related to the gut as a complex anatomical and functional system has been widely debated. Bischoff et al [2] defined the intestinal barrier (IB) as a "*functional entity separating the gut lumen from the inner host and consisting of mechanical elements (mucus, epithelial layer), humoral elements (defensines, IgA), immunological elements (lymphocytes and innate immune cells), muscular and neurological elements*". Differently, intestinal permeability (IP), which contributes to the regulation of solute and fluid exchange between the lumen and tissues, should refer to a key feature of IB that is measurable as a whole or at a given site (e.g. evaluating specific molecules/factors flux rates). IP evaluation can be used to address a normal/stable or disturbed/compromised permeability related with IB function [2]. In this context, it is fundamental to underline that IB integrity and functionality can be affected also by the characteristics of intestinal microbial ecosystem and mucosal immune system.

From an anatomical point of view, a well-organized monolayer of epithelial cells is required to form a selective permeability system mainly controlled by the transcellular and the paracellular pathways [3].

While the absorption and/or transport of nutrients (i.e. sugars, amino acids, vitamins, fatty acids, minerals) occur through specific transporters or membrane channels (transcellular path) [3], a complex

system of junctions crucial for the transport between adjacent cells (i.e. tight junction (TJ), gap junctions (GJ), adherent junctions (AJ), and desmosomes) constitute the paracellular path [4].

TJs have composite molecular structure consisting of multiple protein complexes (with more than 50 proteins identified) that include a series of transmembrane tetra-span proteins, named occludin, claudins and tricellulin, able to develop fibrils crossing the membranes and creating a connection with adjacent cells proteins. In addition, single span transmembrane proteins are included and are mostly represented by junctional adhesion molecules (JAM, belonging to the immunoglobulin superfamily). The claudin proteins are considered to be the structural pillar of TJ [5]. Specifically, TJ sealing, fundamental to avoid paracellular permeability is provided by claudin-1, -3, -4, -5, and -8, while claudin- 2 can form charge-selective pores. Less information is available for the specific activities of claudins-7, -12, -15 and occludin [6].

The transmembrane proteins strictly interact with the intracellular scaffold proteins such as zonula occludens (ZO-1, ZO-2, ZO-3) and cingulin tight-fitting the actin cytoskeleton. In particular, increased paracellular permeability is activated by perijunctional actomyosin ring contraction induced by myosin light chain kinase (MLCK). In addition, other signalling proteins, including protein kinase C (PKC) and mitogen-activated protein kinases (MAPK) together with phosphorylation are involved in the regulation pathways of assembly, disassembly, and maintenance of TJ specific properties [7]. Finally, adherent junctions, together with desmosomes and gap junctions located beneath the TJ are involved in the cell-to-cell adhesion and intracellular signalling but seem not to contribute to paracellular permeability [8].

By considering the complex interplay of functions and activities of TJ proteins and signals regulating the fluxes/exchanges of molecules between the lumen and the environment, it is clear that TJ barrier integrity is essential for human health and metabolic homeostasis.

In fact, an impairment or defect in IB function can lead to modest (i.e. sub-clinical) but chronic immune system activation that might contribute to the pathogenesis of intestinal diseases such as

inflammatory bowel disease [4], celiac disease [9], intestinal bowel syndrome [10] up to colon cancer [11]. In addition, recent research showed a possible correlation of IB dysfunction with several clinical conditions such as metabolic syndrome, obesity, Non-alcoholic Fatty Liver Disease (NAFLD) [12], diabetes [13] , inflammatory joint diseases [14] but also neurological conditions, such as major depression and degenerative disorders such as Parkinson's disease [15] and multiple sclerosis (MS), involving the central nervous system (CNS) [16].

It is noteworthy that emerging experimental evidence suggests that an alteration of IB function and/or increased IP can actually occur also during aging, thus, potentially representing a further mechanism underpinning the activation of the low-grade systemic inflammation process (also named inflammaging) identified in older subjects [17]. The alterations can take place at different levels of the intestinal barrier: for example, induced by impairment of the epithelium (physical barrier) and/or of the immune cells/function, or by an alteration of the chemical barrier consisting in the thick mucus layer able to reduce the passage of bacteria through the epithelium (i.e. mucin secretion) or due to an inefficient/inadequate microbial barrier (represented by the commensal "protective" bacteria). In this regard, it has been demonstrated that age-associated microbial dysbiosis can increase gut microbiota lipopolysaccharide (LPS) production, promote IP with increased risk of systemic endotoxemia and inflammation. In particular, bacteria LPS has been demonstrated to activate nuclear factor kappa b (NF-kB) and mitogen-activated protein kinase (MAPK) by triggering the toll-like receptor 4 (TLR4) inflammatory cascade in immune cells (e.g. macrophages, monocytes)[18].

In addition, dysbiosis is not only an age-associated characteristic but it can be found in different clinical conditions associated with inflammation (e.g. obesity, diabetes, NAFLD).

Thus, intestinal microbiota can be considered a critical regulator of the IP. Gut microorganisms may act directly on IP by affecting tight junction properties and activities and indirectly by modulating inflammation, which is a well-recognized factor promoting IP impairment [19]. Consequently, the

manipulation of the complex intestinal microbial ecosystem has been proposed as a novel strategy to restore IP [2].

Diet and IP

An adequate nutritional status is fundamental to maintain normal IB function (being able to affect all the components of IB) and accordingly, malnutrition is associated with increased IP [20]. For example, Guerriero et al [21] showed that a depletion of glutamine, tryptophan and zinc could lead to increased IP.

Overall, it has been demonstrated that dietary patterns are a dominant factor in shaping the intestinal microbiota [22]. Hence, strategies to modify the relative abundance of specific bacterial groups by means of dietary interventions has been proposed with the aim also to modulate the concentrations of microbial metabolites in the gut affecting inflammation [23].

It has been demonstrated that the Western diet, characterized by high-energy and high-fat intake or high fructose consumption, can alter IP by affecting the gut microbiota composition [21]. In addition, this dietary pattern often involves the consumption of food components like specific fatty acids, alcohol, additives, gliadin, chitosan and food processing methods that are known to alter IB physical structure homeostasis and/or commensal microbial homeostasis. On the other hand, a healthy dietary pattern, such as the Mediterranean diet (MD) rich in fruit, vegetables, legumes and unrefined cereals has been suggested to positively affect IP and related conditions [21]. This may be related to an increased production of short chain fatty acids (SCFAs) including acetate, propionate, butyrate and valerate[2] by gut commensal bacteria following fiber degradation provided by MD dietary pattern. These metabolites have been suggested to play an important role as substrate for a functional colonic epithelium and the maintenance of the intestinal barrier. For example, butyrate showed to affect tight junction integrity but also inhibit TNF-α release and inflammation [23]. In addition, butyrate has shown

to increase expression of claudin-1 and Zonula Occludens-1 (ZO-1), to reverse the aberrant expression of ZO-1 and decrease LPS translocation leading to inhibition of macrophage activation and pro-inflammatory cytokine production[24]. Moreover, plant based dietary patterns including MD are also commonly abundant of bioactive compounds such as polyphenols that have been recently on the spotlight of research for their potential modulatory properties with respect to IP [25].

Rationale for polyphenols contribution to a protective dietary pattern in the context of IP

Polyphenols (PPs) are secondary metabolites of plants, widely distributed in fruits, vegetables and plant-derived foods. A diet rich in fruits, vegetables and plant-based beverages has been estimated to provide about 1 g of polyphenols/day [26], with significant variations depending also on the extent of consumption of beverages rich in polyphenols (tea, wine, coffee, fruit juices). The basic monomer in polyphenols is the phenolic ring. Phenols can be mainly classified into phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavonoids (flavons, flavanones, flavanols, flavonols, isoflavones and anthocyanidins), stilbenes (i.e. resveratrol) and lignans. PPs are recognized to be poorly bioavailable, rapidly absorbed and extensively metabolized by gut microbiota [27]. Additional biotransformation can occur in liver and kidney through methylation, glucuronidation and sulfation reactions of phenolic hydroxyl groups [28] or these reasons, the concentration of the native compounds in the blood is low compared to their metabolic derivates (from nanomoles up to micromoles per liter).

PPs and their metabolites are widely studied for their numerous biological activities, including antimicrobial, antiproliferative, antioxidant and anti-inflammatory function [29]. These effects are exerted both at intestinal and systemic levels. In particular, PPs may exert their effects by down regulating inflammatory genes (i.e. nuclear factor-kB, NF-kB) and up-regulating cytoprotective and antioxidant genes (i.e. nuclear factor erythroid 2–related factor 2, Nrf-2). This modulation may bring to a reduction of cytokines production (e.g., IL-8, IL-1β, and TNF-α) and boost the bodies' own antioxidant status (HO-1, SOD, and GPx) [30]. Furthermore, recent reviews [31,32] have shown that PPs

may affect, either in a positive or negative way, pattern recognition receptors such as Toll-like receptors and nucleotide-binding oligomerization domain proteins, whose activation in epithelial cells may lead to intestinal inflammation. Moreover, PPs seem to be involved in the regulation of epigenetic factors through interaction with the enzymes responsible for DNA methylation and acetylation by reducing intestinal inflammation [32].

Several studies documented the effects of PPs in the modulation of intestinal microbial ecosystem. However, the mechanisms by which these compounds modulate the gut microbiota remain unclear. Some studies report that the interaction between PPs and microbiota may involve interference with enzymatic expression and activity, and modulation of specific pathways related to anti-oxidant and anti-inflammatory activity [33]. In addition, PPs has been proposed to exert a prebiotic effect potentially inhibiting the pathogenic bacteria and stimulating the growth of beneficial microbes [34–36]. In fact, the microbiota can extensively metabolize PPs in numerous derivatives that could affect not only the composition of microbiota but also specific signalling pathways [33]. Another important aspect regards the possible involvement of PPs in the metabolism of colonic products, such as short chain fatty acids (SCFA), sterols (cholesterol and bile acids), and microbial products of non-absorbed proteins which may directly or indirectly counteract or suppress pro-oxidant and/or pro-inflammatory responses with an overall improvement of gut health [37].

To unravel the complex scenario related with PP-microbiota interaction in vivo, a combination of metabolomic, microbiome and metagenomic approaches are strongly demanded [33].

Finally, in the last few decades, specific research has been devoted to the evaluation of PPs as promising protective factors and regulators of the epithelial homeostasis and intestinal barrier function. In particular, a direct/indirect effect of regulation of tight junction proteins has been investigated.

Mechanisms of polyphenols regulation of IP

At present, the exact mechanisms linking PPs with intestinal epithelial barrier function have not been established yet (**Figure 1**). Some studies hypothesized a direct/indirect involvement of nuclear factor-κB (NF-κB) signalling in the onset of IP. This pathway is recognized as one of the most important mediators of the inflammation; cytokines and interleukins have shown to activate NF-κB and impair the epithelial barrier function by tight junction disassembly. Conversely, PPs have documented to block NF-κB activation by inhibiting IKK (kinase) phosphorylation and/or preventing proteasomal degradation of IκB [38].

Other important factors potentially involved in increasing IP are the multiple protein kinases such as mitogen-activated protein kinases (MAPK), phosphoinositide-3-kinases (PI3K)/Akt, protein kinase C (PKC), tyrosine kinases, myosin light chain kinase (MLCK) and AMP-activated protein kinase (AMPK). Most of them are regulators of fundamental biological processes in epithelial cells, including barrier function, primarily through regulating TJ expression. Some PPs (e.g. quercetin, curcumin, epigallocathechin3-gallate, myricetine) have shown to improve epithelial barrier function through the inhibition of different kinases (PKC and MLCK) involved in phosphorylation of target proteins controlling IP [3,30,39].

In order to ascertain the availability of data supporting the role of PPs on IP, a literature search has been performed using the following terms “intestinal permeability” OR “intestinal barrier” AND “polyphenols” OR “bioactives” OR “phenolics” as keywords in PubMED. The use of the word “polyphenols” as specific keyword consistently reduced the number of results. On the contrary, a more appropriate search with single PP subclasses AND “intestinal permeability” provided a larger number of in vitro and animal studies mainly summarized in **Tables (1-2)** and an apparent lack of human intervention studies.

In vitro studies

The main lines of evidence on the *in vitro* effects of PPs in the modulation of the potential mediators and regulatory pathways involved in the IP are reported in **Table 1.** Most of the studies are performed

194 on Caco-2 cell line [38,40–58], as a model of the intestinal barrier, followed by T84, HT29/B6 cells
195 (colonic adenocarcinoma cell line) [59–63], IPEC-J2 cells (intestinal porcine enterocytes) and ECV304
196 cells (human endothelial cell line) [64,65]. The main evidence of protection are available for berberine,
197 quercetin and catechin tested in a range of concentration between 10 and 200 μM (from physiological
198 to pharmacological concentrations). Other PPs tested included genistein, anthocyanins, resveratrol,
199 theaflavin and mix of PPs. Most the studies have shown an increase in transepithelial electrical
200 resistance (TEER) across a cellular monolayer confirming the integrity and functional permeability of
201 the membranes [38,43–49,53–55,57,58,62,65,66]. In addition, most the PPs tested have shown to increase the
202 expression and/or production of numerous TJ proteins including zonula occludens (ZO)-1, occludin,
203 and the family of claudins whose alteration may result in increased paracellular permeability
204 [41,42,44,53,55–57,63,65]. Finally, some studies have reported the capacity of PP to counteract inflammatory
205 process induced by TNF-α and IFN-γ down-regulating the expression of several interleukins such as
206 IL-8 and IL-6 [48,67].

207

208 ***Animal studies***

209 In **Table 2** are reported the effects of PPs and PP-rich extracts in the modulation of IP in animal
210 models [44,49,67–74]. Most of the studies were performed in healthy rat models (i.e. Wistar rats, Sprague-
211 Dawley rats) and IP was induced by stimuli such high fat diets, mannitol, inflammatory cytokines, or
212 chemicals [44,72,74]. Two studies used mice with IL-10 deficiency in order to test the effect on IP [69,70].

213 The main PPs used were obtained from grape seed extracts (1% GSE; g GSE per g dry food weight)
214 [69,70] and grape seed proanthocyanidin extracts (5-50 mg/kg) [74]. Other studies included berberine (200
215 mg/kg) [68], (-)-epicatechin (2-20 mg/kg) [49] and epigallocatechin-3-gallate (about 3 mg/ml) [73]. Some
216 studies were performed by testing anthocyanins-rich raspberry extract, polyphenol-rich propolis
217 extract, and oregano essential oil [44,72]. The doses administered ranged from nearly physiological

(epicatechin) up to supra- physiological (i.e. berberine). The duration of the intervention varied from few days (3-10 days) up to several weeks (15-16 weeks).

On the whole, the results obtained support an improvement of IP following the intervention with PPs and PP-rich extracts. In particular, the studies showed the capacity of PPs to up-regulate some important genes such as AMPK and ERK and down-regulate NF-kB as pathways involved in the inflammation process. In line with the observations reported in the *in vitro* studies, the compounds tested have shown to increase the expression of zonula occludens (ZO)-1, occludin, and several claudins involved in the functioning of tight junctions.

Human studies

The number of human intervention studies with IP as primary or secondary outcome increased in the last years as also documented by the number of trials made available and reported in public registers (i.e. ISRCTN, ClinicalTrial.gov).

Most of these studies were performed, or are ongoing, by using probiotics, prebiotic fibers, dietary supplements, and sugars. Only 4 studies seem to have explored the potential beneficial effects of PPs/PP-rich foods on IP in humans **(Table 3)** [75–78]. The studies differ in terms of population (overweight/obese, cyclists, older subjects), foods administered (green tea, flavonoid-rich beverage, mix of PP-rich foods), dose of bioactives (650 mg of flavonoids, 750 mg of PPs), duration of intervention (from 2 weeks up to 8 weeks), marker of IP selected (endotoxin, lactulose:mannitol ratio, zonulin levels). The trials are still ongoing, and the results will be useful to increase understanding on the actual role of PPs and PP-rich foods in humans where a large number of factors can interact affecting IP. For example, it is well recognized that PPs are poorly bioavailable and are biotransformed by gut microbiota into metabolites that can be absorbed in the colon. At the same time, PPs may modulate the composition of the gut microbial community shaping towards a protective symbionts and reducing pathobionts. The complex and not fully elucidated two way

242 interaction between PPs and gut microbiota is postulated to play a potential direct/indirect role on IP
243 regulation.

244 In this context, the MaPLE project (Microbiome mAnipulation through Polyphenols for managing
245 gut Leakiness in the Elderly) has been developed with the aim to test the hypothesis that changing
246 the diet of older subjects with established enhanced IP by increasing their PPs consumption can alter
247 IME in a way that is beneficial for IB function, resulting in reduced IP and decreased translocation
248 of inflammogenic bacterial factors from the digestive tract into the bloodstream [78]. To test this
249 hypothesis, a multidisciplinary approach has been used (i) to evaluate the impact of a PP-rich dietary
250 pattern on IB, IP and IME in a target group of older subjects; and (ii) to investigate the possible
251 mechanisms involved in the PP-microbiota-IP interactions through *in vitro* and animal models.
252 Findings obtained from our and other studies will be "pivotal" for the development of new and
253 advanced hypothesis and experimental approaches in this complex area of research.
254

255 **Some considerations on IP assessments in different contexts**

256 IP can be evaluated through numerous methodologies and consequently data obtained can differ
257 among studies. The techniques vary depending on the setting (in vitro, ex-vivo or in vivo models),
258 the models (cells, animals, and humans), the markers (i.e. ions, macromolecules, bacteria and
259 bacterial products) but also the compartments (i.e. tissues, blood, urines). The measurement of IP can
260 be performed through *ex vivo* and *in vivo* approaches [79]. An example of ex vivo approach includes
261 the use of an Ussing chamber able to measure the transport of ions and molecules (i.e. nutrients,
262 drugs) across various epithelial tissues by using fresh intestinal tissue. In vivo, the assessment of IP
263 can be performed through permeability assays (i.e. evaluation of ratio lactulose/mannitol, sucralose,
264 sucrose, polyethylene glycols or 52Cr-EDTA in urines), analysis of bacterial related markers (i.e.
265 endotoxin test, EndoCAb, D-lactate, butyrate production), markers of epithelial damage (i.e. citrullin,
266 fatty acid binding protein, cludin-3), and/or other related markers (i.e. faecal calprotectin). Finally,

histological approaches measuring for example Globet cell analysis, shedding of epithelium or Paneth cell loss, can be performed [2].

On the whole, based on revised literature, it can be assumed that current *in vitro* permeability models are still far from reflecting an *in vivo* situation. This limits the relevance of data obtained within cell culture and the possibility to transfer the results to humans. In fact, the comparison between *in vitro* and *in vivo* permeability data is difficult and dependent on numerous factors, including the type of cells used, the molecule under study, the transport route evaluated and the method used for the assessment of intestinal barrier function and permeability (i.e. mainly TEER and biomarkers of epithelial integrity) which can significantly affect the results obtained making it difficult to identify the best approach.

A novel biomarker of IP *in vivo* is zonulin, a protein secreted by enterocytes but also from other type of cells (i.e. epithelial cells), known to be a physiological modulator and thus to control IP reversibly *via* intercellular TJs [80]. Increased zonulin serum levels have been observed in many gut-related diseases and emerging evidence suggests an increased zonulin level in specific subjects (e.g. older persons) [81] and in different diseases or condition (e.g. diabetes, obesity) [82,83]. The reliability and accuracy of the different markers to assess IP is clearly a fundamental part of the recent discussion and a hot topic considering the increasing demand for non-invasive diagnosis tools [84]. In this regard, it seems highly recommendable the concurrent evaluation of different markers of IP to improve reliability of findings on intestinal barrier function.

Conclusion and future perspectives

There is increasing demand for non-invasive strategies able to modulate critical regulatory functions for human health such as IP, which can play a role in the pathogenesis of intestinal and systemic diseases. The improvement or manipulation of the diet, for example increasing or reducing specific nutrients and/or including food bioactives such as PPs is recognised as a potential powerful tool to be

explored also in the context of IP. From data available PPs activity seems to be plausibly a consequence of multiple mechanisms which may also depend on the type and amount of compounds considered. The results from *in vitro* studies have shown the capacity of PPs to increase the expression and/or production of numerous TJ proteins and to reduce the release of several interleukins/cytokines. These results are partially in line with the findings obtained in the animal models showing the capacity of PPs to up-regulate/down-regulate some important genes involved in the inflammatory process. Regarding human studies, recent literature suggests that PPs may modulate IP through a number of direct and indirect effects including the impact on intestinal ecosystem and immune system. This type of research is still in its infancy by considering the few human studies available. Future research should be targeted to identify the PPs and/or their metabolites eventually involved in the modulation of IP while demonstrating also their specific dose-dependent mechanisms of action. Meanwhile, in vivo studies should be performed to increase understanding of the diet-microbiota-intestinal permeability axis possibly through the development of well controlled dietary intervention studies. Finally, by considering the wide discussion in literature on IP evaluation, a further effort is needed to better define the reliability of the already available IP biomarkers and the potential exploitation of new and/or improved candidate biomarkers.

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FIGURE CAPTION

Figure 1: Putative effects of polyphenols on IP at different physiological levels

Figure Caption: 1 – Intraluminal Level: Modulation of microbiota composition, endotoxin and/or short-chain fatty acid (SCFA) production, redox status, dietary component absorption and/or activity; 2 – Intracellular Level: Regulation of expression of tight junction, adherens junction, gap junction and desmosome proteins, upregulation of kinases and nuclear factor erythroid 2-related factor 2 (Nrf-2), dowregulation of nuclear factor kappa B (NF-kB) and toll-like receptor 4 (TLR4); 3 – Systemic Level:Maintenance of functional immune system and regulation of inflammatory processes (towards a reduced pro-inflammatory status).

Table 1- Summary of the Main in Vitro Studies Highlighting the Mechanisms of Action of Polyphenol Compounds in the Modulation of Barrier Integrity and Function

Reference	Cells	Stimulation	Polyphenol source and dose	Signaling Pathway	Response/Marker	Effect
Atkinson and Rao 2001 [40]	Caco-2	Acetaldehyde	Genistein (30–300 μM)	↓ tyrosine kinase	[a]TEER, occludin, [b]ZO-1	↑ TEER ↑ occludin ↑ ZO-1
Watson et al., 2004 [59]	T84	[c]IFN-γ	Epigallocatechin gallate (100 μM)	↓[d]STAT-1 ↓[e]MAPK	TEER	↑ TEER
Amasheh et al., 2008 [51]	Caco-2	-	Quercetin (0-200 μM)	↓[f]MLCK, [g]PKC	TEER, occludin, claudin-1, claudin-3, claudin-4, claudin-7	↑ TEER ↑ claudin-4 = claudin-1 = claudin-3 = claudin-7 = occludin

Suzuki and Hara 2009 [52]	Caco-2	-	Quercetin (0-100 μM)	↓ PKCδ	ZO-2, occludin, claudin-1, claudin-4	↑ ZO-2 ↑ occludin ↑ claudin-1 ↑ claudin-4
Amasheh et al., 2010 [60]	HT29/B6	[h]TNF-α	Berberine (50 μM)	↓[i]NF-Kb, [j]PI3K/Akt, tyrosine kinase	Claudin-1, claudin-2	↑ claudin 1 ↓ claudin 2
Chuenkitiyanon et al., 2010 [64]	ECV304	[m]H_2O_2	Quercetin (10 μM)	↓[n]p38	ZO-1, occludin	↑ ZO-1 ↑ occludin
Rogoll et al., 2010 [61]	T84	-	(+)-Catechin (10 μM) (-)-epicatechin (10 μM) Quercetins (10 μM) Phloretins (20 μM)	↓Tight junction permeability	TEER, ZO-1, occludin, claudin-4	↑TEER ↑ ZO-1 ↑ occludin ↑ claudin-4

			D-(-)-quinic acids (10-50 μM) p-coumaric acids (10 μM) caffeic acids (20 μM)		
Shin et al., 2011 [66]	HCT-116	-	Anthocyanin mixture (45 μg/mL; delphinidin, cyanidin, petunidin, delphinidin, malvdin, peonidin-3,5-diglucoside, cyanidin, petunidin, peonidin, malvidin-3-glucoside)	↑p38	TEER, claudin-1, claudin-3, claudin-4 ↑ TEER ↓ claudin 1 ↓ claudin 3 ↓ claudin 4

Suzuki et al., 2011 [53]	Caco-2	-	Kaempferol (100 μM)	↓Tight junction permeability	TEER, ZO-1, ZO-2, occludin, claudin-1, claudin-3, and claudin-4	↑ TEER ↑ occludin ↑ claudin 1 ↑ claudin 3 ↑ claudin 4 ↑ ZO-1 ↑ ZO-2
Noda et al., 2012 [54]	Caco-2	-	Chrysin, daidzein, genistein, hesperetin, luteolin, morin, and naringenin (100 μM)	↓Tight junction permeability	TEER, ZO-1, ZO-2, JAM1, claudin-1, claudin-3, claudin-4	↑TEER (negative effect for chrysin) Effect on tight junction proteins was compound dependent

Amasheh et al., 2012 [62]	HT-29/B6	IFN-γ, TNF-α	Quercetin (200 μM)	↓Tight junction permeability	TEER, claudin-1, claudin-2, claudin-3, claudin-4, claudin-7, occludin	↑ TEER ↓claudin-2 ↓claudin-3 = claudin-1 = claudin-4 =claudin-7 =occludin
Noda et al., 2013 [55]	Caco-2	-	Naringenin (100 μM)	↑[o]Sp1-dependent transcriptional regulation ↓Tight junction permeability	TEER, ZO-1, ZO-2, occludin, [p]JAM-A, claudin-1, claudin-3, claudin-4	↑ TEER ↑claudin-1 ↑claudin-4 ↑occludin = ZO-1 = JAM-A

Cao et al., 2013 [56]	Caco-2	IFN-γ, TNF-α	Berberine (100 μM)	↓MLCK	Occludin, claudin-1, ZO-1, intestinal permeability	↑ Occludin ↑ claudin-1 ↑ ZO-1 ↓intestinal permeability
Carrasco-Pozo et al., 2013 [57]	Caco-2	Indomethacin	Mix of quercetin (33μM), resveratrol (438μM), rutin (164μM), epigallocatechin gallate (218μM)	↑epithelial barrier function	TEER, [q]FD4, ZO-1, occludin	↑TEER (no effect with rutin) ↓FD4 (no effect with rutin) ↑ ZO-1 after quercetin ↑ occludin after quercetin

Piegholdt et al., 2014 [58]	Caco-2	TNF-α	Biochanin A (50 μM), prunetin (50 μM)	↓NF-Kb, [r)]ERK, tyrosine kinase	TEER, claudin 1, occludin, ZO-1, E-cadherin	↑ TEER = claudin 1 = ZO-1 = E-cadherin
Park et al., 2015 [41]	Caco-2	-	Theaflavins-3'-0-gallate (20 μM)	↓MLCK	Occludin, claudin-1, ZO-1	↑occludin ↑claudin-1 ↑ZO-1
Contreras et al., 2015 [42]	Caco-2	TNF-α	(-)-Epicatechin (0.5–5 μM)	↓NF-Kb, [s)]p-IKKα, p-IkBα, MLCK	Occludin, ZO-1, claudin-2	↑ZO-1 = occludin =claudin-2
Valenzano et al., 2015 [43]	Caco-2	-	Berberine (50-200 μM) Quercetin (100-400 μM)	↑epithelial barrier function	TEER, claudin-1 claudin-2, claudin-3	↑TEER (only berberine) Quercetin (↑claudin 2,

					claudin-4, claudin-5 claudin-7, occludin tricellulin, D-mannitol	claudin-4, claudin-5, ↓tricellulin) Berberin (↓claudin-2, D-mannitol)
Ling et al., 2016 [65]	IPEC-J2	Deoxynivalenol	Resveratrol (0-200 μM)	↓p38, ERK, ^{ij}p-JNK	TEER, FD4, Claudin-1, Claudin-3, Claudin-4, Claudin-7, occludin, ZO-1	↑ TEER ↑ occludin ↑ claudin-3 ↑ claudin-4 ↓FD4 = claudin-1 = claudin-7

Wang et al., 2016 [44]	Caco-2	-	Polyphenol-rich propolis extract (25 and 50 μg/mL)	↑[u]AMPK-α, ERK1/2, Akt, p38	ZO-1, occludin	↑ TEER ↑ occludin ↑ ZO-1
Azzini et al., 2016 [45]	Caco-2	-	3 different polyphenol-rich extracts from Chicory (0.2, 1.3, 10, 17, 34, 70 μM)	↑epithelial barrier function	TEER	↑TEER
Luescher et al., 2017 [38]	Caco-2	TNF-α	Xanthohumol (chalcone; 10 μM), isoxanthohumol (prenylflavone; 10 μM)	↓Tight junction permeability	TEER	↑TEER
Cremonini et al., 2017 [46]	Caco-2	TNF-α IFN-γ	cyanidin, delphinidin, malvidin, petunidin, or	↓IKK and p65 phosphorylation	TEER	↑TEER

			peonidin- 3-O-glucoside (0.25–1 μM)			(only cyanidin and delphinidin, and ACN-rich plant extracts)
			crowberry extract (1–10 μg/mL) anthocyanin-rich plant extracts (black chokeberry, black kernel rice, wild blueberry, bilberry, crowberry, domesticated blueberry, red grape (5 μg/mL))			
Rybakovsky et al., 2017 [47]	Caco-2	^{14}C−D-mannitol	Theaflavins (5-20 μg/mL) Quercetin (100-400 μM) Berberine (50-200 μM)	↑ membrane permeability	Claudin-1,claudin-2, Claudin-4, claudin-5	↑TEER (quercetin) ↓Transepithelial Mannitol

						Permeability (quercetin) ↑ claudin-2 = claudin-1 = claudin-4 = claudin-5
Van Buiten et al., 2018 [48]	Caco-2	-	Decaffeinated green tea polyphenols (0-100 µg/mL)	↓paracellular permeability	TEER, [v]IL-6, IL-8	↑TEER ↓IL-6 ↓IL-8
Li et al., 2018 [67]	MODE-K	[z]LPS	Naringin (50-200 µM)	↓NF-kB, MLCK/MLC	TNF-α, IL-10, IL-6, MLCK, p-MLC/MLC, p-p65/p65, p-IkBα/IkBα	↓TNF-α ↓ IL-10 ↓ IL-6 ↓ MLCK ↓ p-MLC/MLC ↓ p-p65/p65

						↓ p-IkBα/IkBα
Cremonini et al., 2018 [49]	Caco-2	TNF-α	(-)-Epicatechin	↑ERK1/2, AMPK, ↓NF-kB	[x)]NOX1/NOX4, [y)]FITC-dextran transport, TEER	↑TEER ↓ FITC ↓NOX1/NOX4
Vazquez-Olivo et al., 2019 [50]	Caco-2	-	4 polyphenol-rich mango extracts (100 µg/mL) Gallic acid (100 µg/mL)	↑ membrane permeability	Papp	↑Improvement of apparent membrane permeability
Nunes et al., 2019 [63]	HT-29	TNF-α, IL-1, IFN-γ	Non-alcoholic polyphenolic red wine extract (catechin, oligomeric procyanidins, anthocyanin, phenolic acids, ethyl cinnamate,	↓paracellular permeability	Occludin, claudin-5, ZO-1	↑ occludin ↑ claudin-5 ↑ ZO-1

condensed tannin); 200, 400 and 600 μg/mL

Note: *[a)]TEER, trans-epithelial eletrical resistance; [b)]ZO-1, zonula occludens; [c)]IFN-γ, interferon gamma; [d)]STAT-1, signal transducer and activator of transcription 1; [e)]MAPK, mitogen-activated protein kinases; [f)]MLCK/MLC, myosin light-chain kinase; [g)]PKC, protein kinase C; [h)]TNF-α, tumor necrosis factor alpha; [i)]NF-kB, nuclear factor-kB; [l)]PI3K/Akt, phosphoinositide 3-kinase; [m)]H_2O_2, hydrogen peroxide; [n)]p38, p38 pathway; [o)]SP-1, specific protein transcription factor-1; [p)]JAM-A, junctional adhesion molecule-A; [q)]FD4, fluorescein isothiocyanate-labeled dextrans; [r)]ERK1/2, extracellular signal–regulated kinases; [s)]p-IKKα, IκB kinase α; [t)]JNK, c-Jun N-terminal kinases; [u)]IL-(6,8,10), interleukin-(6,8,10); [v)]AMPK, 5' AMP-activated protein kinase; [z)]LPS, Lipopolysaccharide; [x)]NOX, nicotinamide adenine dinucleotide oxidase; [y)]FITC, fluorescein*

Table 2- Summary of the Main Evidence from Animal Models Reporting the Effects of PPs and PP-rich Extracts in the Modulation of Barrier Integrity and Function

Reference	Animal model	Diet	Polyphenol source and dose	Signaling Pathway	Response/Marker	Main findings
Gu et al., 2011 [68]	Male C57BL/6 mice	BBR *vs* C LPS-stimulation	BBR: berberine (200 mg/kg) C: control diet 7 days	↓[a)]MLCK	Intestinal permeability Claudin-1 Claudin-4 Occludin [b)]ZO-1	↑ ZO-1 ↑ occludin ↑Claudin-1 ↑Claudin-4 ↓intestinal permeability
Yang et al., 2014 [69]	C57BL/6 (WT) and [c)]IL-10-deficient (IL-10-/-,	GSE *vs* C dextran sulfate sodium-stimulation	GSE: grape seed extract (0 or 1% GSE)* C: standard rodent diet 16 weeks	↓[d)]NF-kB	Claudin-1 Claudin-2	↑claudin-1 ↓claudin-2

	IL10KO) female mice					
Wang et al., 2013 [70]	IL10-deficient mice (IL10KO)	GSE *vs* C dextran sulfate sodium-stimulation	GSE: grape seed extract (0 or 1% GSE)* C: standard rodent diet 16 weeks	↓[e] AMPK	Claudin-1 Claudin-2	↑claudin-1 ↓claudin-2
Li et al., 2014 [71]	BALB/c mice	ARF *vs* C dextran sulfate sodium-stimulation	ARF: Anthocyanin-rich raspberry extract (20 mg/kg) C: Saline solution as control treatment 10 days	↓NF-kB ↓[f] MAPKs	Colonic histological architecture	↑colonic histological architecture

Wei et al., 2015 [72]	Males Wistar rats	OEO vs C Diquat-stimulation	OEO: oregano essential oil (5 or 20 mg/kg BW) C: saline solution as control treatment 14 days	↓[g]SOD ↓[h]GSH-Px	ZO-1 occludin	↑ ZO-1 ↑ occludin
Wang et al., 2016 [44]	Male Sprague-Dawley rats	PPE *vs* C 2,4,6-trinitrobenzenesulfonic acid stimulation	PPE: Polyphenol-rich propolis extract (0.3% w/w)* C: control diet 14 days	↑AMPK ↑[i]ERK	ZO-1 occludin	↑ ZO-1 ↑ occludin
Bitzer et al 2016 [73]	Male CF-1 mice	[l]DSS treatment + D (0.5% citric acid)	EGCG: epigallocatechin-3-gallate (3.2 mg/ml)	--	[m]GLP-2 [n]LAC/RHA	↓ GLP-2 ↓ LAC/RHA

		DE (DDS + EGCG) + D (0.5% citric acid) C-diet	C: control diet 3 days		[o]SUC/ERY	↓ SUC/ERY
Gil-Cardoso et al 2017 [74]	Female Wistar rats	CAF CAF+GSPE C-group	CAF: cafeteria diet* CAF+GSPE: (cafeteria diet + grape seed proanthocyanidin extract 5- 50 mg/kg) C: control diet 15 weeks CAF 3 weeks CAF+GSPE	--	ZO-1 Occludin Claudin-1 [p]JAM-A	↑ZO-1
Cremonini et al 2018 [49]	C57BL/6J mice	HF *vs* C HFE20 *vs* CE	CE: (-)-epicatechin (2-20 mg/kg) C: control diet	↑ERK1/2 ↑NF-kB (p65) ↑AMPK	[q]p65 GLP-2 [r]NOX1/NOX4	↑ p65 (HF) ↑GLP-2 (CE and HFE20)

			HF: high fat diet (60% total calories from fat); HFE20: high fat diet + 20 mg/kg epicatechin 15 weeks		↑NOX1/NOX4 (HF)
Li et al 2018 [67]	Male Kunming mice	[s]CLP + vehicle CLP+ NG (30) CLP+ NG (60)	NG: naringin (30 mg/kg and 60 mg/kg) C: None control diet 24 - 72 h	--	[t]TEM [u]FITC-dextrane D-lactate	↑survival CLP +NG (30-60) ↑IM Impairment CLP + Vehicle CLP↑ FITC-dextrane and D-lactate

CLP + NG ↓ FITC-dextrane (dose-dependent)

Note: [a)]MLCK/MLC, myosin light-chain kinase; [b)]ZO-1, zonula occludens; [c)]IL, interleukin; [d)]NF-kB, nuclear factor-kB; [e)]AMPK, 5′ AMP-activated protein kinase; [f)]MAPKs, mitogen-activated protein kinases; [g)]SOD, superoxide dismutase; [h)]GSH-Px, glutathione peroxidase; [i)]ERK1/2, extracellular signal–regulated kinases; [l)]DSS, dextran sulphate sodium; [m)]GLP-2, glucagon-like peptide-2; [n)]LAC/RHA, lactulose/rhamnose ratio; [o)]SUC/ERY, sucralose/erythritol ratio; [p)]JAM, junctional adhesion molecule; [q)]p65, transcription factor p65; [r)]NOX1/NOX 4, NADPH oxidases; [s)]CLP, cecal ligation and puncture; [t)]TEM, transmission electron microscopy; [w)]FITC, fluorescein

**Data on polyphenol characterization not provided.*

Table 3- Summary of the Ongoing Human Studies Evaluating the Effect of PPs and PP-rich Food on Intestinal Permeability

Title	**Source**	**Subject number/characteristics Inclusion criteria**	**Study design**	**Intervention**	**Duration of intervention**	**Markers understudy**
Dietary green-tea confection for resolving gut permeability-induced metabolic endotoxemia in obese adults	ClinicalTrials.gov NCT03413735 [75]	40 Overweight/obese (BMI = 28-40 kg/m^2) Fasting glucose < 126 mg/dL Normotensive (blood pressure < 140/90 mmHg) Non-dietary supplement user Non-smoker	Randomized parallel design	Test group: green tea extract (GTE)-rich confection Placebo group: no green tea extract-rich confection Dose: daily (no information about the amount provided in term of polyphenols)	4 weeks	Primary outcome: Endotoxin Secondary outcome: Gut Permeability (Lactulose to Mannitol Ratio, and Sucralose to Erythritol Ratio) Microbiota Firmicutes to Bacteroidetes Ratio)

						Calprotectin Green tea polyphenol bioavailability
Effect of flavonoids on gut permeability in cyclists	ClinicalTrials.gov NCT03427879 [76]	22 Male or female of any race or ethnicity between 18 to 49 years of age Competed in a road race or triathlon in past 12 months Free of chronic disease and gut inflammation conditions	Randomized crossover design	Test group: a high flavonoid, sports nutrition recovery beverage will be prepared from milk (78%), sugar (8.6%), maltodextrin (8.6%), blueberry powder (2.4%), cocoa powder	2 weeks	Primary outcome: Urinary lactulose:mannitol ratio Plasma intestinal fatty acid binding protein Secondary outcome: Fecal calprotectin

Train at least 3 times per week, 1 hour at a time on average Willing to prepare and consume provided pre-workout beverage daily Maintain weight (no more/less than 5 kg change) Willing to avoid consumption of high flavonoid foods/supplements, large dose vitamin and mineral supplements, and [a]NSAIDs or other	(1.6%), green tea extract (0.1%), whey protein isolate (0.6%) containing approximately 620 mg flavonoids per serving. <u>Placebo group:</u> a low flavonoid, sports nutrition recovery beverage will be prepared from milk (78%), sugar (8.6%), maltodextrin	Urinary sucralose:mannitol ratio Inflammatory markers ([b]TNF-α, [c]IL-10) Endotoxin Other variables related exercise performance

medications known to affect inflammation during study period	(8.6%), placebo blueberry powder (2.4%), alkalized cocoa powder (1.6%), whey protein isolate (0.6%), containing approximately 5mg flavonoids per serving Dose: 330 mL/ day

Effect of dietary flavonoids on intestinal microbiota, intestinal inflammation and metabolic syndrome	ClinicalTrials.gov NCT02728570 [77]	30 Overweight/obese ([d]BMI = 25-35 kg/m^2)	Randomized crossover design	Test group: Prepared diet with diet high levels of dietary flavonoids (340 mg of flavonoids/1000 Kcals) with a macronutrient composition of 17% en from protein, 30% en from fat and 53% energy from carbohydrate Control group:	6 weeks	Primary outcome: Fecal calprotectin Serum [e]PCR Serum TNF-α Serum insulin Secondary outcome: Fecal microbiome composition, short chain fatty acids, eosinophil protein X, myeloperoxidase Intestinal permeability by four sugar

Prepared diet with diet high levels of dietary flavonoids (10 mg of flavonoids/1000 Kcals) with a macronutrient composition of 17% en from protein, 30% en from fat and 53% energy from carbohydrate	differential absorption test
	Serum endotoxin, IL-6, soluble $^{\beta}$TNFr-2, fasting glucose
	Calculated Homeostatic Model Assessment-Insulin Resistance
	Serum C-peptide
	Plasma lipid profile
	Blood pressure

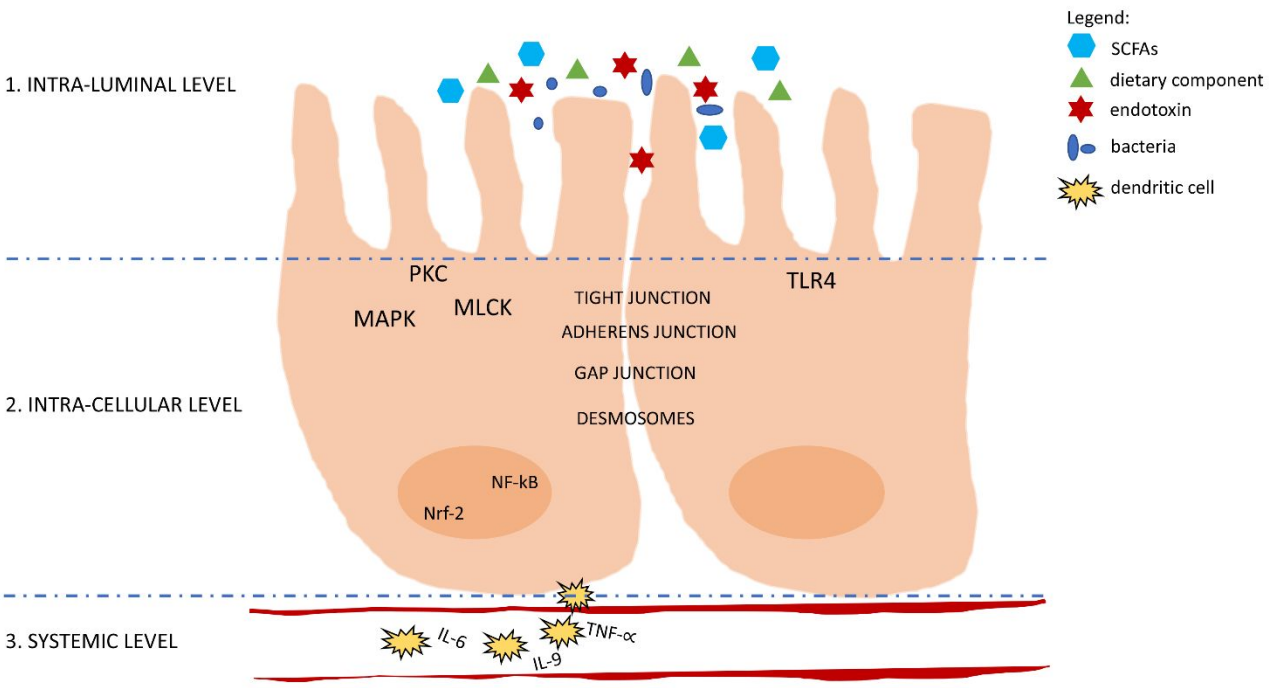
						Other Outcome Measures: Serum resistin, visfatin, adiponectin, leptin Body weight
Effect of a polyphenol-rich diet on leaky gut in the elderly	ISRCTN registry ISRCTN10214981 [78]	60 healthy older subjects Age > 60 years old Intestinal Permeability evaluated by Zonulin serum level	Randomized crossover design	Test group: habitual diet + polyphenol-rich products (berries and derived products, blood oranges and derived products, pomegranate	8 weeks	Primary outcome: Zonulin serum levels Secondary outcome: Total blood bacterial load

Adequate nutritional status evaluated with Mini Nutritional Assessment (MNA) score ≥24	juice, Renetta apple and purée, green tea and dark chocolate products)	Faecal microbiota composition and metabolism
Good cognitive status tested with Mini Mental State Evaluation (MMSE) score ≥24	<u>Control group:</u> comparable diet without the polyphenol-rich products	Short chain fatty acids and polyphenol-derived metabolites
Self-sufficiency assessed with validated tests (e.g. Barthel index - activities of daily living, Tinetti balance assessment)	Dose: three portion of polyphenol-rich food products daily (about 750	Inflammatory, oxidative stress and related markers
		Endotoxin
		[g)]LPS-BP
		Metabolomic markers

		mg of polyphenols)	Metabolic and anthropometric markers

Note: [a)]NSAIDs, nonsteroidal anti-inflammatory drugs; [b)]TNF-α, tumor necrosis factor-alpha; [c)]IL-10, interleukin-10; [d)]BMI, body mass index; [e)]PCR, C-reactive protein; [f)]TNFr-2, tumor necrosis factor receptor-2; [g)]LPS-BP, lipopolysaccharide binding protein

Figure 1


1. INTRA-LUMINAL LEVEL
2. INTRA-CELLULAR LEVEL
3. SYSTEMIC LEVEL
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MAPK
MLCK
TIGHT JUNCTION
ADHERENS JUNCTION
GAP JUNCTION
DESMOSOMES
TLR4
NF-kB
Nrf-2
IL-6
IL-9
TNF-α
Legend:
SCFAs
dietary component
endotoxin
bacteria
dendritic cell

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