Exploring the molecular pathways behind the effects of nutrients and dietary polyphenols on gut microbiota and intestinal permeability in aging by metabolomics: novel approaches for future clinical applications

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Abstract

The gastrointestinal tract hosts the largest microbial population of the human body, which works in symbiosis with the host to provide several important functions and contributes to the maintenance of host health. The diet is one of the factors that can most affect the gut microbiota, with subsequent consequences on host health. One consequence of changes in microbiota is changes in intestinal permeability (IP); disruption of this latter is related to the development of several diseases and is a frequent condition in older people. Nevertheless, the molecular pathways regulating these effects are still unclear, and a comprehensive understanding of the dietary components that can affect IP is lacking. Metabolomics, that has been widely used to study the transformation of nutrients by intestinal microbiota, could be a suitable approach for this purpose. However, up to now, the research has focused mainly on dietary fibers and tryptophan, while the activity of dietary polyphenols remains almost completely unexplored. Hence, the aim here was to review the most recent literature concerning the application of metabolomics in the study of the correlation between diet-induced alterations of gut microbiota and the effects on intestinal permeability, with a particular focus on the discovery of the molecular pathways involved. An additional aim was to give a perspective on the future research involving dietary polyphenols, given that despite their potential implication for the prevention and treatment of several diseases related to increased intestinal permeability, few studies have been reported to date.

Keywords: metabolomics, gut microbiota, intestinal permeability, nutrients, polyphenols

Introduction

The gastrointestinal tract (GI) is responsible for a wide range of functions, including digestion and absorption of nutrients, water and ions, regulation of host immunity, protection against the ingress of pathogenic microorganism, and the the metabolism and detoxification of xenobiotics. The GI also hosts the largest microbial population of the human body, which works in symbiosis with the host to accomplish these various intestinal functions. Gut bacteria are particularly important for host health, being involved in the synthesis of vitamins, secondary bile acids and neurotransmitters, and playing a direct role in the metabolism and degradation of dietary components and drugs, that can affect their bioavailability and absorption [1]. It has been estimated that over than 1,000 different bacterial species populate the intestinal environment, with a genome comprising 100-fold more genes than those found in human genome [2]. The physiological variations in the small intestine and colon, such as the presence of distinct chemical environments, nutrients and host immune activity allow distinct groups of bacterial species to populate the different regions of the lower gastrointestinal tract [3,4], and this variability becomes even more complex considering the inter-individual variations and the influence of host genetics [5-7]. Nevertheless, most human gut microbiota share a core set of resident bacteria and related microbial genes [8,9]. *Firmicutes, Bacteroidetes* and *Actinobacteria* are the three most abundant phyla, among the over 50 that have been identified by metagenomic approaches [10,11]. A synergistic equilibrium among the different species and the maintenance of a microbial diversity are of crucial importance for health, since the microbiota plays a central role on the proper functioning of the intestinal barrier and maintaining appropriate intestinal permeability (IP), which is directly involved in the development of numerous disorders. In this vein, a low diversity and a scarce abundance of species as *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* have been associated

51 with gut disease states, e.g. Crohn's disease [12], type 1, type 2 and gestational diabetes [13-
52 15], celiac disease [16] and obesity [17].
53 Diet, as a source of macro- and micro-nutrients and other bioactive components, is one
54 of the factors that most can affect the microbiota. Among the dietary constituents,
55 polyphenols have been in the spotlight in recent years, due to their particular
56 physicochemical properties and their potential to directly affect microbiota activity and
57 host health. Polyphenols are secondary metabolites of plants, fruits and vegetables, and
58 major components of commonly consumed foods and beverages such as chocolate, tea
59 and coffee [18-20] which, due to their characteristic (poly)hydroxylated phenyl moieties and
60 the presence of ionizable functional groups on their scaffolds, have a low bioavailability
61 and are scarcely absorbed by the intestine [21,22]. Consequently, they are prone to catabolism
62 by the gut microbiota, which leads to the production of smaller molecular weight (MW)
63 compounds that can be absorbed across the intestinal wall, enter the bloodstream and
64 eventually, undergo further transformation and conjugation in the liver [23,24]. It has been
65 estimated that total polyphenol absorption in the small intestine is around 5%–10%, while
66 the remaining 90%–95% transits to the large intestinal lumen and accumulates in the
67 millimolar range [20]. Hence, microbial polyphenol derivatives could be responsible for the
68 biological effects attributed to their parent compounds, or at least contribute to the overall
69 activity. Catechins from green tea, for example, have been reported to exert antioxidant,
70 anti-inflammatory and anti-tumorigenic activities [25-27]. However, the most representative
71 green tea catechin, (−)-epigallocatechin gallate, is scarcely absorbed from the intestine
72 and is extensively metabolized by gut microbiota [28] to form smaller MW derivatives that
73 not only contribute to the observed bioactivities of green tea, but can also exert higher
74 activity than the parent compound [29]. Polyphenols and their microbial metabolites could
75 also exert antimicrobial and bacteriostatic activities, hence regulating the overgrowth of

harmful bacteria on the intestinal and urinary tract epithelia [20,30]. As an example, cranberry (*Vaccinium macrocarpon* Ait.) fruits, rich sources of type-A procyanidins (PAC-A), are known to exert anti-adhesive activity against the uropathogenic bacteria responsible for most of the lower urinary tract infections, although the mechanisms of action are still unknown and the outcomes of in vitro assays and in vivo clinical trials aimed at reducing urinary tract infections are frequently inconsistent [31]. Recent studies show that, after supplementation with dry cranberry extracts, urine samples of both rats and human volunteers exert effective anti-adhesive activity against uropathogenic *E. coli*, despite their negligible contents of intact PAC-A [32,33]. However, the same urine samples were characterized by high amounts of hydroxyphenyl-valeric acid and hydroxyphenyl-valerolactone derivatives, previously reported as end-products of microbial degradation of flavan-3-ols [34], indicating the important contribution of PAC-A microbial metabolites to the observed bioactivity [32,33]. Finally, the effects of polyphenols on microbiota, inflammation and oxidative stress and their capacity to regulate the synthesis and expression of specific proteins on the intestinal epithelium seem to be part of the mechanisms by which these compounds can regulate the permeability of the intestinal barrier [35], whose alterations are related to the development of several diseases, especially in older subjects.

Many efforts have been made to characterize the microbial community colonizing the human intestine, for which the widespread use of metataxonomics based on 16S rRNA gene profiling and metagenomics (microbiomics) has been particularly important. However, although representing powerful tools for bacterial identification and classification, microbiomics does not allow to obtain information about fluctuations in metabolic activities [1]. To this purpose, metabolomics is the most suitable approach, and numerous reports based on metabolomic analysis have been reported over the last decade

[36]. Focusing on the application of metabolomics in the study of diet-microbiota interactions and searching for the keywords "metabolomics AND diet AND microbiota" in PubMed, we found that the number of publications almost doubled from 2014 to 2018, as an index of the popularity that metabolomics gained during the recent years (Fig. 1). Metabolomic approaches have been widely used to study the transformation of nutrients and xenobiotics by intestinal microbiota [37-42], thus allowing the characterization of hundreds of metabolites derived from macro- and micronutrients and polyphenols coming from fruits and vegetables. In 2009, Jacobs published a first review article regarding the role of colonic microbiota in the degradation of non-digestible food ingredients and their impact on gut health and immunity [43]. For the first time, the importance of metabolomics in the study of the links between the bioconversion of non-digestible food ingredients, their bioavailability and their downstream effects on microbiota composition and host metabolism was recognized [43]. More recently, the use of integrated multi-omics approaches has faxcilitated the study of the molecular interactions between diet and microbiota, and has led to the identification of several metabolites that are produced as a result of microbial metabolism of various dietary constituents. Nevertheless, considering the challenges to study the mutual relationship between gut microbiota and the host, its tight connection with diet, environment and lifestyle, and the still incomplete characterization of the huge microbial metabolome, the path to assess precise and validated metabolites to link the microbial activity to specific effects on health is just starting. In a way to find a clinical relevance of metabolomics data and offer to clinicians a robust tool to predict, prevent and treat several diseases, further progress is necessary.

The aim of this work was to review the most recent literature regarding the application of metabolomics in the study of the interactions between food components and gut microbiota and the effects on IP, with a particular focus on the elucidation of the

126 molecular pathways involved. Since to date the research has mainly focused on the
127 degradation of non-digestible fibers and tryptophan and on the bioactivity of their
128 metabolites, a major part of the work will be dedicated to these important dietary
129 components. Additionally, a perspective on the future research involving the role of
130 dietary polyphenols in modulating the activity and composition of gut microbiota and the
131 effects on IP will be discussed, given that, despite their potential implication in the
132 prevention and treatment of several diseases, few clinical studies have been performed up
133 to now.
134
135 **The role of microbiota and microbiota-derived dietary metabolites in regulating**
136 **intestinal permeability**
137 The intestinal wall represents a barrier that selectively transports nutrients, ions and water
138 from the lumen to the bloodstream, via passive and active mechanisms. A layer of
139 epithelial cells constitutes the main physical barrier between the intestinal lumen and the
140 mucosal tissues [44]. Tight junctions (TJ), composed of transmembrane proteins and
141 junctional adhesion molecules that regulate the flow of water, ions and small molecules,
142 seal the paracellular spaces [45]. Several distinct proteins contribute to form the TJ,
143 including mainly occludins and claudins, depending on the tissue and location that
144 interlink within the paracellular space [46]. Although highly cross-linked, the structure of
145 TJ is dynamic, so that it can be 'opened' and 'closed' following specific stimuli [47].
146 Physiological stimuli could shrink the TJ to prevent the diffusion of toxins, viruses or
147 bacterial fragments to the mucosal layer, while they can open the paracellular space to
148 allow the diffusion of nutrients [48]. For instance, the activation of the sodium dependent
149 glucose transporter led to the opening of TJ and allowed the diffusion of small molecules
150 and peptides with MW < 40,000 Da [49]. On the other hand, the physiological structure and

dynamism of TJ could be altered due to pathological states [50], leading to a condition of increased IP, also known as "leaky gut". Celiac disease, inflammatory bowel disease and type I diabetes are three of the principal pathological causes of leaky gut [51], which leads to the permeation of potentially harmful molecules, organisms or microbial fragments from the intestinal lumen to the mucosal layer, inducing a cascade of events that result in immune activation and local or systemic inflammation. Older people are frequently affected by decreased intestinal barrier function and consequently leaky gut [52]. Among the causes, the aging-related decline of immune function (namely immune-senescence), the remodeling of intestinal epithelium and the alterations of gut microbiota composition are thought to be the most important ones [52-54]. As observed in disease-associated increased IP, the dysfunction of the intestinal barrier in older subjects facilitates the diffusion of toxic substances or peptides and microbial fragments to the mucosal layer and to the bloodstream and the triggering of a systemic inflammatory response [55].

As previously stated, diet plays an important role in the maintenance of the gut barrier integrity and is hence determinant for IP. The short-chain fatty acids (SCFAs), produced by the degradation of dietary fibers by several bacteria in the gut (including *Clostridium, Eubacterium*, and *Butyrivibrio*), have been the most studied microbial catabolites involved in the regulation of IP to date. Among them, butyrate has been identified as a marker of the positive effects of non-digestible dietary fiber consumption on microbiota composition and intestinal permeability. It exerts several activities on the intestinal wall, such as controlling inflammation by altering the expression of pro-inflammatory cytokines [56], preserving the intestinal barrier function by inducing the expression of TJ proteins claudin-1 and claudin-2 [57], and modulating composition of gut microbiota by inhibiting the growth of pathogenic bacteria [58]. Food is the only source of non-digestible carbohydrates, and alterations in diet lead to variations in the production of intestinal

butyrate. In aged mice, the increased butyrate production after the consumption of high doses of soluble fiber was associated with an induced expression of the TJ proteins Tjp2 and Ffar2 and to a counterbalance of the age-related microbiota dysbiosis, with a significant amelioration of the increased IP condition typical of older individuals [59]. Similar effects of a high fiber diet were also observed in mice affected by autoimmune hepatitis, characterized by an imbalance of Treg/Th17 cells and increased IP [60]. After dietary intervention, the levels of butyrate were increased in feces, and the expression of TJ proteins ZO-1, occludin and claudin-1 was induced in the ileum, with consequent increased intestinal barrier function and decreased translocation of bacterial components through the intestinal wall [60]. The same effects were also observed in mice treated with sodium butyrate, indicating a direct involvement of this bacterial metabolite in the regulation of IP [60].

Microbial tryptophan metabolites also play an important role in regulating barrier functions and gut microbiota activity. A metabolomic approach allowed to obtain preliminary elucidations about the role of tryptophan and its microbial and endogenous derivatives in the regulation of immune tolerance toward intestinal microbiota [61]. Starting from these findings, further research has elucidated the role of other microbial-derived tryptophan metabolites in the regulation of gut permeability, by direct effects on epithelial cells. Venkatesh et al. showed that indole-3-propionic acid (IPA), produced by the firmicute *Clostridium sporogenes*, regulates mucosal integrity and intestinal barrier function by activating the pregnane X receptor (PXR) and upregulating junctional protein-coding mRNAs [62]. More recently, Dodd et al. Used an integrated targeted-untargeted approach to identify 12 microbial metabolites derived from the reductive activity of *C. sporogenes* on aromatic amino acids (phenylalanine, tyrosyne and tryptophan), of which nine (lactate, acrylate and propionate derivatives) were reported to

accumulate in host plasma [63]. The authors particularly focused on IPA and its effects on gut barrier and the mucosal immune system, and their results supported the findings of Venkatesh and coll. about the PXR-mediated effect on gut permeability [62,63]. A treatment with 20 mg kg^{-1} IPA for four consecutive days was shown to significantly decrease the IP in HFD-fed obese T2D mice [64], which, prior to treatment, were characterized by higher IP and lower circulating IPA levels compared to lean animals. Plasma IPA amounts were also reported to increase in the same obese model 3 months after Roux-en-Y gastric bypass (RYGB) surgery [64], indicating, once again, the direct involvement of gut microbiota in the maintenance of the intestinal barrier functions. Furthermore, results from *in vitro* assays reported by the same authors showed that IPA could reduce the permeability of T84 cell monolayer compromised by pro-inflammatory cytokines [64]. Other metabolites derived from the same degradation pathway of tryptophan, i.e. indole (produced by *Escherichia coli, Clostridium bifermentans, Proteus vulgaris, Paracolobactrum coliforme, Achromobacter liquefaciens*, and *Bacteroides* spp.) [65], indole-3-acetic acid (produced by *C. sporogenes*) and tryptamine (produced by *C. sporogenes* and *Ruminococcus gnavus*) [66], were also reported to exert anti-inflammatory activity both in the intestinal lumen and in the liver [66,67], and to up-regulate the expression of several proteins involved in the trans-epithelial cells linkage on the intestinal wall, such as tight junction proteins TJP1, TJP3, and TJP4, and gap junction proteins GJE1, GJB3, GJB4, and GJA8, among others [65].

In recent years, polyphenols have been widely considered for their beneficial effects on health and polyphenol-rich diets have been evaluated for the prevention of several chronic diseases, ranging from metabolic disorders to inflammation and cancer. Some studies have also evaluated the consumption of polyphenol-rich food for the prevention of diseases associated to aging, such as cognitive impairment [68] and depression [69], although

226 up to now the reported effects have been inconsistent. However, numerous in vitro and
227 animal studies show that the consumption of polyphenol-rich food could positively affect
228 IP, reinforcing the barrier properties of the intestinal epithelium by direct influence on the
229 synthesis and expression of tight junction proteins [70,71] or by interaction with gut
230 microbiota. As previously described, this latter is directly involved in the metabolic
231 transformation of plant polyphenols and in the production of smaller MW derivatives [72],
232 which in turn contributes to the maintenance of barrier function and drives changes in gut
233 microbiome constituents [73,74], with important effects for host health. However, although
234 several molecular targets of dietary polyphenols and their metabolites on the intestinal
235 epithelium have been elucidated [75], it is unclear how the interaction of the same
236 compounds with gut microbiota leads to beneficial effects on the intestinal barrier, and
237 further efforts are required to fill this gap. Mice fed a high-fat diet supplemented with 4%
238 w/w powdered green tea leaves rich in flavanols showed an increased intestinal
239 population of *Akkermansia* spp. after 22 weeks [76], a bacterium that has been implied in
240 the maintenance of a functional intestinal barrier through the preservation of mucus layer
241 thickness [77]. More recently, Li et al. reported that the consumption of a medium-dose (20
242 mg/kg per day) of bilberry anthocyanin extract (BAE) promoted the generation of SCFAs
243 (acetic acid, propionic acid and butyric acid) in aging rats, through the regulation of the
244 intestinal microbiota [78]. Specifically, several starch-utilizing and butyrate-producing
245 bacteria (among whom *Lactobacillus* and *Bacteroides*) were induced by BAE, while
246 harmful species such as *Verrucomicrobia* and *Euryarchaeota* where inhibited. These
247 variations, associated with decreased levels of TNF-α and IL-6 in the colon induced by
248 BAE consumption, contributed to the restoring of the intestinal barrier function typically
249 altered in older individual [78]. Overall, these results indicate that the effects of polyphenols
250 on IP are related to both direct effects on the expression of TJ proteins and to changes

induced to the intestinal microbiota, with an increase in the prevalence of species that can preserve barrier functions through the production of active metabolites or by direct action on the mucous layer. Nevertheless, the data supporting these observations are still scarce, and up to now only few compounds (e.g. butyrate) correlating the diet-induced modifications of gut microbiota to the effects on the intestinal integrity and permeability have been discovered.

Conclusion and future perspective

It is well known that a healthy microbiota is associated with good host health, and diet plays a crucial role in regulating this equilibrium. Although the study of the effects of dietary interventions on gut microbiota and IP and investigations of the mechanisms of action have begun only recently, it appears clear that appropriate dietary habits and the regular consumption of vegetables and fruits rich in fibers and polyphenols play an important role in the maintenance of proper intestinal functions. The precursors of SCFAs and of several indole or phenolic derivatives produced by bacterial catabolism in the intestinal lumen, for example, are abundant constituents of both plant-derived foods, as cereals, nuts, fruits and vegetables rich in non-digestible fibers [79], and animal-based foods such as dairy products, eggs and meat, which are rich sources of tryptophan [80]. Thanks to the employment of integrated multi-omics approaches, the involvement of several partners (food components, microbiota and microbial-derived compounds) in the maintenance of the intestinal barrier function and the molecular pathways behind this activity are being gradually elucidated, although further efforts are required to link specific food components and their metabolites to specific mechanisms of action.

In conclusion, the studies reviewed in this work could be considered as a starting point for further research, with the final goal being identification of precise biomarkers. These

biomarkers, once validated for clinical relevance, will be novel instruments available to clinicians for the development of dietary plans aimed at managing and preventing diseases directly linked to increased IP, as chronic inflammation and immunological disorders, which are determinant for the gradual decline of health in older subjects.

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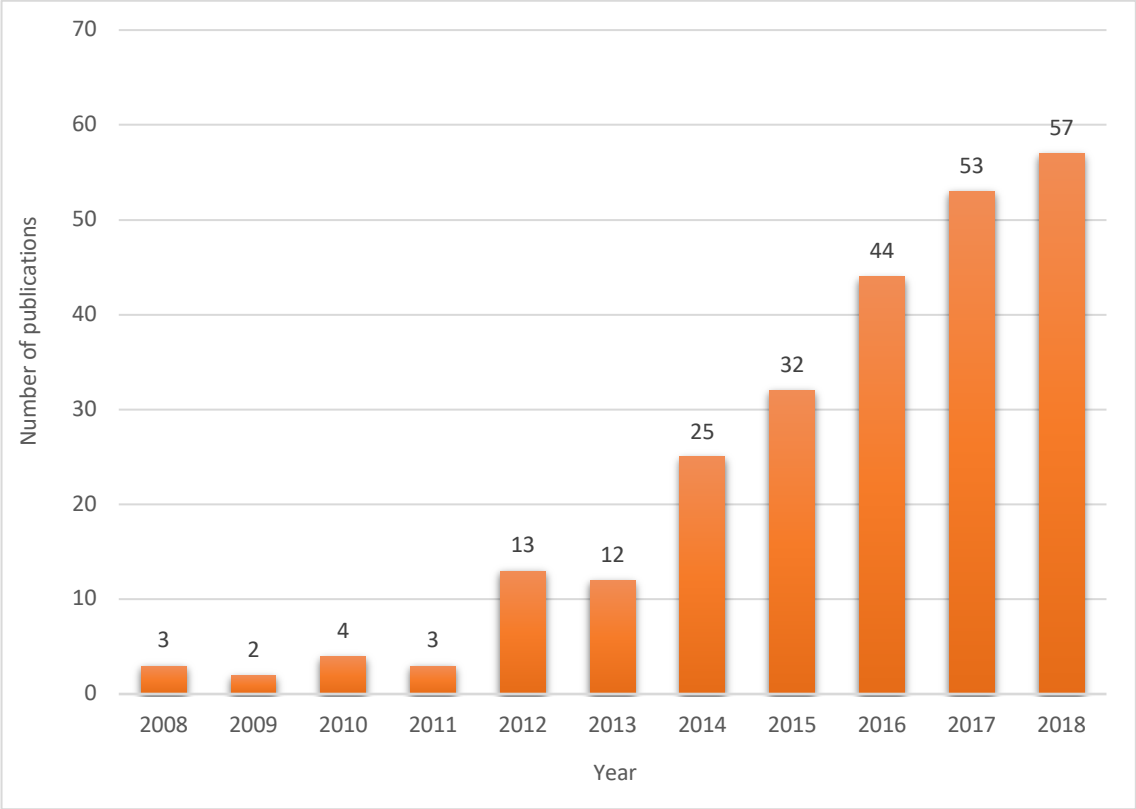

Figure 1. The increase of the scientific literature regarding the use of metabolomics in the study of the interactions between diet and gut microbiota during the last 11 years. Source: PubMed (https://www.ncbi.nlm.nih.gov/pubmed/).

GRAPHICAL ABSTRACT (TOC)