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# The deleterious effect of p-cresol on human colonic epithelial cells is prevented by proanthocyanidin-containing polyphenol extracts from fruits and proanthocyanidin bacterial meta...

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# Deleterious Effect of *p*-Cresol on Human Colonic Epithelial Cells Prevented by Proanthocyanidin-Containing Polyphenol Extracts from Fruits and Proanthocyanidin Bacterial Metabolites

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**ABSTRACT:** The protective effect of proanthocyanidin-containing polyphenol extracts from apples, avocados, cranberries, grapes, or proanthocyanidin microbial metabolites was evaluated in colonic epithelial cells exposed to *p*-cresol, a deleterious compound produced by the colonic microbiota from L-tyrosine. In HT29  $\text{Glc}^{\pm}$  cells, *p*-cresol significantly increased LDH leakage and decreased ATP contents, whereas in Caco-2 cell monolayers, it significantly decreased the transepithelial electrical resistance and increased the paracellular transport of FITC-dextran. The alterations induced by *p*-cresol in HT29  $\text{Glc}^{\pm}$  cells were prevented by the extracts from cranberries and avocados, whereas they became worse by extracts from apples and grapes. The proanthocyanidin bacterial metabolites decreased LDH leakage, ameliorating cell viability without improving intracellular ATP. All of the polyphenol extracts and proanthocyanidin bacterial metabolites prevented the *p*-cresol-induced alterations of barrier function. These results suggest that proanthocyanidin-containing polyphenol extracts and proanthocyanidin metabolites likely contribute to the protection of the colonic mucosa against the deleterious effects of *p*-cresol.

**KEYWORDS:** *p*-cresol, mitochondria, proanthocyanidins, colonic cells, microbiota, avocado, apple, cranberry, grapes, gut barrier function

## INTRODUCTION

Polyphenols are secondary metabolites including phenolic acids and flavonoid compounds (flavanols, flavonols, and anthocyanins) that are implicated in the protection of plants against predators, infection, and diverse types of stress. Because of their chemical structure, polyphenols exhibit a great number of health-promoting properties, such as antioxidant, antibacterial, anti-inflammatory, antihypertensive, antiproliferative, regulatory of mitochondrial function, and so forth.<sup>1</sup> For this reason, polyphenols are considered as beneficial dietary non-nutrients whose intake, in the form of fruits and vegetables, is recommended to decrease the risk of chronic noncommunicable diseases in the population. Among dietary polyphenols, proanthocyanidins (PACs), also known as condensed tannins, are oligomeric forms of flavan-3-ols abundant in plant-derived foodstuffs such as red wine, green tea, grape seeds and skin, cocoa, avocados, cranberries, peanut cuticles, cinnamon, and apples.<sup>2,3</sup> In the U.S. population, they are the second most abundant phenolic compounds in the diet after lignin with an estimated daily PAC intake of approximately 224 mg.<sup>2,3</sup> Dietary PACs with a degree of polymerization higher than 3 are in general poorly absorbed in the intestine, thus reaching the colon where they are metabolized by the colonic microbiota.<sup>4–7</sup> Studies in humans and animals consuming cocoa, red wine, apples, or green tea have shown that the PACs present in these foodstuffs stimulate the growth of bacterial populations from

the autochthonous microbiota in the colon, including *C. histolyticum*, *E. rectale Lactobacillus*, and *Bifidobacterium* spp.<sup>8–13</sup> It is therefore possible that this prebiotic-like effect explains some of their health-promoting properties. The bacterial metabolism of dietary PACs generates aromatic acids such as 3-hydroxyphenyl acetic acid (3-HPAA), 4-hydroxyphenyl valeric acid (4-HPVA), 4-hydroxyphenyl acetic acid (4-HPAA), 3,4-dihydroxyphenyl propionic acid (3,4-DHPPA), 3-(3-hydroxyphenyl) propionic acid (3-HPPA), and 3-phenylpropionic acid (3-PPA) that may be detected in high concentrations in the stool or fecal water of healthy subjects.<sup>6,8,13,14</sup> These compounds may be absorbed in the colon, and eventually, they may be detected in the bloodstream at micromolar concentrations (2–50  $\mu\text{M}$ ) and exert systemic activities. For example, 3,3-DHPPA has been shown to act as a potent vasodilator in rats, whereas 3,4-DHPPA exhibits cytotoxic and anti-inflammatory properties.<sup>15–17</sup>

Constrastingly, the colonic microbiota also expresses proteases and peptidases capable of partially degrading dietary and endogenous proteins that remain in the small intestine and reach the colon. The phenylalanine, tyrosine, and tryptophan

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70 released through this process are subsequently fermented,  
71 resulting in the formation of phenolic metabolites including *p*-  
72 cresol (4-methylphenol).<sup>18,19</sup> This compound is specifically  
73 produced from L-tyrosine by anaerobic bacteria, a process that  
74 occurs preferentially in the distal colon where protein  
75 breakdown is more intense, and then, the concentrations of  
76 *p*-cresol are more elevated.<sup>20</sup> *p*-Cresol is absorbed by the  
77 epithelial colonic cells, transferred into the bloodstream,  
78 metabolized in the liver, and excreted in the urine where it  
79 represents more than 90% of the phenolic compounds  
80 recovered.<sup>21</sup> Interestingly, higher urinary concentrations of *p*-  
81 cresol have been described in patients with colorectal cancer.<sup>22</sup>  
82 In this respect, we recently showed that *p*-cresol negatively  
83 affects human colonic epithelial cells (HT-29  $\text{Gle}^{\pm}$ ) **in vitro**,  
84 inhibiting their proliferation and respiration, decreasing their  
85 ATP content, and increasing DNA damage.<sup>23</sup> Additionally, *p*-  
86 cresol is also recognized as a uremic toxin, and in patients with  
87 chronic kidney disease, its accumulation or that of its conjugate,  
88 *p*-cresyl-sulfate, contributes to the development of renal and  
89 cardiovascular complications.<sup>24–26</sup> It is noteworthy that the  
90 intracolonic concentrations of *p*-cresol, and eventually its  
91 subsequent deleterious effects in the epithelium, increased in  
92 individuals consuming high protein diets,<sup>27</sup> whereas they  
93 decreased in those consuming diets rich in resistant starch or  
94 pre- and probiotics.<sup>28,29</sup> Interestingly, a decrease of *p*-cresol has  
95 also been reported in the colon of rats fed a diet supplemented  
96 with epigallocatechin gallate (EGCG), the main phenolic  
97 compound present in green tea.<sup>30</sup>

98 In this context, the aim of the present study was to determine  
99 whether polyphenol extracts containing PACs from apples,  
100 cranberries, grapes, avocados, or PAC microbial metabolites  
101 may protect colonic epithelial cells from the deleterious effects  
102 of *p*-cresol in human colonic cell lines in terms of cell viability,  
103 mitochondrial function, and epithelial integrity.

## 104 ■ MATERIALS AND METHODS

105 **Chemicals.** CytoTox-ONE homogeneous membrane integrity  
106 assay kits and CellTiter-Glo luminescent cell viability assay kits were  
107 from Promega (Madison, USA). Flavonol glycosides quercetin 3-*O*-  
108 galactoside (hyperoside), quercetin 3-*O*-glucoside (isoquercitrin), and  
109 quercetin 3-*O*-rhamnoside (quercitrin) were from Roth (Karlsruhe,  
110 Germany). 5-Caffeoyl quinic acid and 4-caffeoyl quinic acid were  
111 obtained from Phytolab (Vestenbergsgreuth, Germany). All solvents  
112 (HPLC-grade acetonitrile, methanol, and TFA) were purchased from  
113 Merck (Darmstadt, Germany). *p*-Cresol, 4-hydroxyphenylacetic acid  
114 (4-HPAA), 3,4-dihydroxyphenyl propionic acid (3,4-DHPPA), 3-  
115 phenylpropionic acid (3-PPA), fluorescein isothiocyanate-dextran  
116 (FD-4), rhodamine 123 quercetin, rutin, apigenin, kaempferol,  
117 myricetin, isorhamnetin, epicatechin, catechin, epicatechin gallate,  
118 catechin gallate, phloretin-2'-xyloglucoside, phloretin-2'-glucoside  
119 (phloridzin), cyanidin-3-*O*-glucoside, cyanidin-*O*-arabinoside, peoni-  
120 din-*O*-arabinoside, gallic acid, syringic acid, vanillic acid, chlorogenic  
121 acid, caffeic acid, ellagic acid, sinapic acid, ferulic acid, anisic acid, *p*-  
122 coumaric acid, cinnamic acid, protocatechuic acid, *p*-hydroxybenzoic  
123 acid, *m*-hydroxybenzoic acid, caffeoyl glucoside, feruloyl glucoside,  
124 coumaroyl glucoside, and the Folin–Ciocalteu reagent were purchased  
125 from Sigma-Aldrich (St. Louis, MO, USA).

126 **Preparation of the Polyphenol Extracts from Fruits.** The  
127 polyphenol extracts from cranberries, grapes, and apples, and avocados  
128 were prepared from concentrated cranberry juice (Agricola Cran Chile  
129 Ltd., Lanco, Chile), grape skin and seeds (Cabernet Sauvignon variety,  
130 De Neira Vinyard, Guarilhue, Chile), apple peels (Granny Smith  
131 variety, Surfrut Ltd., Chile), and avocado peels (Hass variety,  
132 purchased at a local market), respectively.<sup>31</sup> First, 500 g of frozen  
133 samples ( $-20\text{ }^{\circ}\text{C}$ ) of grape, apple, and avocado peels were mixed with  
134 hot water (5 min,  $90\text{ }^{\circ}\text{C}$ ) and filtered on a sintered glass funnel (70–

100  $\mu\text{m}$ ). Marcs were homogenized for one min with an Ultraturrax 135  
and extracted again with water at  $65\text{ }^{\circ}\text{C}$ . After 60 min of maceration, 136  
the pH of the pooled extracts was adjusted to 2.5 with formic acid, and 137  
these were poured on a glass column (150 mm i.d.  $\times$  300 mm) packed 138  
with Sepabeads SP-850 (Supelco, Bellefonte, USA) preconditioned 139  
with acidic water. Sugars and other water-soluble compounds were 140  
removed by washing with 5 L of distilled water, and the polyphenol 141  
extracts were obtained after elution with absolute ethanol.<sup>26</sup> Cranberry 142  
extract was obtained from concentrated juice (500 mL) by 5-fold 143  
dilution with acidic water (pH 2.5) and adsorption on an Amberlite 144  
XAD7HP column. The ethanolic fractions were evaporated under 145  
vacuum ( $<40\text{ }^{\circ}\text{C}$ ) followed by freeze-drying of the aqueous remnants. 146  
Dried polyphenol-rich extracts were stored at  $-70\text{ }^{\circ}\text{C}$  until use. 147

**High-Performance Liquid Chromatography.** The identification 148  
and quantification of the polyphenols present in the four extracts were 149  
carried out as previously described by Pastene et al.<sup>31,32</sup> Polyphenols 150  
were analyzed by RP-HPLC using a Waters Alliance 2695 system 151  
equipped with a C18 column (Kromasil 250  $\times$  4.6 mm, 5  $\mu\text{m}$ ) and a 152  
C18 precolumn (Nova-Pak Waters, 22  $\times$  3.9 mm, 4  $\mu\text{m}$ ) (Milford, 153  
MA) at  $30\text{ }^{\circ}\text{C}$ . An elution gradient was performed by varying the 154  
mobile phase from 0 to 25 min, 10–30% B; 25–30 min, 30–75% B; 155  
30–35 min, 75–10% B at a rate flow of 0.8 mL/min with a final 156  
stabilization period of 10 min. Known compounds were identified by 157  
matching their retention times ( $t_{\text{R}}$ ) and online UV spectra with those 158  
of reference compounds. Quantification was performed by using a 159  
UV–vis chromatogram acquired at 350 nm for flavonols, 320 nm for 160  
phenolic acids, 280 nm for flavan-3-ols, and 520 nm for anthocyanins. 161  
When available, standard compounds were used to construct the 162  
respective external calibration curves. Rutin, quercetin, myricetin, 163  
isorhamnetin, kaempferol, cyanidin, malvidin, delphinidin, peonidin, 164  
and pelargonidin were used when authentic standards were not 165  
available. All results were expressed as grams per 100 g of polyphenols. 166  
Total polyphenolic contents (TPC) were determined with Folin– 167  
Ciocalteu reagent and expressed as mg of gallic acid equivalents 168  
(GAE) per gram of dried extract. 169

**Determination of the Mean Degree of Polymerization of** 170  
**Proanthocyanidins.** Phloroglucinolysis was used for estimating 171  
proanthocyanidin contents and their mean degree of polymerization.<sup>32</sup> 172  
Briefly, 10 mg of the extracts were dissolved in 2 mL of a solution of 173  
0.1 M HCl in methanol containing 50 mg/mL of phloroglucinol and 174  
10 mg/mL of ascorbic acid. The reaction mixture was incubated at 50 175  
 $^{\circ}\text{C}$  for 20 min, and 10 mL of 40 mM aqueous sodium acetate was 176  
added to stop the reaction. The formed phloroglucinol adducts were 177  
analyzed by the above-described method of RP-HPLC. Quantitative 178  
determination of (–)-epicatechin, (+)-catechin, and phloroglucinol 179  
adduct products was performed using external standards. Phloroglu- 180  
cinol-adducts were prepared from *Peumus boldus* and apple peel 181  
extracts as a source of catechin and epicatechin polymeric proanthocyanidins 182  
according to Köhler and Winterhalter as well as Pastene et al.<sup>33,34</sup> The 183  
proanthocyanidin contents were calculated by summing the masses of all 184  
subunits (excluding the phloroglucinol portion of the phloroglucinol 185  
adducts). The mean degree of polymerization was obtained summing 186  
the extension subunits and then dividing it by the sum of terminal 187  
subunits. Native (–)-epicatechin and (+)-catechin in the samples were 188  
quantified in extracts not treated with the nucleophile, and the 189  
corresponding values were subtracted for calculations. 190

**Cells and Cell Culture Conditions.** The human colonic 191  
adenocarcinoma cell line HT29  $\text{Gle}^{\pm}$  (passages 46–56) was used in 192  
the studies of cell viability and mitochondrial function, and the human 193  
colonic adenocarcinoma cell line Caco-2 (passages 6–20) (ATCC, 194  
USA) was used in the studies of barrier function. Both cell lines were 195  
cultured in Dulbecco's modified Eagle's medium (DMEM) 196  
supplemented with 4.5 g/L of glucose and L-glutamine (Corning), 197  
10% heat inactivated fetal calf serum (Gibco, USA), and 1% penicillin/ 198  
streptomycin (Hyclone). Caco-2 cells were also supplemented with a 199  
1% nonessential amino acids solution (Gibco). Cell cultures were 200  
grown at  $37\text{ }^{\circ}\text{C}$  in a 5%  $\text{CO}_2$  humidified atmosphere. On day 0, HT29 201  
 $\text{Gle}^{\pm}$  cells were seeded at density of  $2 \times 10^4$  cells/ $\text{cm}^2$  in 96-well plates. 202  
The culture medium was changed on day 3, and each test was started 203  
on day 4. Cells were coincubated with 0.8 mM *p*-cresol (freshly 204

Table 1. Phenolic Composition of the Fruit Extracts

compound (%)	apple peel <sup>a</sup>	cranberry <sup>a</sup>	grape <sup>a</sup>	avocado peel <sup>a</sup>
Flavonoids				
hyperoside	22.12 ± 1.23	2.96 ± 0.06	ND	ND
isoquercitrin	6.58 ± 0.98	3.88 ± 0.13	ND	ND
quercitrin	8.23 ± 1.02	9.07 ± 0.11	ND	ND
quercetin	0.09 ± 0.01	4.02 ± 0.18	1.77 ± 0.07	1.23 ± 0.02
quercetin- <i>O</i> -pentosides (≠ of rutin) <sup>b</sup>	19.21 ± 2.66	1.03 ± 0.09	4.79 ± 0.05	ND
rutin	4.01 ± 0.12	ND	0.96 ± 0.01	1.09 ± 0.04
apigenin	ND	ND	ND	12.80 ± 0.12
kaempferol	ND	ND	1.81 ± 0.09	ND
kaempferol derivatives <sup>c</sup>	ND	1.43 ± 0.01	2.03 ± 0.05	ND
myricetin	ND	1.78 ± 0.03	ND	ND
myricetin hexosides <sup>d</sup>	ND	5.19 ± 0.09	ND	ND
isorhamnetin	ND	0.68 ± 0.36	ND	ND
isoramnetin derivatives <sup>e</sup>	ND	ND	3.75 ± 0.05	ND
∑ flavonoids	60.24 ± 1.00	30.04 ± 0.12	15.11 ± 0.05	15.12 ± 0.06
Flavan-3-ol Monomers				
epicatechin	6.08 ± 0.98	1.02 ± 0.01	3.66 ± 0.20	12.09 ± 0.38
catechin	0.03 ± 0.01	1.44 ± 0.03	2.04 ± 0.13	1.92 ± 0.01
epicatechin gallate	ND	ND	1.74 ± 0.09	ND
catechin gallate	ND	ND	0.89 ± 0.02	ND
∑ flavan-3-ol monomers	6.11 ± 0.50	2.46 ± 0.02	8.33 ± 0.11	14.01 ± 0.20
∑ total procyanidins <sup>f</sup>	22.01 ± 2.88	32.01 ± 0.36	41.01 ± 0.36	29.08 ± 1.01
Dihydrochalcones				
phloretin-2'-glucoside	10.34 ± 3.88	0.18 ± 0.01	ND	ND
phloretin-2'-xyloglucoside	0.22 ± 0.11	ND	ND	ND
∑ dihydrochalcones	10.56 ± 2.00	0.18 ± 0.01	ND	ND
Anthocyanins				
cyanidin- <i>O</i> -hexosides <sup>g</sup>	ND	5.23 ± 0.03	1.23 ± 0.02	ND
cyanidin- <i>O</i> -arabinoside <sup>g</sup>	ND	5.01 ± 0.03	ND	ND
cyanidin-3- <i>O</i> -glucoside <sup>g</sup>	ND	ND	ND	3.00 ± 0.03
peonidin- <i>O</i> -hexosides <sup>h</sup>	ND	8.46 ± 0.01	1.01 ± 0.01	ND
peonidin- <i>O</i> -arabinoside <sup>h</sup>	ND	4.97 ± 0.06	ND	ND
malvidin- <i>O</i> -hexosides <sup>i</sup>	ND	ND	12.05 ± 0.01	ND
delphinidin- <i>O</i> -hexosides <sup>j</sup>	ND	ND	4.53 ± 0.08	ND
petunidin- <i>O</i> -hexosides <sup>k</sup>	ND	ND	3.01 ± 0.13	ND
∑ total anthocyanins	ND	23.67 ± 0.03	20.83 ± 0.03	3.00 ± 0.03
Phenolic Acids				
gallic acid	ND	ND	4.11 ± 0.01	ND
syringic acid	ND	ND	0.63 ± 0.03	ND
vanillic acid	ND	ND	3.94 ± 0.13	3.16 ± 0.01
chlorogenic acid	1.02 ± 0.05	ND	ND	1.00 ± 0.02
caffeic acid	0.06 ± 0.01	0.07 ± 0.01	1.96 ± 0.01	8.01 ± 0.01
ellagic acid	ND	1.94 ± 0.11	ND	ND
sinapic acid	ND	0.28 ± 0.09	ND	0.25 ± 0.02
ferulic acid	ND	0.09 ± 0.01	0.72 ± 0.06	6.54 ± 0.02
anisic acid	ND	ND	ND	2.28 ± 0.02
<i>p</i> -coumaric acid	ND	1.52 ± 0.18	1.16 ± 0.03	ND
cinnamic acid	ND	0.38 ± 0.02	ND	ND
5-caffeoyl quinic acid	ND	3.83 ± 0.12	ND	ND
4-caffeoyl quinic acid	ND	0.23 ± 0.01	ND	ND
protocatechuic acid	ND	0.78 ± 0.03	2.20 ± 0.02	0.98 ± 0.09
<i>p</i> -hydroxybenzoic acid	ND	0.98 ± 0.02	ND	9.92 ± 0.08
<i>m</i> -hydroxybenzoic acid	ND	ND	ND	6.65 ± 0.10
caffeoyl glucoside	ND	0.89 ± 0.01	ND	ND
feruloyl glucoside	ND	0.19 ± 0.01	ND	ND
coumaroyl glucoside	ND	0.46 ± 0.02	ND	ND
∑ phenolic acids	1.08 ± 0.03	11.64 ± 0.05	14.72 ± 0.04	38.79 ± 0.04
total	100 ± 1.28	100 ± 0.10	100 ± 0.12	100 ± 0.27

<sup>a</sup>Data are average (±SD) of triplicates determined by the HPLC method and expressed as the percentage of the total polyphenol content measured by the Folin–Ciocalteu method. <sup>b</sup>Calculated on the basis of rutin measured by RP-HPLC. <sup>c</sup>Calculated on the basis of kaempferol measured by RP-HPLC. <sup>d</sup>Calculated on the basis of myricetin measured by RP-HPLC. <sup>e</sup>Calculated on the basis of isorhamnetin measured by RP-HPLC. <sup>f</sup>Estimated

Table 1. continued

by RP-HPLC method after phloroglucinolysis. <sup>g</sup>Calculated on the basis of cyanidin measured by RP-HPLC. <sup>h</sup>Calculated on the basis of peonidin measured by RP-HPLC. <sup>i</sup>Calculated on the basis of malvidin measured by RP-HPLC. <sup>j</sup>Calculated on the basis of delphinidin measured by RP-HPLC. <sup>k</sup>Calculated on the basis of petunidin measured by RP-HPLC. ND = not detected.

Table 2. Effect of PAC-Containing Polyphenol Extracts and PAC Metabolites on Cell Integrity (LDH Leakage) and Mitochondrial Function (Cellular ATP Levels) in HT29 Glc<sup>+</sup> Cells Cultured without or with *p*-Cresol<sup>a</sup>

	concentration	LDH leakage (% of control without <i>p</i> -cresol)		ATP level (% of control without <i>p</i> -cresol)	
		without <i>p</i> -cresol	0.8 mM <i>p</i> -cresol	without <i>p</i> -cresol	0.8 mM <i>p</i> -cresol
control cells		100	128 ± 21 <sup>c</sup>	100	87 ± 13 <sup>c</sup>
cranberry (μg/mL)	12.5	94 ± 4 <sup>b</sup>	103 ± 6 <sup>d</sup>	135 ± 19 <sup>c</sup>	125 ± 13 <sup>c,e</sup>
	25	92 ± 6 <sup>c</sup>	96 ± 6 <sup>e</sup>	135 ± 14 <sup>c</sup>	127 ± 14 <sup>e</sup>
	50	91 ± 4 <sup>c</sup>	97 ± 5 <sup>e</sup>	133 ± 19 <sup>c</sup>	122 ± 11 <sup>b,e</sup>
avocado (μg/mL)	12.5	93 ± 4 <sup>c</sup>	100 ± 5 <sup>e</sup>	144 ± 15 <sup>c</sup>	125 ± 13 <sup>c,e</sup>
	25	91 ± 6 <sup>c</sup>	99 ± 5 <sup>e</sup>	144 ± 17 <sup>c</sup>	127 ± 14 <sup>c,e</sup>
	50	88 ± 4 <sup>c</sup>	96 ± 5	132 ± 14 <sup>c</sup>	122 ± 11 <sup>c,e</sup>
grape (μg/mL)	12.5	101 ± 5	174 ± 10 <sup>b,e</sup>	98 ± 7	84 ± 3 <sup>c</sup>
	25	107 ± 11	185 ± 13 <sup>c,e</sup>	103 ± 8	81 ± 5 <sup>c</sup>
	50	117 ± 8 <sup>c</sup>	225 ± 18 <sup>c,e</sup>	102 ± 7	53 ± 9 <sup>c,e</sup>
apple (μg/mL)	12.5	101 ± 10	170 ± 14 <sup>c</sup>	100 ± 10	82 ± 3 <sup>c</sup>
	25	105 ± 8	175 ± 12 <sup>c</sup>	103 ± 6	84 ± 2 <sup>c</sup>
	50	105 ± 7	176 ± 22 <sup>b</sup>	101 ± 10	82 ± 4 <sup>c</sup>
3-PPA (μM)	50	108 ± 3 <sup>c</sup>	105 ± 4	79 ± 9 <sup>c</sup>	81 ± 3 <sup>c</sup>
	200	107 ± 3 <sup>c</sup>	105 ± 9	95 ± 12	92 ± 9
3,4-DHPPA (μM)	30	112 ± 4 <sup>c</sup>	104 ± 6	81 ± 7 <sup>c</sup>	92 ± 6
	100	108 ± 3 <sup>c</sup>	108 ± 3	79 ± 5 <sup>c</sup>	92 ± 12
4-HPPA (μM)	10	107 ± 4 <sup>b</sup>	107 ± 3	77 ± 13 <sup>c</sup>	95 ± 8 <sup>b</sup>
	50	107 ± 4 <sup>b</sup>	108 ± 6	81 ± 7 <sup>c</sup>	89 ± 7 <sup>b</sup>

<sup>a</sup>Values represent the mean ± SD of 3 independent experiments. <sup>b</sup>*p* < 0.05 compared with untreated control cells. <sup>c</sup>*p* < 0.01 compared with untreated control cells. <sup>d</sup>*p* < 0.05 compared with cells treated only with 0.8 mM *p*-cresol. <sup>e</sup>*p* < 0.01 compared with cells treated only with 0.8 mM *p*-cresol.

205 dissolved in culture medium) and the PAC-containing polyphenol  
206 extracts (12.5, 25, and 50 μg/mL, i.e., in concentrations achievable in  
207 the gut lumen after the intake of a standard diet) or the PAC bacterial  
208 metabolites 3-PPA (50 and 200 μM), 3,4-DHPPA (30 and 100 μM),  
209 4-HPPA (10 and 50 μM), i.e., in concentrations similar to those  
210 reported by Jenner et al.<sup>8</sup> in the fecal water of human subjects. The  
211 culture medium was changed daily from days 4 to 7. Caco-2 cells were  
212 cultured at a density of 10<sup>5</sup> cells/cm<sup>2</sup> on Transwell polycarbonate  
213 filters (12 mm diameter; 0.4 μm pore size) (Corning-Costar, USA)  
214 previously treated with 0.044 μg/μL of collagen (Thermo) in 0.01 M  
215 HCL. The basolateral and apical compartments of the transwell filters  
216 were filled with 1.5 and 0.5 mL of culture medium, respectively. The  
217 culture medium was changed three times per week; the cell  
218 monolayers were used when their transepithelial electrical resistance  
219 was stabilized, usually 10–15 days after reaching confluence.

220 **Cell Viability.** The cell viability of HT29 Glc<sup>+</sup> cells was measured  
221 using the CytoTox-One kit. Cells were incubated with the polyphenol  
222 extracts or PAC metabolites in the presence or absence of 0.8 mM *p*-  
223 cresol until day 7. LDH released into the culture medium (in relative  
224 fluorescence units, RFU) was determined with a multimode  
225 microplate reader (Synergy HT, Biotek) (λ<sub>ex/em</sub> = 560/590 nm).  
226 Results were expressed as percentage of LDH leakage in comparison  
227 to that of the untreated control cells.

228 **Intracellular ATP Contents.** The intracellular concentrations of  
229 ATP of HT29 Glc<sup>+</sup> cells were measured using a CellTiter-Glo kit.  
230 Cells were incubated with the polyphenol extracts or PAC metabolites  
231 in the presence or absence of 0.8 mM *p*-cresol until day 7. ATP levels  
232 were assayed according to the manufacturer's instructions, and  
233 luminescence was expressed as relative luminescence units (RLU)  
234 measured with the multimode microplate reader. Results were  
235 expressed as the percentage of ATP levels relative to that of the  
236 untreated control cells.

**Mitochondrial Membrane Potential.** The mitochondrial mem- 237  
brane potential (MMP) was quantified using Rhodamine-123 (R-123), 238  
which diffuses easily across membranes due to its lipophilic nature, and 239  
accumulates in the negatively charged mitochondrial matrix. HT29 240  
Glc<sup>+</sup> cells were incubated with the polyphenol extracts or PAC 241  
metabolites in the presence or absence of 0.8 mM *p*-cresol until day 7. 242  
For the experiment, cells were exposed to 1 μg/mL of R-123 for 30 243  
min to allow its loading within the cells; these were subsequently 244  
washed twice with PBS to remove the R-123 excess and lysed with 2% 245  
Triton-X100. Finally, MMP was determined with a multimode 246  
microplate reader (λ<sub>ex/em</sub> = 500/530 nm). Results were expressed as 247  
percentage of MMP relative to that of the control untreated cells. 248

**Intestinal Barrier Function.** Changes in the intestinal barrier 249  
function were determined only in Caco-2 cells as HT29 Glc<sup>+</sup> cells 250  
develop a low transepithelial electrical resistance (TEER). TEER, as a 251  
reflection of the integrity of the epithelial paracellular pathway, was 252  
measured in the Caco-2 cell monolayers grown in a Transwell filter 253  
with an ohm/voltmeter (EVOM, WPI, USA) as previously 254  
described.<sup>35</sup> Cell monolayers were used after confluence when their 255  
TEER was stabilized. Measurements were carried out at 24 h of 256  
coincubation with the different concentrations of the polyphenol 257  
extracts or PAC metabolites and 3.2 mM *p*-cresol. Results were 258  
expressed as percentage of the TEER value obtained at time 0. 259

Changes in the barrier function of the Caco-2 cell monolayers were 260  
also determined by measuring the paracellular passage of fluorescein 261  
isothiocyanate-conjugated dextran (FD-4) from the apical to the 262  
serosal side of the monolayer. FD-4 (4.4 kD MW, 0.8 mg/mL 263  
dissolved in culture medium) was added to the apical compartment in 264  
the absence or presence of the polyphenol extracts or PAC metabolites 265  
at different concentrations with or without the addition of 3.2 mM *p*- 266  
cresol. The amount of FD-4 transferred to the basolateral side after 24 267  
h exposure was determined in the multimode microplate reader (λ<sub>ex/em</sub> 268

269 = 490/520 nm). Results were expressed as percentage of the basal  
270 value.

271 **Statistical Analysis.** Data were analyzed using GraphPad Prism 6  
272 statistical software. Values represent the means of three independent  
273 experiments each conducted in triplicate. Data distribution of the  
274 variables was first analyzed by the Shapiro–Wilk test and parametric  
275 (ANOVA) or nonparametric analysis of variance (Kruskal–Wallis)  
276 was used according to the results of the test. When the result of the  
277 analysis of variances was significant ( $p < 0.05$ ), a posthoc Tukey test  
278 or a Dunn's test was carried out.

## 279 ■ RESULTS

280 The polyphenolic composition of the fruit polyphenol extracts  
281 is described in Table 1. The grape extract had the highest PAC  
282 content (41.0%), followed by that from cranberry, avocado, and  
283 apple (32.0, 29.1, and 22.0%, respectively). The mean degree of  
284 polymerization of the grape PACs was also higher (9.8) than  
285 that of cranberry (7.3), avocado (6.1), and apple (3.2). In  
286 addition, other types of polyphenol were also present in the  
287 extracts: the apple extract was characterized by its high content  
288 of flavonoids and dihydrochalcones (60.2 and 10.6%,  
289 respectively) and the cranberry extract by its high content of  
290 anthocyanins (23.7%), and the avocado extract was rich in  
291 monomeric flavan-3-ols and phenolic acids (14 and 38.8%,  
292 respectively).

293 The effects of the polyphenol extracts and their bacterial  
294 metabolites on cell viability and mitochondrial function was  
295 first evaluated in HT29  $\text{Glc}^{\mp}$  cells. As shown in Table 2, extracts  
296 from cranberries, avocados, and apples did not increase LDH  
297 leakage used as a marker of membrane integrity and cell  
298 viability; on the contrary, they slightly decreased the basal  
299 release of LDH. Although this parameter was not affected by  
300 the grape extract at the lowest concentrations, it was  
301 significantly increased at the higher concentration (50  $\mu\text{g}/$   
302 mL) ( $p < 0.01$ ). When the impact on the mitochondrial  
303 function of the polyphenol extracts was evaluated by measuring  
304 intracellular ATP contents, a significant increase was observed  
305 with cranberry and avocado extracts ( $p < 0.01$ ), whereas no  
306 change was observed with grape and apple extracts. At all of the  
307 concentrations tested, PAC metabolites induced a small  
308 (between 7 and 12%) but significant increase of LDH release  
309 by HT29  $\text{Glc}^{\mp}$  cells and a decrease (between 16 and 21%) of  
310 their cellular ATP content; however, this latter parameter was  
311 not affected by the addition of 3-PPA at 200  $\mu\text{M}$ .

312 Table 2 also shows that exposure of HT29  $\text{Glc}^{\mp}$  cells to *p*-  
313 cresol significantly increased LDH release ( $p < 0.01$ ) while at  
314 the same time it decreased their ATP content ( $p < 0.01$ ). The  
315 deleterious effect of *p*-cresol on LDH leakage was prevented by  
316 polyphenol extracts from cranberries and avocados. However,  
317 no protective effects were observed with the extracts from  
318 apples or grapes; on the contrary, they increase even more the  
319 *p*-cresol-induced LDH release; in the case of grape PACs, this  
320 deleterious effect was concentration-dependent. PACs from  
321 cranberries and avocados not only prevented the decrease of  
322 ATP content induced by *p*-cresol but also significantly  
323 increased ATP levels compared with cells not treated with *p*-  
324 cresol. Polyphenol extracts from apples and grapes did not  
325 exhibit any protective effect on the ATP content of the cells  
326 treated with *p*-cresol. In addition, the cellular ATP content  
327 decreased concentration-dependently with PACs from grapes,  
328 such that at the higher concentration of this extract the ATP  
329 values were lower compared with those of the control cells  
330 treated with *p*-cresol. The bacterial metabolites of PACs  
331 prevented the increase of LDH release induced by *p*-cresol.

However, these compounds did not exhibit effects on the  
cellular ATP content of the *p*-cresol-treated cells.

Changes in the mitochondrial function of HT29  $\text{Glc}^{\mp}$  cells  
were also evaluated through the determination of MMP; the  
results indicate that neither *p*-cresol nor the polyphenol extracts  
and PAC metabolites affect this parameter (data not shown).

For determining their effect on gut barrier function, extracts  
were placed on the apical side of Caco-2 cell monolayers and  
incubated for 24 h in the presence or absence of *p*-cresol. TEER  
and FD-4 transport across the epithelial monolayer were  
regularly measured. None of the polyphenol extracts affected  
the TEER and FD-4 transport across Caco-2 cell monolayers  
after 24 h of incubation regardless of the concentration tested  
(data not shown). As shown in Figure 1, cell exposure to

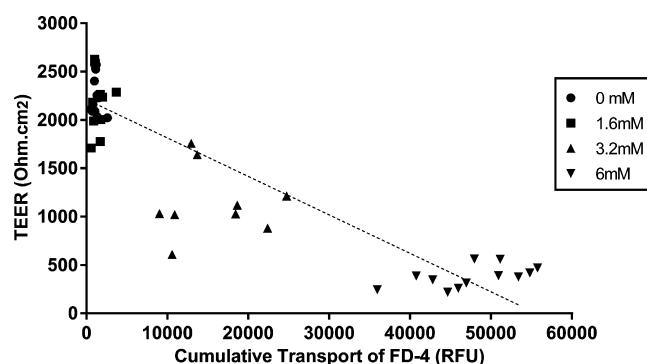


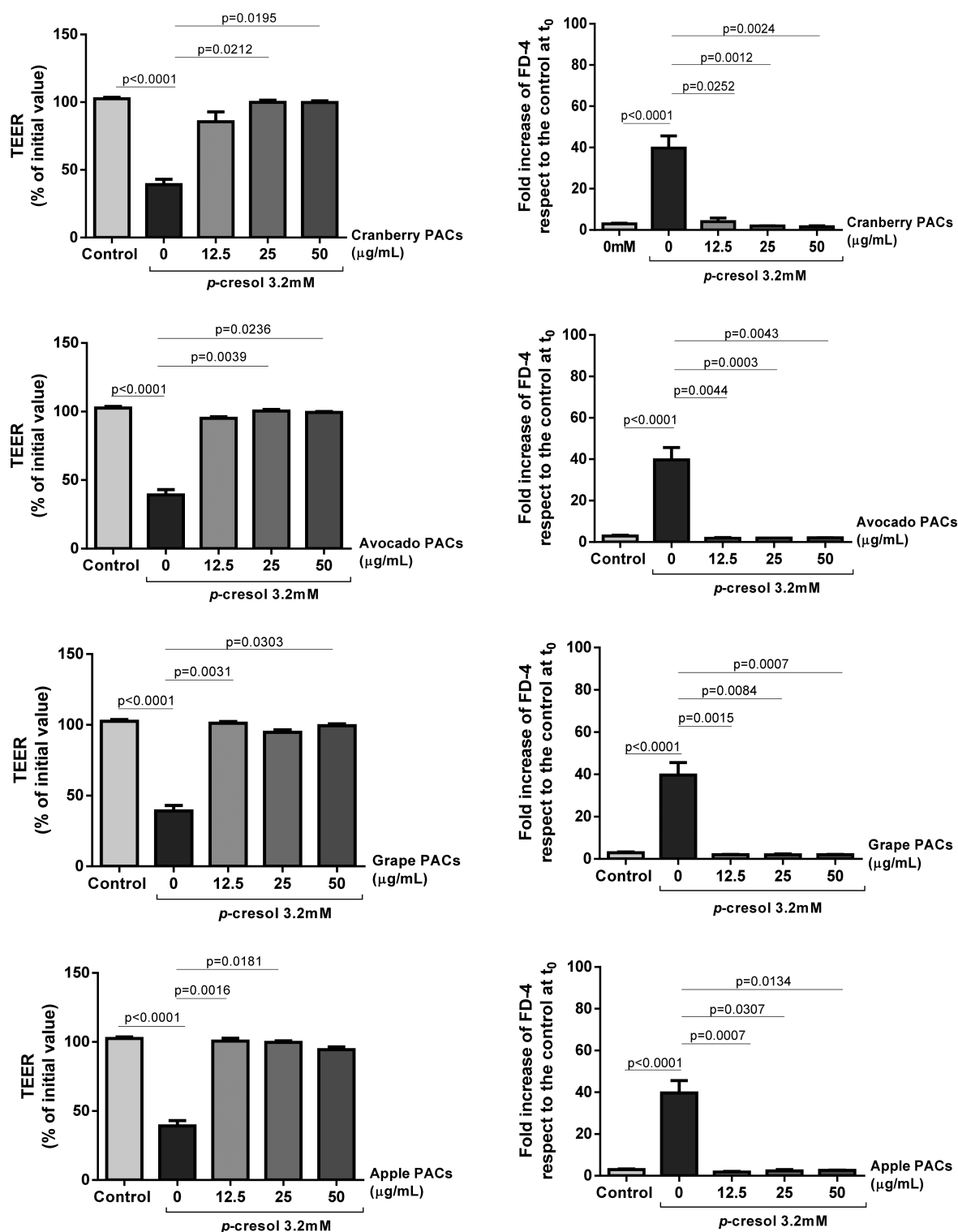
Figure 1. Correlation between transepithelial electrical resistance (TEER) and paracellular permeability to FD-4 in Caco-2 cell monolayers exposed for 24 h to different concentrations of *p*-cresol (Pearson  $r = -0.9018$ ;  $p < 0.0001$ ).

different *p*-cresol concentrations significantly decreased TEER  
values while correlatively increasing FD-4 transport ( $r = -0.90$ ;  
 $p < 0.0001$ ). The subsequent experiments were carried out  
incubating Caco-2 cell monolayers with 3.2 mM *p*-cresol for 24  
h. Under these conditions, TEER decreased 2.6-fold while FD-  
4 transport was increased 40-fold compared with that of  
untreated control monolayers at time 0. The protective effects  
of polyphenol extracts are shown in Figure 2. Results indicate  
that the extracts fully prevented the decrease of TEER induced  
by *p*-cresol, usually at concentrations of 25 and 50  $\mu\text{g}/$   
mL. In addition, PACs also protected cell monolayers against the  
increase of FD-4 transport at all of the concentrations tested.  
Results showing the protective effects of bacterial PAC  
metabolites are depicted in Figure 3. They indicate that all of  
the metabolites fully prevented the increased FD-4 transport  
induced by *p*-cresol after a 24 h incubation. *p*-Cresol-induced  
TEER decrease was prevented by 3,4-DHPPA at 100  $\mu\text{M}$ , 3-  
PPA at 50 and 200  $\mu\text{M}$ , and 4-HPPA at 10  $\mu\text{M}$ .

## ■ DISCUSSION

*p*-Cresol is an aromatic compound produced by the  
fermentation of tyrosine by anaerobic and aerobic bacteria  
from the human gut microbiota.<sup>18,19</sup> The concentration of free  
(unbound) *p*-cresol in the colonic lumen, although difficult to  
quantitate with exactitude, is more elevated in subjects  
consuming high-protein diets.<sup>27</sup> High intracolonic and plasma  
concentrations of *p*-cresol are considered risk factors for several  
pathological conditions. In fact, *p*-cresol is recognized as a  
uremic toxin contributing to the development of renal and  
cardiovascular complications in patients with chronic renal



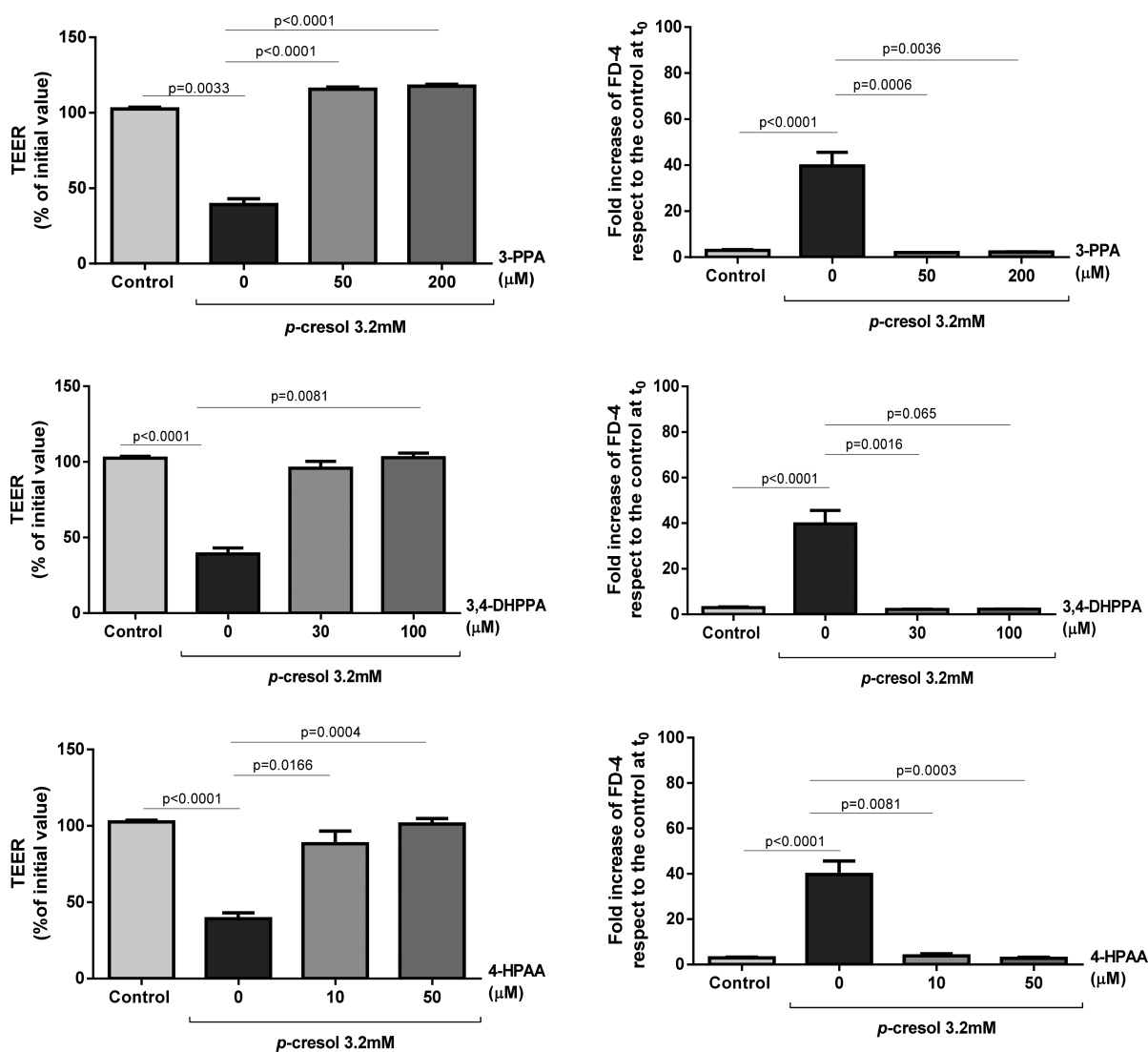


**Figure 2.** Protective effect of the different polyphenol extracts against the alterations of transepithelial electrical resistance (TEER) (left column) and paracellular permeability to FD-4 (right column) in Caco-2 cell monolayers exposed to 3.2 mM *p*-cresol for 24 h (mean  $\pm$  SEM).

375 diseases.<sup>24–26</sup> In addition, excess *p*-cresol has also been  
 376 reported to be hepatotoxic because it induces mitochondrial  
 377 dysfunction<sup>36</sup> and has been found to be associated with autism  
 378 in young patients.<sup>37</sup> We recently reported that, in the colon, *p*-  
 379 cresol affects cell proliferation and increases DNA damage in  
 380 human colonic cells in *in vitro* experiments.<sup>23</sup> In the present  
 381 study, we evaluated whether PAC-containing polyphenol  
 382 extracts from different fruits as well as PAC bacterial

metabolites protect human colonic epithelial cell lines against 383  
 the deleterious effects induced by *p*-cresol. 384

Our results showed that the exposure of HT29 C<sub>12</sub> cells to 385  
*p*-cresol resulted in the alteration of their cell membrane 386  
 integrity (evaluated through the LDH assay) and decreases of 387  
 ATP synthesis. This latter event suggests mitochondrial 388  
 dysfunction; however, no changes in MMP were detected 389  
 under our experimental conditions. This confirms the results 390



**Figure 3.** Protective effect of bacterial PAC metabolites against the alterations of transepithelial electrical resistance (TEER) (left column) and paracellular permeability to FD-4 (right column) in Caco-2 cell monolayers exposed to 3.2 mM *p*-cresol for 24 h (mean  $\pm$  SEM).

391 from our previous study indicating that the decreased ATP  
 392 synthesis was probably, at least in part, the underlying  
 393 mechanism explaining the inhibitory effect of *p*-cresol on  
 394 cellular proliferation.<sup>23</sup> In addition, our current results also  
 395 indicate that the barrier function of Caco-2 cell monolayers was  
 396 strongly altered after a 24 h incubation with *p*-cresol as reflected  
 397 by the decrease of TEER and the correlative increase of the  
 398 paracellular permeability of FD-4. In humans as well as in  
 399 laboratory animals, such alterations of the gut barrier function  
 400 are generally considered as an initial step previous to the  
 401 development of local and systemic pathological events. For  
 402 example, increased gut permeability is associated with the  
 403 formation of gastrointestinal ulcers in rats treated with  
 404 indomethacin, a nonsteroidal anti-inflammatory drug that  
 405 induces mitochondrial dysfunction in intestinal epithelial  
 406 cells<sup>38,39</sup> and in animal models of colorectal cancer. Increased  
 407 gut permeability is accompanied by higher plasma endotoxin  
 408 concentrations, mesenteric lymph node hypertrophy, and the  
 409 progression of cachexia.<sup>40</sup>

410 After further characterization of the deleterious impact of *p*-  
 411 cresol on the cultured intestinal cells, we evaluated whether this  
 412 might be prevented by our characterized PAC-containing

polyphenol extracts. Relative to the changes in cell membrane  
 413 integrity and ATP contents of HT29  $Glc^+$  cells, our results  
 414 were relatively variable according to the fruit source of the  
 415 extracts. Those from cranberries and avocados did not affect  
 416 LDH release and increased the intracellular ATP content,  
 417 whereas the extracts from apples and grapes had no effect on  
 418 the cellular ATP content but tended to increment LDH release.  
 419 Such differences between extracts were also observed when  
 420 they were incubated with the cells in the presence of *p*-cresol.  
 421 Avocado and cranberry extracts prevented the LDH release  
 422 induced by *p*-cresol whereas the apple and grapes extracts  
 423 magnified it. Similar results were observed on the cellular ATP  
 424 content. The reason for these differences in the activity of the  
 425 extracts remains unclear. However, it is interesting to note that  
 426 apples and grapes mainly contain type-B PACs whereas  
 427 avocados and cranberries mostly contain type-A PACs, whose  
 428 constituent monomers are also linked through an additional  
 429 ether bond between C2 and C7.<sup>1-3</sup> It has been shown that this  
 430 chemical structure confers specific biological activities to type-A  
 431 PACs that are not observed with the type-B PACs. This is the  
 432 case, for example, of the potent antiadhesive activity exhibited  
 433 by cranberry PACs against pathogens.<sup>41</sup> Therefore, it is possible  
 434

435 that this molecular feature may explain the better protective  
436 effect of the cranberry and avocado extracts on the membrane  
437 integrity and ATP content of *p*-cresol-exposed cells. It must  
438 also be stated that cytotoxicity induced by grape PACs has  
439 already been reported by Shao et al., who observed an increase  
440 of LDH release in cardiomyocytes exposed to grape seed PACs  
441 accompanied by increased free radical production, higher levels  
442 of nitric oxide, and intracellular glutathione depletion.<sup>42,43</sup>  
443 However, we did not find any data related to the differential  
444 toxicity of type-A and type-B PACs.

445 No differences between extracts were detected in their ability  
446 to prevent the decrease of TEER and the increased FD-4  
447 transport induced by *p*-cresol in Caco-2 monolayers; the  
448 disturbance of the barrier function observed after 24 h of  
449 incubation with *p*-cresol was totally prevented by all of the  
450 extracts. These results confirm our previous studies and those  
451 of other authors reporting that gut barrier alterations in animals  
452 and intestinal cell lines are prevented by apple peel polyphenol  
453 extract, cocoa PACs, grape seed PACs, and epigallocatechin-  
454 gallate.<sup>35,38,44–48</sup> Such a protective effect could be related to the  
455 ability of some PACs to bind lipid rafts in the apical membrane  
456 of intestinal cells, a phenomenon associated with the regulation  
457 of some cell signaling cascade events.<sup>46</sup>

458 Finally, we also evaluated in *p*-cresol-treated cells the  
459 protective effect of three metabolites, 4-HPAA, 3,4-DHPPA,  
460 and 3-PPA, originating from PAC degradation by the  
461 microbiota. It is worth noting that one of these (4-HPAA)  
462 may also be generated by the microbiota through protein  
463 fermentation.<sup>49</sup> Bacterial metabolites of polyphenols, and more  
464 specifically proanthocyanidins, may be detected in urine of  
465 human subjects after the intake of polyphenol-rich foodstuffs,  
466 suggesting that these compounds are absorbed in the colon and  
467 that they could exert biological activities in colonocytes as well  
468 as in a number of other tissues and organs.<sup>50,51</sup> Various studies  
469 have shown that these metabolites may be found in high  
470 concentrations in the colonic lumen,<sup>8</sup> confirming that they are  
471 poorly absorbed in the small intestine. Accordingly, they could  
472 directly act on epithelial cells, modulating signaling events and  
473 contributing to the homeostasis of the colonic mucosa. We  
474 observed that, when incubated with HT29  $\text{Glc}^{\pm}$  cells in the  
475 absence of *p*-cresol, these compounds slightly increased LDH  
476 release and decreased cellular ATP content. Such effects  
477 suggest that these metabolites might eventually induce cell  
478 toxicity. This could be due to the fact that HT-29  $\text{Glc}^{\pm}$  cells,  
479 being derived from a human colonic adenocarcinoma, may be  
480 more sensitive to these compounds compared with non-  
481 transformed cells.<sup>52</sup> In the presence of *p*-cresol, the bacterial  
482 PAC metabolites prevented the increase of LDH release by  
483 HT29  $\text{Glc}^{\pm}$  cells without affecting the cellular ATP content,  
484 and when incubated with Caco-2 cell monolayers, they totally  
485 prevented the alterations of barrier function induced by *p*-  
486 cresol. Although studies exploring the biological activities of  
487 PAC metabolites are scarce, it has been shown that 3,4-DHPPA  
488 upregulates the expression of glutathione S-transferase T2  
489 (GSTT2) and downregulates that of cyclooxygenase-2 (COX-  
490 2) in LT97 colonic cells.<sup>53</sup> COX-2 inhibition in HT29  $\text{Glc}^{\pm}$   
491 cells has also been reported after incubation with human fecal  
492 water with high levels of phenolic compounds.<sup>52</sup> The inhibition  
493 of cyclooxygenase-2 may explain the protective effect against  
494 gut barrier alterations we observed in Caco-2 cell monolayers,  
495 as previously described by Gentile et al.<sup>54</sup>

496 In conclusion, our present study indicates that exposure of  
497 colonic epithelial cells to high and therefore abnormal *p*-cresol

concentrations affects cell integrity, the intestinal barrier, and  
mitochondrial function. Our data indicate that PAC-containing  
polyphenol extracts and PAC bacterial metabolites prevent to a  
considerable extent some of these alterations with those from  
cranberries and avocados (characterized by the presence of  
type-A PACs) apparently being more effective. These *in vitro*  
results need further assessment in *in vivo* conditions to explain  
the presumed beneficial effect of diets rich in fruits and  
vegetables against colorectal cancer in humans.

## AUTHOR INFORMATION

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### Notes

The authors declare no competing financial interest.

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