Modulation of (-)-epicatechin metabolism by co-administration with other polyphenols in Caco-2 cell model

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Abbreviations:
3’-G, epicatechin-3’-O-β-D-glucuronide; 3’M, 3’-O-methyl-epicatechin; 3’M-4’S, 3’-O-methyl-4’-O-sulfate-epicatechin; 3’M-5S, 3’-O-methyl-5-O-sulfate-epicatechin; 3’M-7S, 3’-O-methyl-7-O-sulfate-epicatechin; 3’-S, 3’-O-sulfate-epicatechin;
4'-G, epicatechin-4’-O-β-D-glucuronide; 4’M-5-S, 4’-O-methyl-5-O-sulfate-epicatechin; 4’M-7-S, 4’-O-methyl-7-O-sulfate; 4’-S, 4’-O-sulfate-epicatechin; 5-S, 5-O-sulfate-epicatechin; 7-G, epicatechin-7-O-β-D-glucuronide; 7-S, 7-O-sulfate-epicatechin; AUC, area under the curve; DCNP, 2,6 dichloro-4-nitrophenol; DMEM, Dulbecco’s modified eagle medium; EC, (-)-epicatechin; EGC, (-)-epigallocatechin; EGCG, (-)-epigallocatechin-3-gallate; HBSS, Hank’s Balanced Salt; LOD, limit of detection; LOQ, limit of quantification; PB2, procyanidin B2; U-G, umbelliferone glucuronide; U-S, umbelliferone sulfate.
ABSTRACT

Widely consumed beverages such as red wine, tea and cocoa-derived products are a great source of flavanols. Epidemiological and interventional studies suggest that cocoa flavanols such as (-)-epicatechin may reduce the risk of cardiovascular diseases. The interaction of (-)-epicatechin with food components including other polyphenols could modify its absorption, metabolism and finally its bioactivity. In the present study we investigate (-)-epicatechin absorption and metabolism when co-exposed with other polyphenols in the intestinal absorptive Caco-2™ cell model. Depending on the type of polyphenols co-administered, the total amount of 3'-O-methyl-epicatechin and 3'-O-sulfate-epicatechin conjugates found both in apical and basal compartments ranged from 19 to 801 nM and from 6 to 432 nM, respectively. The co-incubation of (-)-epicatechin with flavanols, chlorogenic acid and umbelliferone resulted in similar amounts of 3'-O-methyl-epicatechin effluxed into the apical compartment relative to control. Co-incubation with isorhamnetin, kaempferol, diosmetin, nevadensin, chrysin, equol, genistein and hesperitin promoted the transport of 3'-O-methyl-epicatechin towards the basolateral side while decreasing the apical efflux. Quercetin and luteolin considerably inhibited the appearance of this (-)-epicatechin conjugate both in the apical and basolateral compartments. In conclusion, we could demonstrate that the efflux of (-)-epicatechin conjugates to the apical or basal compartments of Caco-2™ cells is modulated by certain classes of polyphenols and their amount. Ingesting (-)-epicatechin with specific polyphenols, could be a strategy to increase the bioavailability of (-)-epicatechin and to modulate its metabolic profile.
Introduction

Among the different classes of polyphenols, flavanols represent one of the most abundant compounds found in human diets (de Pascual-Teresa et al., 2000; Scalbert and Williamson, 2000). Flavanols are present in large amounts in beverages such as red wine, tea, and cocoa-derived products which are widely consumed throughout the world (Scalbert and Williamson, 2000). A number of epidemiological and interventional studies have suggested an inverse relationship between chronic consumption of flavanol-rich foods and the risk of cardiovascular diseases (Hertog et al., 1993; Hertog et al., 1997; Arts et al., 2001; Mink et al., 2007; Shrime et al., 2011; Hooper et al., 2012). (-)-Epicatechin, the major flavanol in cocoa extracts, has been identified as one of the bioactive compounds (Schroeter et al., 2006; Loke et al., 2008).

Cocoa extracts are typically consumed in form of beverages and confectionery products. Several studies have shown that less than 30% of the (-)-epicatechin dose consumed is absorbed (Manach et al., 2005; Borges et al., 2010; Actis-Goretta et al., 2012; Actis-Goretta et al., 2013). Following absorption in the intestinal tract, (-)-epicatechin is rapidly metabolized into glucuronide, sulfate, and/or methyl conjugates (Harada et al., 1999; Donovan et al., 2001; Actis-Goretta et al., 2012; Actis-Goretta et al., 2013) (Figure 1). These metabolites, chemically different from (-)-epicatechin, are the compounds circulating in the bloodstream and reaching the target organs (Heiss et al., 2005; Schroeter et al., 2006; Tinahones et al., 2008; Borges et al., 2010). As the (-)-epicatechin benefits are dependent of the amount of its metabolites appearing in the bloodstream, factors affecting the absorption and modulation of metabolites represent important considerations for increasing its bioefficacy.
Recently, we demonstrated that the plasma metabolic profile was different when (-)-epicatechin was consumed as chocolate or perfused as purified compound in the intestine (Actis-Goreta et al., 2012; Actis-Goreta et al., 2013). Therefore, it could be suggested that other food ingredients could modify the metabolism of (-)-epicatechin in vivo (Schramm et al., 2003; Neilson and Ferruzzi, 2011). Previous reports showed that co-exposure of hesperetin with specific compounds modulates the amounts of hesperetin metabolites both in apical and basolateral chamber in a Caco-2\textsuperscript{TM} cell in vitro model (Brand et al., 2010). Other Structure-related polyphenols consumed with foods have been shown to be metabolized by the same enzymes as (-)-epicatechin (Morimitsu et al., 2004). Similarly, the co-administration of polyphenols with (-)-epicatechin could modify the metabolic profile.

To our knowledge, previous data about the effect of other polyphenols over the transport and generation of (-)-epicatechin metabolites has not been reported. Therefore, we aimed at investigating the modulation of (-)-epicatechin conjugates in Caco-2\textsuperscript{TM} cell monolayers, a well-recognized model for studying the intestinal transport of nutrients and drugs (Hidalgo et al., 1989; Yamashita et al., 2000), by co-incubation with different dietary polyphenols. Altogether, understanding the interaction of polyphenols with the profile of (-)-epicatechin metabolites could provide a future insight for increasing its bioavailability by addition or elimination of other polyphenols in the food products.
Materials and methods

Chemicals

(−)-Epicatechin, quercetin, isoharmetin, kaempferol, lutein, diosmetin, chrysin, genistein, catechin, EGC, EGCG and chlorogenic acid were purchased from Extrasynthèse (Genay, France). Nevadensin was purchased from Apin Chemicals (Abingdon, Oxon, UK) while equol and umbelliferone, umbelliferone glucuronide and umbelliferone sulfate were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Procyanidin B2, 3'-O-methyl-epicatechin, 4'-O-methyl-epicatechin, 4'-O-methyl-catechin, epicatechin-3'–β-D-glucuronide, epicatechin-4'–β-D-glucuronide, 3'-O-methyl-4'-sulfate-epicatechin and hesperitin-7-glucoside were produced by complete chemical synthesis at the Nestlé Research Center (Lausanne, Switzerland). 3'-sulfate-epicatechin and 4'-sulfate-epicatechin were synthesized as a mixture of compounds and then separated according to the method of Gonzalez-Manzano et al. (Gonzalez-Manzano et al., 2009). Acetonitrile ultra-gradient HPLC grade was purchased from J. T. Baker Europe (Deventer, The Netherlands). HPLC grade water was prepared using a Millipore Milli-Q purification system (Bedford, MA, USA). All other solvents HPLC grade and reagents were purchased from Merck (Darmstadt, Germany). 2,6 Dichloro-4-nitrophenol was purchased from Sigma-Aldrich (Basel, Switzerland).

Cell culture experiments

Caco-2(TM) cell lines are immortalized cells of human malignant colon cancer origin. Caco-2(TM) cells were obtained from America Type Culture Collection (ATCC, USA under perpetual license agreement between NaviCyte Scientific and Nestec Ltd.). For maintenance, Caco-2(TM) cells were seeded at a density of 40x10^3 cells/cm² and cultured in a humidified incubator at 5% CO₂ and 37 °C in Dulbecco’s modified...
Eagle’s medium (DMEM) containing 4.5 g glucose/ L, 20 % heat inactivated fetal bovine serum (FBS), 1 % non-essential amino acids, 2 mM L-glutamine, 1 µg/ mL amphotericin B, 100 U/ mL penicillin, and 100 µg/ mL streptomycin. Medium was changed every two days.

To obtain differentiated monolayers, Caco-2™ cells were seeded at a density of 20x10³ cells/ cm² in 12-well inserts and cultured for 21 days. Medium was changed every two days. Cell culture media without phenol red was used during the differentiation period.

On the experiment day, medium was removed and replaced by Hank’s Balanced Salt (HBSS) supplemented with 25 mM glucose, 10 mM HEPES and 1.8 mM CaCl₂, 189U/ ml Catalase and 0.5 mM ascorbic acid, referred to as exposure medium below. (–)-Epicatechin (100 µM) was placed in the apical compartment of the cell monolayers and incubated for 2 h. Aglycone and conjugated compounds were detected in the cell culture media in the apical and basolateral sides using the analytical conditions described below. Trans-epithelial electrical resistance (TEER) was measured before and after the transport study, and indicated that the integrity of the monolayer was not affected by the transport experiment. Co-incubation experiments were performed by adding (–)-epicatechin (100 µM) in the presence or absence of other polyphenols (50 µM, 10 µM, 5 µM or 1 µM) to the apical side of the Caco-2™ cell monolayer. The compounds were stocked in DMSO and diluted to the right concentration into the exposure medium. The concentration of DMSO at the apical side was kept at 0.05 % in each experiment.

Cell culture media preparation.
After 2 h incubation, the whole media in both chambers was collected and centrifuged for 5 min. An aliquot of 400 µL of supernatant was transferred into a 96-well plate, and 800 µL of 2 % formic acid in H₂O added. Afterwards, 10 µL of 16 µM internal standard mixture (4′-O-methyl-catechin, umbelliferone sulfate and umbelliferone glucuronide) was added to each well. Salt contained in the exposure medium significantly influenced the shape of the peaks of some targeted compounds. To avoid this interference, the cell culture samples were cleaned-up and concentrated using a solid phase extraction (SPE) step (Strata-X, Phenomenex, USA). The mixture was transferred into a pre-conditioned (1 mL Methanol, followed by 1 mL water) 96-well SPE plate and allowed to pass through. The wells were washed with 1 mL water and epicatechin metabolites were eluted with 1 ml methanol:pyridine (1:1) into a 96-well collection plate. The eluates were dried under a flow of nitrogen gas at room temperature. Fifty µL of solution of 8% acetonitrile and 0.1% CH₃COOH in H₂O was added to each well to reconstitute the sample. Ten µL was injected into UPLC-MS for analysis.

**Analytical methodology**

The analytical assessment of (-)-epicatechin metabolites was conducted with an ACQUITY Ultra Performance Liquid Chromatography– Mass Spectrometry (UPLC–MS) system (Waters® Corporation, Switzerland). Separations were performed on an ACQUITY UPLC HSS C18 2.1×100 mm column (Waters® Corporation, Switzerland). Mobile phases A and B consisted of 0.1% (v/v) acetic acid in water and 0.1% (v/v) acetic acid in acetonitrile, respectively. Analysis was completed within 14 min with a flow rate of 0.5 mL/ min. The following gradient was applied: 0–1 min isocratic at 8%
Column effluent was perfused into a Quattro micro bench-top triple quadruple mass spectrometer (Micromass; Manchester, UK) through its ESI source working in negative mode. The source temperature, capillary voltage, desolvation gas flow and temperature were set up as described elsewhere (Actis-Goretta et al., 2012; Actis-Goretta et al., 2013). It allowed us to detect and quantify the epicatechin metabolites following their m/z transitions with specific cone voltage and collision energy in MRM mode. The molecular transitions were m/z 289 to m/z 245 for (−)-epicatechin aglycone, m/z 303 to m/z 244 for 3’-O-methyl-(−)-epicatechin, m/z 303 to m/z 137 for 4’-O-methyl-(−)-epicatechin and 4’-O-methyl-(+)-catechin (IS), m/z 465 to m/z 289 for glucuronidated (−)-epicatechin metabolites, m/z 369 to m/z 289 for sulfated (−)-epicatechin metabolites, m/z 479 to m/z 303 for glucuronidated O-methyl-(−)-epicatechin metabolites, m/z 383 to m/z 303 for sulfated O-methyl-(−)-epicatechin metabolites, m/z 241 to 161 for umbelliferone sulfate (IS) and m/z 337 to 161 for umbelliferone glucuronide (IS). MassLynx software (v 4.0; Micromass) was used to control the instruments and for data acquisition and processing.

**Data analysis**

The apparent permeability coefficients (Papp) were calculated using the following equation $P_{app} = \frac{\Delta Q/\Delta t}{A \times C_0}$, where $\Delta Q/\Delta t$ is the linear appearance rate of the compound on the receiver side (in mM/ s), $A$ is the membrane surface area (cm$^2$), and $C_0$ is the initial concentration in the donor (Apical) compartment (in mM/ cm$^3$). All samples were analyzed at least in triplicate. Statistical analyses were conducted with GraphPad Prism version 6.04 (GraphPad Software, San Diego, CA, USA).
are shown as mean ± SD. Statistical differences were determined by one way ANOVA. A p value < 0.05 was considered to be statistically significant.
Results

Transport of (-)-epicatechin in Caco-2™ cell culture model

Although some in vitro studies are describing a passive diffusion transport (Vaidyanathan and Walle, 2001), others reported the relevance of the facilitated mechanism for flavanols absorption (Vaidyanathan and Walle, 2001; Zhang et al., 2004; Chan et al., 2007). With the objective of investigating its transport across Caco-2™ cells, (-)-epicatechin was placed either in the apical or basal compartments. Results showed a similar linear correlation between the amount of (-)-epicatechin transported from apical to basal than basal to apical at different times suggesting a passive diffusion or paracellular mechanism of transport (Supplemental Figure 1A). Moreover, it was not possible to saturate the (-)-epicatechin transport by increasing the concentration up to 35 mM (Supplemental Figure 1B).

In addition, Papp was calculated under our experimental conditions where \( \Delta Q = 1.25 \mu M \), \( \Delta t = 7200s \), \( A = 1.12 \text{ cm}^2 \), and \( C_0 = 250 \mu M \), therefore \( P_{app} = 0.6 \times 10^{-6} \text{ cm/ s} \).

The calculated value was similar to the one reported by Tian et al (Tian et al., 2009) \( (0.60\pm0.05 \times 10^{-6} \text{ cm/ s} ) \) confirming the hypothesis of passive diffusion or paracellular transport of (-)-epicatechin.

Identification of (-)-epicatechin metabolites in Caco-2™ cell model

(-)-Epicatechin metabolites were identified in the exposure media both at the apical and basolateral compartments. The concentration of conjugates measured in apical compartment augmented with increasing concentrations of (-)-epicatechin (10, 50 and 100 \( \mu M \)) incubated with Caco-2™ cells (Figure 2A). 3'-O-methyl-epicatechin, 3'-O-sulfate-epicatechin and 3'-O-methyl-5-O-sulfate-epicatechin were identified as the main metabolites of (-)-epicatechin both at the apical and basolateral compartments after incubation of this compound with Caco-2™ cells for 2 h, (Figure 2B). The 3'-O-
methyl-4’-O-sulfate-epicatechin and 4’-O-methyl-epicatechin conjugate were quantified solely in apical compartment while other (-)-epicatechin metabolites such as 3’-O-methyl-7-O-sulfate-epicatechin and 4’-O-methyl-5-O-sulfate-epicatechin were identified at concentrations lower than the limit of quantification of 5 nM and have not been reported in the figure.

In our previous results Caco-2™ cells generated relevant amounts of umbelliferone glucuronide indicating the ability to glucuronidate other compounds but the lack of a specific isoform able to glucuronidate (-)-epicatechin (Actis-Goretta et al., 2013).

**Modulation of the metabolic profile of (-)-epicatechin by other polyphenols.**

To evaluate the effect of other food components on the modulation of (-)-epicatechin metabolism, 16 polyphenols were chosen to conduct co-incubation experiments (Table 1). These compounds were selected according to their presence in dietary foods and belonging to different polyphenol groups. The initial working concentration for these polyphenols was set at 50 µM (1:2 ratio to (-)-epicatechin). In the presence of other polyphenols, the conjugates of (-)-epicatechin identified were as before i.e. 3’-methyl-epicatechin, 3’-O-sulfate-epicatechin, 3’-O-methyl-5-O-sulfate-epicatechin and 4’-O-methyl-epicatechin but their amount and distribution varied according to the polyphenol/(-)-epicatechin combination tested.

The total amount of 3’-O-methyl-epicatechin, 3’-O-sulfate-epicatechin and 3’-O-methyl-5-O-sulfate-epicatechin conjugates found both in apical and basal compartments ranged from 19 to 801 nM, from 6 to 432 nM and from lower than 5 (limit of detection) to 176 nM respectively (Figure 3). The co-incubation of (-)-epicatechin with flavanols, chlorogenic acid and umbelliferone resulted in similar amounts of 3’-O-methyl-epicatechin effluxed into the apical compartment relative to
the control (Figure 4). Co-incubation with isorhamnetin, kaempferol, diosmetin, nevadensin, chrysin, equol, genistein and hesperitin promoted the transport of 3'-O-methyl-epicatechin towards the basolateral side while decreasing the apical efflux. Quercetin and luteolin considerably inhibited the appearance of this (-)-epicatechin conjugate both in the apical and basolateral compartments. These findings suggest that certain polyphenols families could compete for or inhibit the basolateral transporter of the (-)-epicatechin metabolites.

The appearance of 3'-O-sulfate-epicatechin in the apical and basolateral compartments is illustrated in Figure 5. DCNP, a highly affinity substrate for sulfation, (Fayz et al., 1984; Morimitsu et al., 2004), inhibited the production of 3'-O-sulfate-epicatechin to a fourth of the control in the apical compartment and completely in the basolateral compartment. As described for 3'-O-methyl-epicatechin, flavanols and chlorogenic acid do not modify the efflux of 3'-O-sulfate-epicatechin to the apical compartment. The co-incubation of (-)-epicatechin with flavonols (quercetin, isorhamnetin and kaempferol), flavones (diosmetin, luteolin, nevadensin and chrysin), hesperetin and umbelliferone significantly inhibited the apical distribution of 3'-O-sulfate-epicatechin. Isoflavones, like equol and genistein significantly diminished the efflux of 3'-O-sulfate-epicatechin to the apical side while increasing its basolateral transport. Basolateral concentrations of 3-O-sulfate-epicatechin were close to the limit of detection of 5 nM.

**Increasing doses of polyphenols influence the metabolism of (-)-epicatechin**

The results described above suggest that certain compounds might exert specific modulation effects on the metabolism of (-)-epicatechin in either apical or basolateral compartments of the Caco-2™ cell model. Genistein, hesperitin,
nevadensin and chrysin were some of the compounds found to increase the concentration of 3'-O-methyl-epicatechin in the basal compartment. We performed co-incubation experiments with (-)-epicatechin (100 µM) and the above cited compounds at different concentration (1, 5, 10 and 50 µM) (Figure 6). The efflux of 3'-O-methyl-epicatechin to the apical side was reduced and transport to the basolateral compartment was the same or slightly higher than the control when (-)-epicatechin was co-incubated with 1 µM of these polyphenols (ration 1:100). The transport of 3'-O-methyl-epicatechin to the basolateral compartment increased with increasing doses of genistein, hesperitin, nevadensin and chrysin. The efflux mechanism seemed to be less sensitive to the concentration change and remained constant with increasing concentrations of polyphenols while still lower when compared to the control experiment.
Discussion

Bioavailability of polyphenols is subject to transport and metabolism by phase II enzymes in intestinal cells (Scalbert and Williamson, 2000; Manach et al., 2005). With the use of a multilumen perfusion catheter in humans, it was recently demonstrated that nature and substitution position of (-)-epicatechin conjugation are major determinants of the metabolic fate in the body, influencing whether the compound is effluxed into the lumen or absorbed into the blood (Actis-Goretta et al., 2013). Previous \textit{in vitro} studies using Caco-2\textsuperscript{TM} cell monolayers as intestinal barrier model highlighted the effect of co-administration of different flavonoids on the apical and basolateral efflux of hesperetin and hydroxycinnamic acid (Brand et al., 2010; Wong and Williamson, 2013). The objective of our study was to investigate the extent of competition of (-)-epicatechin with other polyphenols to better understand its absorption and metabolism.

The results obtained in this study suggest a passive diffusion of (-)-epicatechin to the basolateral side of the Caco-2\textsuperscript{TM} cell monolayer and imply that co-administration of other polyphenols should not compete for the transport of (-)-epicatechin.

In Caco-2\textsuperscript{TM} cells (-)-epicatechin was metabolized into 3'-O-methyl-epicatechin, 3'-O-sulfate-epicatechin, 3'-O-methyl-5-O-sulfate-epicatechin, 4'-O-methyl-epicatechin, and 3'-O-methyl-4'-O-sulfate-epicatechin which were mainly effluxed to the apical compartment of the cellular model. \textit{In vivo}, the main metabolites effluxed into the lumen were 3'-O-sulfate-epicatechin, epicatechin-3'-O-glucuronide, 3'-O-methyl-5-O-sulfate-epicatechin and 7'-O-sulfate-epicatechin (Actis-Goretta et al., 2013). The nature of the substitution of (-)-epicatechin was different in the two models, while sulfation was favoured \textit{in vivo} with 73% of metabolites sulfated,
methylation was the main conjugation \textit{in vitro} with 77.4\% of metabolites methylated. Fifteen percent of metabolites were glucuronidated \textit{in vivo} whereas such substitution could not be detected in the Caco-2\textsuperscript{TM} cell model suggesting the absence of some specific UGT isoforms in this model as anticipated by Wong and Vaidyanathan (Vaidyanathan and Walle, 2001; Wong and Williamson, 2013).

Under our experimental conditions, the influence of other polyphenols over the (-)-epicatechin metabolism was confirmed. Co-administration of (-)-epicatechin with flavonols, flavones and isoflavones reversed the transport of (-)-epicatechin metabolites to the basolateral side except for quercetin and luteolin which nearly abolished the metabolism and/or transport of (-)-epicatechin either by inhibition of the transporters and metabolic enzymes or by competition with these latter. In contrast, co-incubation of (-)-epicatechin with other flavanols such as (+)-catechin, and EGC did not influence the conjugation profile or the apical and basolateral distribution of (-)-epicatechin metabolites.

Brand et al. (2010) previously suggested that several classes of polyphenols effectively inhibited the breast cancer resistance protein (BCRP) transporters responsible for the efflux of hesperetin. The affinity of polyphenols for the ATP-binding cassette (ABC) trans-membrane transporters, specifically P-glycoprotein (Pgp) and multidrug resistant proteins (MRP1 and MRP2) is also a key factor limiting their intestinal transport (Feng, 2006; Takano et al., 2006). These transporters actively remove xenobiotics from the cell interior to the lumen (Feng, 2006). The affinity of (-)-epicatechin metabolites for these transport systems significantly limits the ability of these compounds to cross into the bloodstream. Therefore, we can suggest that the addition of other polyphenols can generate a competition for these efflux transporters. As a result, some compounds (such as hesperitin, isorhamnetin,
kaempferol, diosmetin, chrysin, nevadensin, equol and genistein) could have more affinity for the efflux transporters increasing the level of (-)-epicatechin metabolites in the bloodstream as demonstrated with co-administration of (-)-epicatechin and increasing doses of genistein, hesperitin, nevadensin and chrysin (Figure 6).

As previously suggested by Brand et al. (2010) for hesperidin and other flavonoids, a structure-activity relationship could be suggested for the inhibition/competition mechanism leading to the presence or absence of 3’-O-methyl-epicatechin in the basolateral compartment of the cellular model. Among the polyphenols chosen for co-administration with (-)-epicatechin, 3 structural groups could be distinguished: a) presence of catechol and benzopyranone group; inhibition of the production of the conjugate compared to the levels observed with (-)-epicatechin treatment, b) presence of catechol and absence of benzopyranone group; no difference in production of conjugate relative to (-)-epicatechin control treatment, c) absence of catechol and benzopyranone group; increase of 3’-O-methyl-epicatechin in basolateral compartment compared to the treatment with (-)-epicatechin (Table 2).

Within all the polyphenols tested in our experiments solely quercetin and luteolin reduced the levels of 3’-O-methyl-epicatechin both in the apical and basal compartments. These findings suggest a higher affinity of the catechol O-methyl-transferases (COMT) for these compounds than (-)-epicatechin. Quercetin and luteolin were previously identified as high affinity substrates for COMT (Zhu and Liehr, 1996; Chen et al., 2011). Quercetin already showed higher affinity to COMT than catecholestrogenos (Zhu and Liehr, 1996), neurotransmisors (Singh et al., 2003) and other flavonoids (Wang et al., 2012).
Under our experimental conditions, the conjugates of (-)-epicatechin in apical and basal compartment represented 1-2% of its initial amount under the control conditions or when (-)-epicatechin was co-administered with other polyphenols. The metabolites produced by the enterocytes had a low impact on the total amount transport across the epithelium. However, the type and quantity of metabolites produced by the enterocytes was different and could promote either the absorption or the efflux of the conjugate highlighting the importance of different metabolic profiles.

In conclusion, using the Caco-2™ cell model we could demonstrate that (-)-the efflux of epicatechin conjugates into the apical or basal compartment is modulated by certain classes of polyphenols and their amount. In vivo, efflux into the apical or basolateral compartment really translates into excretion of metabolites back into the intestinal lumen or absorption and, consequently, bioavailability. Ingesting (-)-epicatechin with specific polyphenols, like genistein or nevadensin, could be a strategy to modulate the bioavailability of (-)-epicatechin or its metabolites. In addition, decreasing the amount of quercetin and luteolin in foods recipe might also contribute to increase the bioavailability of (-)-epicatechin. However, additional in vivo clinical trials would be necessary to test this hypothesis and prove the benefit of the association of (-)-epicatechin and other polyphenols on human health.
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Authorship contribution

Participated in research design: Actis-Goretta.

Conducted experiments: Sanchez-Bridge, Lévèques, Li, Bertschy and Patin

Contributed new reagents or analytic tools: Lévèques and Patin

Performed data analysis: Lévèques, Li and Actis-Goretta.

Wrote or contributed to the writing of the manuscript: Sanchez-Bridge and Actis-Goretta.

All authors read and approved the final manuscript.
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Footnotes:

All of the authors are (LAG, AL, MR), or were (HL) employees of Nestec Ltd, which is a subsidiary of Nestlé Ltd. and provides professional assistance, research, and consulting services for food, dietary, dietetic, and pharmaceutical products of interest to Nestlé Ltd. No other authors declare conflicts of interest.
Legends for Figures

Figure 1: Chemical structure of (-)-epicatechin and epicatechin metabolites.

Figure 2: A) Concentration of (-)-epicatechin metabolites in apical compartment of Caco-2TM cells after 2h incubation with different concentrations of (-)-epicatechin. B) Concentration of (-)-epicatechin metabolites in apical and basal compartment of Caco-2TM cells after incubation with 100 µM of (-)-epicatechin in apical compartment for 2h. Data is presented as mean ± SD (n=3).

Figure 3: Total amount of 3'′-O-methyl-epicatechin (A), 3′-O-sulfate-epicatechin (B) and 3′-O-methyl-5-sulfate-epicatechin (C) in the apical and basolateral compartments of Caco-2TM cell monolayers following co-incubation of (-)-epicatechin (100 µM) with different polyphenol compounds (50 µM) at the apical side. Values are shown as mean ± SD (n=6). * p<0.05. nd: not detected.

Figure 4: Apical efflux and basolateral transport of 3′-O-methyl-epicatechin in Caco-2TM cell monolayers following co-incubation of (-)-epicatechin (100 µM) with different compounds (50 µM) at the apical side. Values are shown as mean ± SD (n=6). * p<0.05.

Figure 5: Apical efflux and basolateral transport of 3′-O-sulfate-epicatechin in Caco-2TM cell monolayers following co-incubation of (-)-epicatechin (100 µM) with different compounds (50 µM) at the apical side. Values are shown as mean ± SD (n=6). * p<0.05. nd: not detected.
**Figure 6:** Apical efflux and basolateral transport of 3’-O-methyl-epicatechin in Caco-2™ cell monolayers following co-incubation of (-)-epicatechin (100 µM) with genistein, hesperitin, nevadensin and chrysin at 1, 5, 10 and 50µM at the apical side for 2h. Values are shown as mean ± SD (n=3). * p<0.05.
**Table 1:** Polyphenols chosen to be tested in co-incubation experiments with epicatechin in Caco-2™ cell model

<table>
<thead>
<tr>
<th>Compound</th>
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<tr>
<td>Kaempferol</td>
<td>Flavonol</td>
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<tr>
<td>Luteolin</td>
<td>Flavone</td>
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<tr>
<td>Diosmetin</td>
<td>Flavone</td>
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<table>
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<tr>
<th>Compound</th>
<th>Family</th>
<th>Structure</th>
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<tr>
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<td>Flavone</td>
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<tr>
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<td>Flavanol</td>
<td><img src="image" alt="Epigallocatechin" /></td>
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<tr>
<td>Epigallocatechin gallate</td>
<td>Flavanol</td>
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<tr>
<td>Procyanidin B2</td>
<td>Flavanol</td>
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<tr>
<td>Chlorogenic acid</td>
<td>Phenolic acids</td>
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<tr>
<td>Nevadensin</td>
<td>Flavone</td>
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<tr>
<td>Equol</td>
<td>Isoflavone</td>
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<tr>
<td>Genistein</td>
<td>Isoflavone</td>
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Table 2: Structure-relationship for the inhibition of 3'-O-methyl-epicatechin in the basolateral compartment of Caco-2™ cell model.

<table>
<thead>
<tr>
<th></th>
<th>Catechol group</th>
<th>Benzopyranone group</th>
<th>Ratio* of 3'M in basolateral compartment</th>
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<tbody>
<tr>
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<td>Yes</td>
<td>&lt;1</td>
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<tr>
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<tr>
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<td>No</td>
<td>≈1</td>
</tr>
<tr>
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<td>≈1</td>
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<td>≈1</td>
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<tr>
<td>Chlorogenic acid</td>
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<tr>
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<tr>
<td>Umbelliferone</td>
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*Ratio between the level of 3'-O-methyl-epicatechin in the basolateral compartment obtained by co-incubating (-)-epicatechin with the corresponding polyphenols and the level of 3'M in the basolateral compartment obtained by incubating only (-)-epicatechin (control treatment)
Figure 1
Figure 2

A

Apical concentration (nM)

Epicatechin added in apical compartment (μM)

- 3'-S
- 3'M-4'S
- 3'M-5S
- 3'-M
- 4'-M

B

Apical concentration (nM)

Epicatechin metabolites

- 3'-S
- 3'M-4'S
- 3'M-5S
- 3'-M
- 4'-M
- EC

Basal concentration (nM)