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# Influence of apple polyphenols on the intestinal barrier in a colonic cell model

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## Summary

Apples (*Malus* spp., Rosaceae) and apple-derived foods contain polyphenols that are associated with various desirable health attributes. Amongst other effects, *in vivo* studies with rodent models have shown that these substances can help to prevent and aid the treatment of intestinal inflammation and other lesions linked to reductions in intestinal barrier function (and thus adverse effects on the status of tight junctions, TJs). In the study presented here we investigated effects of apple polyphenols and their intestinal degradation products on the TJ status (as indicated by the transepithelial resistance, TER), and the mRNA levels of TJ-associated genes (using quantitative real-time PCR) in T84 colon carcinoma cell line monolayers. T84 monolayers were preincubated with sodium caprate (C10) to obtain a model system with decreased barrier function. Polyphenols and their intestinal degradation products significantly increased the TER during 4 h incubations both with and without C10 treatment in comparison to controls. The transcription analyses revealed that polyphenols influenced the transcript levels of all of the tested genes encoding TJ-associated genes. Using physiological concentrations up to 5.7-fold increasing mRNA levels were achieved. Further, apple-specific dihydrochalcones strongly affected both the TER and the expression of tight junction-relevant genes.
Generally, apple polyphenols and their intestinal metabolites appeared to enhance the epithelial barrier functions in the T84 colonic cell monolayer model, indicating that consumption of apples and apple-derived foods may have positive effects on the intestinal barrier in healthy humans and may play an important role in the prevention of inflammatory bowel diseases (IBDs).

## Introduction

Apples (*Malus* spp., Rosaceae) and apple-derived foods (e.g. apple juice) contain various polyphenols, including both flavonoids and hydroxycinnamic acids. Polyphenol contents vary between 5.2 and 27.2 g/kg dry weight in apples (WOJDYLO et al., 2008) and 110 and 459 mg/L in apple juices (KAHLE et al., 2005a). They have several desirable health attributes including anti-oxidative and anti-carcinogenic properties, as well as positive effects on diabetes, cardiovascular and neurodegenerative diseases (BOYER and LIU, 2004; SCALBERT et al., 2005). Furthermore several apple-derived polyphenols have shown anti-inflammatory activity *in vitro*. For instance, Jung et al. found them to have inhibitory effects on inflammatory gene expression in several colonic cell lines (JUNG et al., 2009). In addition, *in vivo* studies with various rodent models have shown that the administration of polyphenols can prevent and treat intestinal inflammation and injury (SHAPIRO et al., 2007; CLARKE and MULLIN, 2008).
Apples are excellent sources of polyphenols, for instance they contain up to 203 and 303 mg/kg dry weight of phloretin 2'-*O*-xyloglucoside and phloretin 2'-*O*-glucoside, respectively. Recently, phloretin 2'-*O*-glucoside has also been identified in strawberries and cranberries, at low levels (HILT et al., 2003; TURNER et al., 2005), but the occurrence of phloretin 2'-*O*-xyloglucoside has not been reported in any plants except apples. Esters of D-(-)-quinic acid with caffeic or *p*-coumaric acid are present in strawberries, cherries (GONCALVES et al., 2004; KULISIC-BILUSIC et al., 2009), elderflower extracts (CHRISTENSEN et al., 2008), tronchuda cabbage (SOUSA et al., 2008), coffee beans (PERRONE et al., 2008), and carrots (KLAIBER et al., 2005), but either their contents are lower than in apples or their average consumption is marginal. Free D-(-)-quinic acid has been found in diverse plant-derived foods including (*inter alia*) apple juice (LEE and WROLSTAD, 1988), coffee (GALLI and BARBAS, 2004), and various vegetables (RUHL and HERRMANN, 1985).
The gastrointestinal availability of apple polyphenols has been studied in ileostomists after consumption of cloudy apple juice when apple polyphenols and their metabolites – such as methyl caffeate, methyl *p*-coumarate, and D-(-)-quinic acid – have been found in ileostomy bags at levels ranging between 1 and 80 µM (KAHLE et al., 2005b; KAHLE et al., 2007). Up to 45% of oral doses was recovered in the ileostomy bags and therefore would reach the colon of healthy subjects (KAHLE et al., 2007). Microbial degradation of polyphenols in the human gastrointestinal tract has also been examined by several authors. Phloroglucinol, 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxytoluene have been reported as colonic metabolites of quercetin (SCALBERT and WILLIAMSON, 2000; LABIB et al., 2004). In the colon 5-caffeoylquinic acid (5-CQA) undergoes cleavage of the ester bond to form caffeic acid followed by the generation of *inter alia* dihydrocaffeic acid and dihydrocinnamic acid (RECHNER et al., 2004). In addition, dihydrocinnamic acid has been characterized as a colonic microbial degradation product of catechins (OLTHOF et al., 2003), and the degradation of phloretin to phloroglucinol and 3-(4-hydroxyphenyl)propionic acid has been described following *ex vivo* incubation with *Eubacterium ramulus* and *Clostridium orbiscindens* isolated from human faeces (SCHNEIDER and BLAUT, 2000; SCHOEFER et al., 2003).
Apple-derived polyphenols and their metabolites reach the colon, where they will encounter the colonic mucosa, the intestinal barrier consisting of a thick secreted mucus layer, a layer of epithelial cells, and nonepithelial mucosal cells (MCGUCKIN et al., 2009). The sealing of the paracellular space between epithelial cells is provided by tight junctions (TJs) that regulate the passage of ions, water, and molecules. Various transmembrane and cellular TJ proteins, including occludin, claudins, and zonula occludens (ZO) have been identified (GONZALEZ-MARISCAL et al., 2003). Defects of the intestinal barrier function are associated with inflammatory bowel diseases (IBDs), such as Crohn's disease and ulcerative colitis (LAUKOETTER et al., 2008). *In vitro* studies have shown that interferon-γ (IFNγ) and tumour necrosis factor-α (TNFα), found at high levels in the intestinal mucosa in IBD cases, can decrease barrier function and lead to the reorganization of tight junction proteins (CLAYBURGH et al., 2004).
The status of the intestinal barrier can be assessed *in vitro* by measuring the transepithelial resistance (TER) and expression of TJ proteins, and several studies have detected enhancement of the TER in a Caco-2 cell line model during incubation with various polyphenols,

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namely: quercetin (AMASHEH et al., 2008; SUZUKI and HARA, 2009), quercetin glycosides (OHNO et al., 2006), a procyanidin mixture (ERLEJMAN et al., 2006) and apple waste extracts (MCCANN et al., 2007). Previous studies in our laboratory confirmed these findings in a T84-cell model (BERGMANN et al., 2009). In addition, increased expression of TJ-associated proteins in Caco-2 cells following incubation with the flavonoid quercetin has been reported (AMASHEH et al., 2008; SUZUKI and HARA, 2009). In contrast, TJ function can be reduced by the addition of sodium caprate (C10), providing a useful means to determine effects of substances on epithelia with decreased barrier function. Incubation with C10 reduces ATP formation, followed by a contraction of the perijunctional actomysin rings (WALLON et al., 2005), leading to dilatations and (hence) structural deformations of the TJs (ANDERBERG et al., 1993).

In the study presented here we used T84 colon epithelial cell monolayers, as a model of differentiated human colon cells (SAMBRUY et al., 2001), to examine the effects of various apple polyphenols and their metabolites on the intestinal barrier at physiological concentrations. The compounds used in the tests included *inter alia* esters of D-(-)-quinic acid with caffeic or *p*-coumaric acid, phloretin, phloretin 2'-*O*-glucoside and phloretin 2'-*O*-xyloglucoside. We measured the TER before and during incubations with these compounds, and both before and after preincubation with C10, and measured levels of mRNAs encoding the TJ-related proteins claudin-4, occludin and ZO-1, to observe the influence of these compounds on the intestinal barrier function.

## Materials and Methods

### Chemicals

Dimethylsulfoxide (DMSO), caffeic acid (3,4-dihydroxycinnamic acid), 5-caffeoylquinic acid (5-CQA, chlorogenic acid), phloretin (2',4',6',4-tetrahydroxydihydrochalcone), phloretin 2'-*O*-glucoside (phloridzin), and (-)-epicatechin (*cis*-5,7,3',4'-tetrahydroxyflavan-3-ol; 2R, 3R) were obtained from Sigma (Steinheim, Germany). 3,4-Dihydroxytoluene, 3,4-dihydroxyphenylacetic acid and D-(-)-quinic acid were purchased from Aldrich (Steinheim, Germany). Quercetin (3,5,7,3',4'-pentahydroxyflavone) was supplied by Merck (Darmstadt, Germany). *p*-Coumaric acid (4-hydroxycinnamic acid), phloroglucinol, 3-(4-hydroxyphenyl)propionic acid and dihydrocaffeic acid were purchased from Fluka (Steinheim, Germany). (+)-Catechin (*trans*-5,7,3',4'-tetrahydroxyflavan-3-ol; 2R, 3S) and quercetin 3-*O*-rhamnoside were purchased from Roth (Karlsruhe, Germany). Dihydrocinnamic acid was purchased from Acros organics (Geel, Belgium). 1-Caffeoylquinic acid (1-CQA) was synthesized from caffeic acid and D-(-)-quinic acid according to published protocols (SEFKOW, 2001; SEFKOW et al., 2001). 3-Caffeoylquinic acid (3-CQA) and 4-caffeoylquinic acid (4-CQA) were isolated after isomerization of 5-CQA (according to TRUGO and MACRAE, 1984). 4-*p*-coumaroylquinic acid (4-*p*-CouQA), 5-*p*-coumaroylquinic acid (5-*p*-CouQA) and phloretin 2'-*O*-xyloglucoside were isolated from extracts of apple juice treated with laccase (according to WILL et al., 2007). Methyl caffeate and methyl *p*-coumarate were kindly provided by P. Schreier (Wuerzburg, Germany).

### Cell culture and TER analysis

T84 colon epithelial cells purchased from ATCC (American Type Culture Collection, Rockville, MD, USA; CCL-248) were grown in a 5% $CO_2$ humidified incubator at 37°C on 1 µm filters (Falcon, Heidelberg, Germany) with medium containing Ham's F-12 nutrient mixture and DMEM (1:1) supplemented with 5% FCS, antibiotics (PenStrep) and 2.5 mM glutamine (all from Gibco, Germany). The transepithelial resistance (TER) of the resulting monolayers was determined in an Endohm 24 chamber (WPI, Berlin, Germany). In all experiments we utilized T84 monolayers of at least 2 weeks confluence when the transepithelial resistance (TER) was reproducible, and the monolayer was intact. Cytotoxicities of the polyphenols were tested in appropriate concentrations by trypan blue staining (PATTERSON, 1979).

### Taqman® probes

Taqman® probes were synthesized together with oligonucleotides by MWG (Ebersberg, Germany). VIC-labelled probes for the specific Taqman® housekeeping control gene, human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were purchased from Applied Biosystems (Darmstadt, Germany). The following primers and probes were designed using Beacon Designer 2.1 (Premier Biosoft, Palo Alto, USA) and overt amplification of DNA: ZO-1 sense, 5'-ATG GTG TCC TAC CTA ATT CAA CTC AT-3'; ZO-1 antisense, 5'-GCC AGC TAC AAA TAT TCC AAC ATC A-3'; ZO-1 probe, 5'-CAC CAG CCA GCC GCA AAC CCA CA-3'; occludin sense, 5'-AAG GTC AAA GAG AAC AGA GCA AGA-3'; occludin antisense, 5'-TAT TCC CTG ATC CAG TCC TCC TC-3'; and OCC probe, 5'-CTC ATC ACA GGA CTC GCC GCC AGT TG-3'. Probes were labelled with 5'-FAM and 3'-TAMRA. Claudin-4 oligonucleotides and probes were applied as a 'ready-to-use' kit from Applied Biosystems.

### Real-time RT PCR

Total RNA from T84 monolayers grown on culture inserts was obtained by lysis using TriFast (Peqlab, Erlangen, Germany) and purified using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Portions (2 µg) of RNA were reversely transcribed using iScript (BioRad, Munich, Germany) then amplified by real-time PCR using an iCycler (BioRad). After activating the polymerase at 95°C for 10 min. the ZO-1, occludin, claudin-4 and GAPDH primers and probes were annealed at 60°C, and transcript levels were semi-quantitatively analyzed, using standard calibration curves obtained from serial dilutions of cDNAs synthesized from known quantities of total RNA from T84 cells. ZO-1, occludin, claudin-4 and GAPDH starting transcript number values for the standard curves were set arbitrarily. ZO-1, occludin, claudin-4 and GAPDH expression levels were then estimated in terms of cycle threshold (Ct) values, and corresponding transcript numbers were read off standard curves as previously described (SCHAUBER et al., 2003). Values for unknown samples were quantified by measuring their Ct and reading the corresponding values off the standard curves. ZO-1, occludin and claudin-4 expression levels were then normalized to GAPDH expression and their expression levels in polyphenol-free medium-treated control cells were considered to be "1". All experiments were conducted in triplicates.

### Ussing chamber experiments

For Ussing chamber experiments with the T84 colon carcinoma cell line, cells were grown in 12 mm diameter permeable transwell cell culture inserts (Corning Inc., Wiesbaden, Germany). After cultivating the cells until confluency these permeable supports were fixed in P2302 Ussing chamber inserts (Hugo Sachs Elektronik, Harvard Apparatus GmbH, March-Hugstetten, Germany), exposing 1.13 $cm^2$ of cell surface to 5 mL of Hanks' Balanced Salt Solution (HBSS) (Sigma, Munich, Germany) and kept at 37°C under constant 95% $O_2$-5% $CO_2$ gassing (Rießner-Gase, Lichtenfels, Germany). After a 30 min. equilibration period to achieve steady-state conditions each of the test polyphenols in DMSO (final DMSO concentration, 0.05%) was added, individually, to apical compartments of the chambers. Incubation concentrations were comparable to those recovered in

ileostomy bags 2 h after consumption of cloudy apple juice (Tab. 1). Intestinal microbial degradation products were incubated in the corresponding concentrations. The transepithelial electrical potential difference (PD) was then measured at 2 min. intervals under current-clamped conditions. Tissue conductance or transepithelial resistance was determined at an applied current of 100 mA, and the short-circuit current (Isc) was calculated using Ohm's law (R = V/I). Cell suspensions were obtained by lysis using TriFast reagent. All experiments were done in triplicate. In order to detect changes in permeability and the influence of the polyphenols on the colonic barrier, the TJ modulator sodium caprate (C10) (Sigma) was added to the apical side for approximately 10 min., following which the transepithelial resistance was ca. 50% of its initial value. The concentration of C10 applied (10 mM) has been reported to increase the paracellular permeability of both human and rat ileum (WALLON et al., 2005). In these experiments the C10 was thinned out using fresh HBSS medium after the 10 min. incubation period before adding the test polyphenols.

### Statistics

Statistical evaluations were performed using Student's paired t-tests, and differences in means were considered significant if $p \leq 0.05$ and highly significant if $p \leq 0.001$. Data are presented as means ± standard deviation (SD).

## Results

### Effects of apple polyphenols on TER

The TER of T84 cell monolayers was monitored for 4 hours during incubation with apple polyphenols at physiological concentrations in the Ussing type chambers. In complementary experiments the T84 cell monolayers were preincubated with C10 in order to reduce the TER to 50% of the initial value. After a wash-out with C10-free HBSS-media the incubation with the apple polyphenols was started.

Measured TER values of confluent monolayers of T84 cells ranged

**Tab. 1:** Transepithelial resistance (TER) of T84 monolayers pretreated with and without caprylic acid (C10) at the end of a 4 h incubation with apple polyphenols (*p<0.05, ***p<0.001). Concentrations of apple polyphenols and their intestinal metabolites [µM] used are in the same range as concentrations found in ileostomy bags 2 h after cloudy apple juice consumption (data not published). *p*-CouQA = *p*-coumaroylquinic acid, CQA = caffeoylquinic acid. Values are means ± SD obtained from three experiments.

| | Conc. [µM] | T84-monolayers | | C10 pretreated T84-monolayers | |
|---|---|---|---|---|---|
| | | % of initial TER | significance | % of initial TER | significance |
| **control** | | 77.4 ± 0.3 | | 53.1 ± 9.4 | |
| **flavonoids** | | | | | |
| (+)-catechin | 10 | 119.7 ± 8.8 | * | 91.4 ± 26.6 | * |
| (-)-epicatechin | 10 | 123.4 ± 7.3 | * | 82.1 ± 35.3 | |
| quercetin | 10 | 111.5 ± 12.8 | * | 90.5 ± 15.0 | * |
| quercetin 3-*O*-rhamnoside | 10 | 127.4 ± 12.8 | *** | 121.1 ± 9.0 | * |
| phloretin | 20 | 135.1 ± 1.1 | *** | 77.1 ± 13.6 | * |
| phloretin 2'-*O*-glucoside | 20 | 120.2 ± 4.9 | * | 78.9 ± 11.2 | * |
| phloretin 2'-*O*-xyloglucoside | 20 | 129.4 ± 2.8 | * | 111.1 ± 8.1 | *** |
| **esters of hydroxycinnamic acids** | | | | | |
| 4-*p*-Cou-QA | 10 | 136.9 ± 3.7 | * | 107.8 ± 12.9 | * |
| 5-*p*-CouQA | 10 | 131.7 ± 6.1 | *** | 99.9 ± 23.6 | * |
| 1-CQA | 20 | 128.5 ± 2.8 | * | 133.0 ± 33.5 | * |
| 3-CQA | 50 | 131.5 ± 0.5 | *** | 137.1 ± 16.7 | * |
| 4-CQA | 10 | 112.2 ± 6.2 | * | 93.9 ± 23.0 | * |
| 5-CQA | 50 | 126.7 ± 2.9 | *** | 93.3 ± 9.3 | * |
| **intestinal degradation products** | | | | | |
| phloroglucinol | 20 | 109.6 ± 10.3 | * | 95.0 ± 23.4 | * |
| 3-(4-hydroxyphenyl)propionic acid | 20 | 117.9 ± 10.0 | * | 69.0 ± 11.3 | |
| dihydrocinnamic acid | 20 | 104.3 ± 22.0 | * | 56.4 ± 20.3 | |
| 3,4-dihydroxyphenylacetic acid | 20 | 126.0 ± 15.5 | * | 90.0 ± 21.4 | * |
| 3,4-dihydroxytoluene | 20 | 59.5 ± 4.1 | | 83.7 ± 17.1 | |
| caffeic acid | 20 | 124.9 ± 23.5 | * | 92.1 ± 23.0 | * |
| *p*-coumaric acid | 20 | 136.1 ± 31.2 | * | 95.1 ± 32.5 | |
| dihydrocaffeic acid | 20 | 107.0 ± 20.4 | * | 105.6 ± 23.2 | * |
| methyl caffeate | 20 | 115.9 ± 32.3 | * | 70.5 ± 21.1 | |
| methyl p-coumarate | 20 | 128.4 ± 15.5 | * | 84.1 ± 19.4 | * |
| D-(-)-quinic acid | 80 | 72.6 ± 2.5 | | 115.1 ± 23.5 | * |

from 400 and 650 $\Omega \cdot cm^2$ in the absence of polyphenols or C10. After a 30 min. equilibration period the polyphenols dissolved in DMSO were added to the apical chamber and the TER values were set to 100%. Control monolayers were treated in the same manner, except that only DMSO was added. The TER of the control monolayers rose slightly during the first hour of the incubation, but then declined to 77.4 ± 0.3% of the initial value. In general, the apple-derived polyphenols used in the study increased the TER in comparison to the control (as illustrated by the time-courses of effects of phloretin and its glycosides on the TER of T84 monolayers during incubation for 4 h shown in Fig. 1). Phloretin, phloretin 2'-*O*-glucoside and phloretin 2'-*O*-xyloglucoside increased the TER of the monolayers until stable levels were reached of 135.1 ± 1.1%, 120.2 ± 4.9%, and 129.4 ± 2.8% of the initial values, respectively. No decreases in the TER were observed. The time courses of TER during incubation with phloretin and its glycosides were representative of those observed in tests with of the polyphenols used here. In Tab. 1 the TER values of T84 monolayers after 4 h of incubation with other apple polyphenols and their intestinal degradation products are summarized. Significantly high effects were observed with physiological concentrations of quercetin 3-*O*-rhamnoside (127.4 ± 12.8%), phloretin (135.1 ± 1.1%), 4-*p*-CouQA (136.9 ± 3.7%), 3-CQA (131.5 ± 0.5%), and 5-CQA (126.7 ± 2.9%). However, no significant changes in TER values were observed after incubation with the intestinal degradation products 3,4-dihydroxytoluene (59.5 ± 4.1%) or D-(-)-quinic acid (72.6 ± 2.5%).

In a complementary experimental setup the T84 monolayers were pretreated with C10 (10 mM) until the TER was 50% of the initial value followed by a wash-out with fresh HBSS media then polyphenols were added. The effects of phloretin, phloretin 2'-*O*-glucoside, and phloretin 2'-*O*-xyloglucoside on TER after C10 treatment are shown in Fig. 2. The TER values recovered to 77.1 ± 13.6%, 78.9 ± 11.2%, and 111.1 ± 8.1% of the initial values, respectively, during incubation with polyphenols, whereas TER values of controls (with no addition of polyphenols) only increased up to 53.1 ± 9.4% of initial values within 4 h. The increase in TER values after incubation with phloretin 2'-*O*-xyloglucoside was highly significant in comparison to controls. TER values of C10-pretreated T84 monolayers after incubation with all polyphenols used in the tests are shown in Tab. 1. No significant enhancement of the TER values in comparison to controls was observed after treatment with (-)-epicatechin, dihydrocinnamic acid, 3,4-dihydroxytoluene, 3-(4-hydroxyphenyl)propionic acid, methyl caffeate and *p*-coumaric acid whereas all other polyphenols induced significant enhancements.

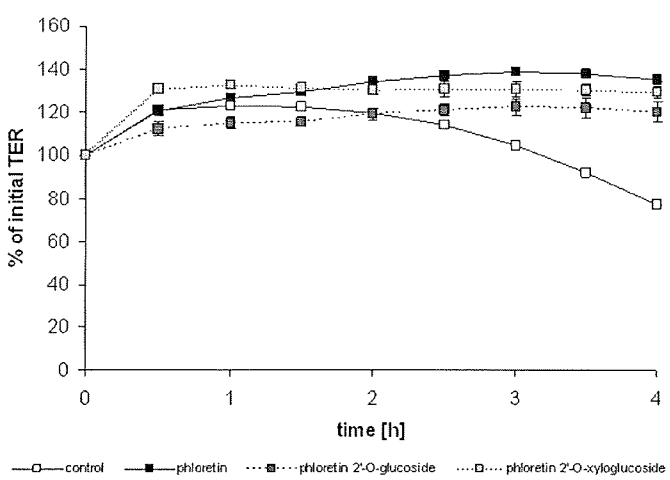


**Fig. 1:** Time-courses of effects of phloretin and its glycosides on the TER of T84 monolayers over 4 h. Values are means ± SD obtained from three independent experiments.

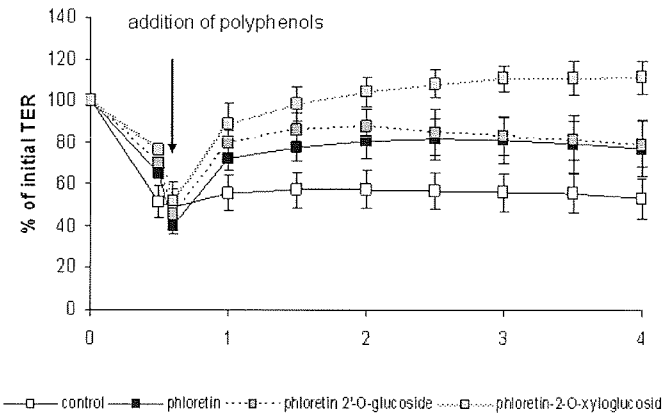


**Fig. 2:** Time-courses of effects of phloretin and its glycosides on the TER of C10-pretreated T84 monolayers over 4 h. T84 monolayers were pre-incubated with C10 followed by a wash-out (first 30 min.). Addition of polyphenols is indicated with an arrow. Values are means ± SD obtained from three experiments.

**Effects of apple polyphenols on mRNA expression of TJ proteins**

The levels of mRNAs encoding the TJ proteins ZO-1, occludin and claudin-4 in the monolayers following incubation with apple polyphenols for 4, 8 and 24 h were quantified by TaqMan® qRT-PCR with specific primers, and normalized to GAPDH signals. The results show that incubations with the apple polyphenols had varying effects on the expression of genes encoding these proteins. Results are shown in Fig. 3, 4, and 5.

The PCR assays revealed that the following apple flavonoids increased ZO-1 mRNA levels at physiological concentrations (10 or 20 µM): (+)-catechin, (-)-epicatechin, quercetin, phloretin, phloretin 2'-*O*-glucoside and phloretin 2'-*O*-xyloglucoside. However, 10 µM quercetin 3-*O*-rhamnoside had no effect on ZO-1 expression. Occludin mRNA levels were increased after incubation with (+)-catechin, quercetin, phloretin, and phloretin 2'-*O*-xyloglucoside. Incubation with phloretin 2'-*O*-glucoside resulted in decreased occludin mRNA levels after 4 h, but increased levels after 8 h, while (-)-epicatechin and quercetin 3-*O*-rhamnoside had no apparent effects on its expression. Claudin-4 mRNA levels were increased after incubation with (+)-catechin, quercetin, quercetin 3-*O*-rhamnoside and phloretin, but incubation with (-)-epicatechin, phloretin 2'-*O*-glucoside, and phloretin 2'-*O*-xyloglucoside had no detected effects

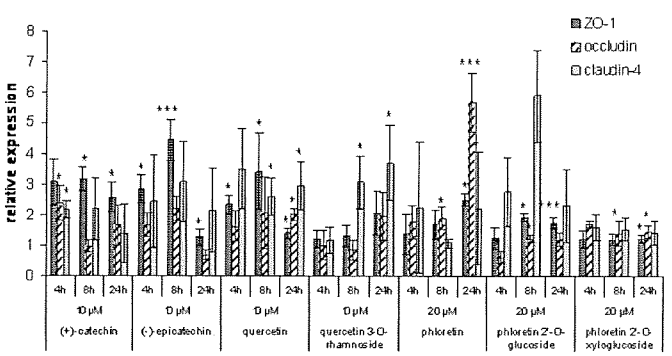


**Fig. 3:** Expression levels of ZO-1, occludin and claudin-4 mRNA in T84 monolayers after incubation with apple flavonoids. mRNA levels are normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and data are expressed as ~fold changes in mRNA transcript levels relative to those of T84 controls (*p<0.05, ***p<0.001). Each value is the mean ± SD obtained from three experiments.

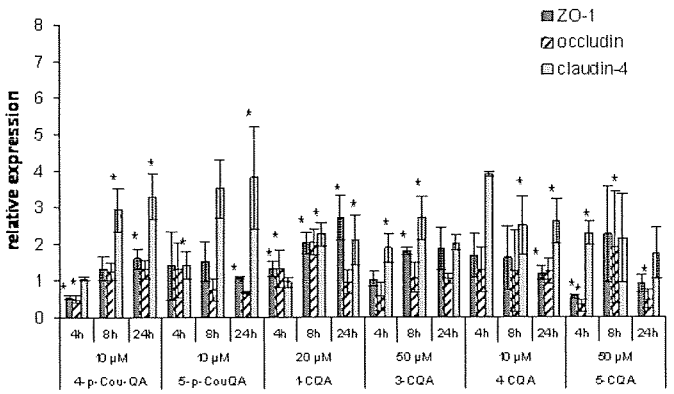


**Fig. 4:** Expression levels of ZO-1, occludin and claudin-4 mRNA in T84 monolayers after incubation with esters of D-(-)-quinic acid and caffeic or *p*-coumaric acid at physiological concentrations. mRNA levels are normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and data are expressed as ~fold changes in mRNA transcript levels relative to those of T84 controls. CQA = caffeoylquinic acid, *p*-CouQA = *p*-coumaroylquinic acid (*p<0.05, ***p<0.001). Each value is the mean ± SD obtained from three experiments.

on them at physiological concentrations. Fold changes in transcript levels of these mRNA species, during incubations with the listed compounds, are shown in Fig. 3.

Quantification of transcript levels during and after incubations with hydroxycinnamic acid esters revealed that 5-*p*-CouQA, 1-CQA, 3-CQA and 4-CQA all increased ZO-1 mRNA levels at some time points, whereas 5-CQA reduced them. Incubation with 4-*p*-CouQA resulted in significant reductions in ZO-1 mRNA levels after 4 h, no significant difference from control levels after 8 h, and a significant increase after 24 h. mRNA levels of occludin were decreased after incubation with 4-*p*-CouQA, 5-*p*-CouQA, and 5-CQA. Increases in occludin mRNA levels were observed after incubation with 1-CQA and 5-CQA, but 3-CQA and 4-CQA had no significant effects on these transcript levels. In addition, the transcription of claudin-4 mRNA was increased after incubation with 4-*p*-CouQA, 5-*p*-CouQA, 1-CQA, 3-CQA, 4-CQA, and 5-CQA. Fold changes observed in the transcription of these genes during incubations with these compounds are illustrated in Fig. 4.

Phloroglucinol, 3,4-dihydroxytoluene, methyl *p*-coumarate, dihydrocinnamic acid, dihydrocaffeic acid and D-(-)-quinic acid all induced increases in ZO-1 mRNA. 3-(4-Hydroxyphenyl)propionic acid induced an increase after 4 h and a decrease after 24 h. 3,4-dihydroxyphenylacetic acid, methyl caffeate, *p*-coumaric acid and caffeic acid had no apparent effects on ZO-1 expression. mRNA levels of occludin were increased after incubation with phloroglucinol, methyl *p*-coumarate, dihydrocinnamic acid, and dihydrocaffeic acid, but decreased after incubation with 3-(4-hydroxyphenyl)propionic acid and caffeic acid). 3,4-Dihydroxytoluene, 3,4-dihydroxyphenylacetic acid, methyl caffeate, *p*-coumaric acid and D-(-)-quinic acid had no detected effects on occludin mRNA levels. Transcription levels of claudin-4 mRNA were increased after incubation with 3,4-dihydroxytoluene, dihydrocaffeic acid and D-(-)-quinic acid, whereas phloroglucinol, 3,4-dihydroxyphenylacetic acid, 3-(4-hydroxyphenyl)propionic acid, methyl *p*-coumarate, methyl caffeate, dihydrocinnamic acid, *p*-coumaric acid and caffeic acid had no significant effects on them. Fold changes observed in the transcription of these genes during incubations with these compounds are illustrated in Fig. 5.

## Discussion

The intestinal barrier serves as a filter with selective permeability allowing the passage of nutrients and preventing the penetration of harmful objects including microorganisms, luminal antigens, and luminal pro-inflammatory factors (FARHADI et al., 2003). Defects in the intestinal barrier are associated with IBDs (LAUKOETTER et al., 2008). Since TJs are the most important intercellular barriers in all epithelia (POWELL, 1981) the effects of food ingredients and their metabolites on them are of great importance. In this study we investigated the influence of apple polyphenols and their intestinal metabolites at physiological concentrations on TJs, by measuring both the TER and the mRNA expression of TJ related proteins in the *in vitro* T84 cell model during incubations with the compounds.

All tested apple polyphenols and metabolites, except the metabolites 3,4-dihydroxytoluene and D-(-)-quinic acid, significantly increased the TER of T84 cell monolayers in comparison to controls during 4 h incubations (Tab. 1). Accordingly, previous studies have found

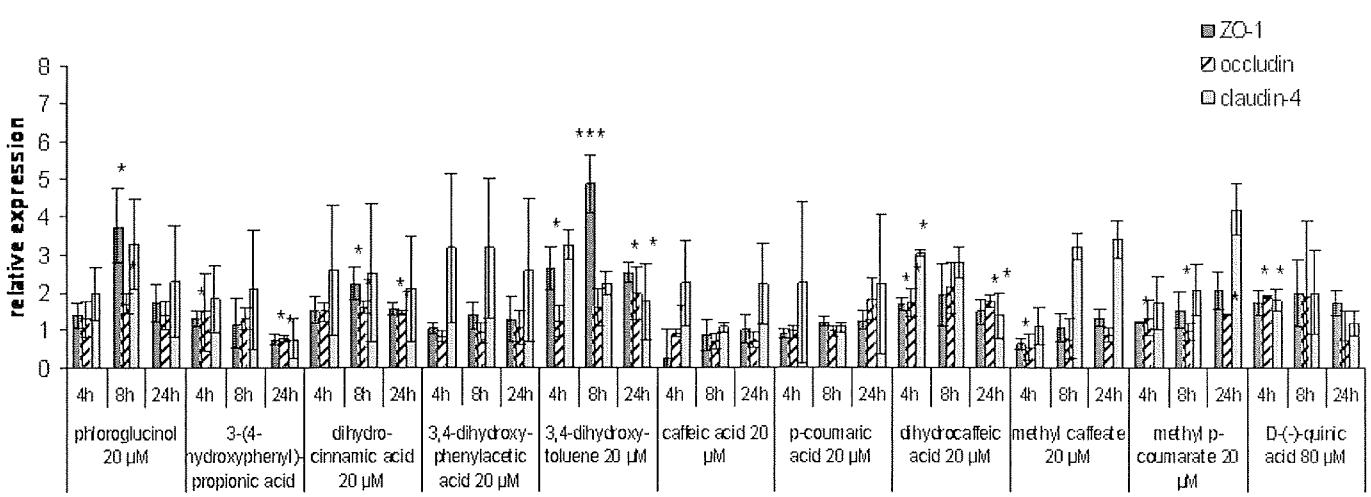


**Fig. 5:** Expression levels of ZO-1, occludin and claudin-4 mRNA in T84 monolayers after incubation with intestinal apple polyphenol degradation products at physiological concentrations. mRNA levels are normalized to those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and data are expressed as ~fold changes in mRNA transcript levels relative to those of T84 controls (*p<0.05, ***p<0.001). Each value is the mean ± SD obtained from three experiments.

quercetin (AMASHEH et al., 2008; SUZUKI and HARA, 2009), apple waste extracts (MCCANN et al., 2007), quercetin glycosides (OHNO et al., 2006), and a mixture of procyanindins (ERLEJMAN et al., 2006) to have positive effects on TER in the colonic cell line Caco-2. In contrast, raspberry extracts – containing various anthocyanins, ellagitannins and hydroxycinnamic acid derivatives – showed no significant effects on the TER in this model in a study by COATES et al. (2007). For illustration, the effects of phloretin and its glycosides on the TER of T84 monolayers observed at various time-points in our study are shown in Fig. 1. No data on the influence of dihydrochalcones on the TER are reported.

Several noxious agents have been used in studies of intestinal injury and repair in attempts to obtain information about mechanisms whereby the colonic epithelium may recover from injury (SCHEPPACH et al., 1996). One of these agents is sodium caprate (C10), which we used here as a TER-reducing agent and is known to affect TJs by reducing ATP formation and thus causing contraction of the perijunctional actomysin rings (WALLON et al., 2005). Structural deformations of the TJs in the form of dilations have also been reported in Caco-2 cells in the presence of 10 mM C10 (ANDERBERG et al., 1993). The cytokines TNFα and IFNγ, which are present in patients suffering from IBDs may alter the TJs via actomyosin contraction too (PRASAD et al., 2005). These observations indicate that treatment of T84 monolayers with C10 provides a good model system for imitating IBDs *in vitro*.

During tests with most of the applied substances the TER of T84 cell monolayers pre-treated with C10 was enhanced within the 4 h incubations in comparison to control monolayers (Tab. 1).

To our knowledge this is the first study to demonstrate the enhancement of the TER in down-modulated cell monolayers by polyphenols, although SCHEPPACH et al. (1996) found that incubating rat colonic mucosa after acid-induced injury with L-glutamine increased the epithelial integrity while n-butyrate had no apparent effects, in comparison to controls.

TJs regulating the passage of ions, water, and molecules are maintained by transmembrane barrier-forming proteins including occludin and claudins. Occludin is adhesive and changes in its expression affect the permeability of the barrier, while claudins (of which 20 have been identified) polymerize into linear fibrils generating extensive networks and exhibit stronger adhesion than occludin. Transmembrane proteins are involved in cytoplasmic regulation, vesicle targeting, and cell polarity in conjunction with membrane-associated proteins (ZO), which provide direct links between the actin cytoskeleton and the sealing proteins (MITIC et al., 2000).

Determinations of the mRNA levels of ZO-1, occludin and claudin-4 after incubation of the T84 monolayers with the polyphenols revealed that they had varying effects, including both increases and decreases at various times (Fig. 3, 4, and 5). However, the expression of TJ proteins was predominantly increased by the test polyphenols. ZO-1 expression was most strongly induced by (-)-epicatechin (4.5-fold after 8 h), and the degradation products phloroglucinol (3.8-fold at 8 h), and 3,4-dihydroxytoluene (2.6-fold at 4 h, 4.9-fold at 8 h). Occludin mRNA levels were most strongly increased by phloretin (5.7-fold at 24 h), while 4-*p*-CouQA (2.9-fold at 8 h, 3.3-fold at 24 h), 5-*p*-CouQA (3.8-fold at 24 h), and quercetin 3-*O*-rhamnoside (3.1-fold at 8h, 3.7-fold 24 at h) had the strongest effects on claudin 4 mRNA expression. Since physiological concentrations were used, the detected increases in expression induced by the typical apple dihydrochalcone phloretin (which is liberated in the gastrointestinal tract from phloretin 2'-O-glucoside as reported by KAHLE et al. (2007) were only up to 5.7-fold.

Taken together, our mRNA and TER data provide evidence that incubation with apple polyphenols *in vitro* has positive effects on the intestinal barrier. The TER values of T84 monolayers were enhanced after incubation with almost all of the polyphenols used in the tests. In addition, the polyphenols generally had effects on levels of the monitored mRNAs at various time points that correlated with the TER data, although conflicting results were obtained with two of the test compounds. During incubation with 3,4-dihydroxytoluene and D-(-)-quinic acid the TER was decreased in comparison to controls, whereas at the transcript level the contents of the monitored mRNAs were significantly increased. Phloretin and its glycosides enhanced TER values and increased contents of all the monitored mRNAs at all sampling time points, except occludin mRNA levels after 4 h incubation with phloretin 2'-*O*-glucoside. TER values and mRNA data obtained in tests with hydroxycinnamic acid esters correlate well.

Effects of the flavonoid quercetin on TJ proteins have previously been described by AMASHEH et al. (2008) and SUZUKI and HARA (2009), who detected increases in levels of various TJ proteins in Caco-2 cells during incubations with quercetin. Suzuki and Hara also reported a direct correlation between TJ protein expression and the TER of Caco-2 monolayers, in accordance with our findings for quercetin.

However, this study is the first to show that esters of D-(-)-quinic acid with caffeic or *p*-coumaric acid, phloretin, phloretin 2'-*O*-glucoside and phloretin 2'-*O*-xyloglucoside are among the most potent polyphenols in apples and apple-derived foods in terms of their effects on TER and TJ protein expression.

Our study has shown that apple polyphenols and their intestinal metabolites enhanced the epithelial barrier functions of T84 colonic cell monolayers. Given the links between epithelial barrier functions and IBDs our findings suggest that the consumption of apples and apple-derived food such as apple juice has positive effects on the intestinal barrier in healthy humans and may prevent IBDs.

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## References

AMASHEH, M., SCHLICHTER, S., AMASHEH, S., MANKERTZ, J., ZEITZ, M., FROMM, M., SCHULZKE, J.D., 2008: Quercetin enhances epithelial barrier function and increases claudin-4 expression in Caco-2 cells. J. Nutr. 138, 1067-1073.

ANDERBERG, E.K., LINDMARK, T., ARTURSSON, P., 1993: Sodium caprate elicits dilatations in human intestinal tight junctions and enhances drug absorption by the paracellular route. Pharm. Res. 10, 857-864.

BERGMANN, H., ROGOLL, D., SCHEPPACH, W., MELCHER, R., RICHLING, E., 2009: The Ussing type chamber model to study the intestinal transport and modulation of specific tight-junction genes using a colonic cell line. Mol. Nutr. Food Res. 53, 1211-1225.

BOYER, J., LIU, R.H., 2004: Apple phytochemicals and their health benefits. Nutr. J. 3, 5.

CHRISTENSEN, L.P., KAACK, K., FRETTE, X.C., 2008: Selection of elderberry (*Sambucus nigra* L.) genotypes best suited for the preparation of elderflower extracts rich in flavonoids and phenolic acids. Eur. Food Res. Technol. 227, 293-305.

CLARKE, J.O., MULLIN, G.E., 2008: A review of complementary and alternative approaches to immunomodulation. Nutr. Clin. Pract. 23, 49-62.

CLAYBURGH, D.R., SHEN, L., TURNER, J.R., 2004: A porous defense: the leaky epithelial barrier in intestinal disease. Lab. Invest. 84, 282-291.

COATES, E.M., POPA, G., GILL, C.I., MCCANN, M.J., MCDOUGALL, G.J., STEWART, D., ROWLAND, I., 2007: Colon-available raspberry polyphenols

exhibit anti-cancer effects on in vitro models of colon cancer. J. Carcinog. 6, 4.

Erlejman, A.G., Fraga, C.G., Oteiza, P.I., 2006: Procyanidins protect Caco-2 cells from bile acid- and oxidant-induced damage. Free Radic. Biol. Med. 41, 1247-1256.

Farhadi, A., Banan, A., Fields, J., Keshavarzian, A., 2003: Intestinal barrier: an interface between health and disease. J. Gastroenterol. Hepatol. 18, 479-497.

Galli, V., Barbas, C., 2004: Capillary electrophoresis for the analysis of short-chain organic acids in coffee. J. Chromatogr. A 1032, 299-304.

Goncalves, B., Landbo, A.K., Knudsen, D., Silva, A.P., Moutinho-Pereira, J., Rosa, E., Meyer, A.S., 2004: Effect of ripeness and post-harvest storage on the phenolic profiles of Cherries (*Prunus avium* L.). J. Agric. Food Chem. 52, 523-530.

Gonzalez-Mariscal, L., Betanzos, A., Nava, P., Jaramillo, B.E., 2003: Tight junction proteins. Prog. Biophys. Mol. Biol. 81, 1-44.

Hilt, P., Schieber, A., Yildirim, C., Arnold, G., Klaiber, I., Conrad, J., Beifuss, U., Carle, R., 2003: Detection of phloridzin in strawberries (*Fragaria x ananassa* Duch.) by HPLC-PDA-MS/MS and NMR spectroscopy. J. Agric. Food Chem. 51, 2896-2899.

Jung, M., Triebel, S., Anke, T., Richling, E., Erkel, G., 2009: Influence of apple polyphenols on inflammatory gene expression. Mol. Nutr. Food Res. 53, 1263-1280.

Kahle, K., Huemmer, W., Kempf, M., Scheppach, W., Erk, T., Richling, E., 2007: Polyphenols are intensively metabolized in the human gastrointestinal tract after apple juice consumption. J. Agric. Food Chem. 55, 10605-10614.

Kahle, K., Kraus, M., Richling, E., 2005a: Polyphenol profiles of apple juices. Mol. Nutr. Food Res. 49, 797-806.

Kahle, K., Kraus, M., Scheppach, W., Richling, E., 2005b: Colonic availability of apple polyphenols – a study in ileostomy subjects. Mol. Nutr. Food Res. 49, 1143-1150.

Klaiber, R.G., Baur, S., Koblo, A., Carle, R., 2005: Influence of washing treatment and storage atmosphere on phenylalanine ammonia-lyase activity and phenolic acid content of minimally processed carrot sticks. J. Agric. Food Chem. 53, 1065-1072.

Kulisic-Bilusic, T., Schnabele, K., Schmoller, I., Dragovic-Uzelac, V., Krisko, A., Dejanovic, B., Milos, M., Pifat, G., 2009: Antioxidant activity versus cytotoxic and nuclear factor kappa B regulatory activities on HT-29 cells by natural fruit juices. Eur. Food Res. Technol. 228, 417-424.

Labib, S., Erb, A., Kraus, M., Wickert, T., Richling, E., 2004: The pig caecum model: a suitable tool to study the intestinal metabolism of flavonoids. Mol. Nutr. Food Res. 48, 326-332.

Laukoetter, M.G., Nava, P., Nusrat, A., 2008: Role of the intestinal barrier in inflammatory bowel disease. World J. Gastroenterol. 14, 401-407.

Lee, H.S., Wrolstad, R.E., 1988: Apple juice composition: sugar, nonvolatile acid, and phenolic profiles. J. Assoc. Off. Anal. Chem. 71, 789-794.

Mccann, M.J., Gill, C.I., O'Brien, G., Rao, J.R., Mcroberts, W.C., Hughes, P., Mcentee, R., Rowland, I.R., 2007: Anti-cancer properties of phenolics from apple waste on colon carcinogenesis in vitro. Food Chem. Toxicol. 45, 1224-1230.

Mcguckin, M.A., Eri, R., Simms, L.A., Florin, T.H., Radford-Smith, G., 2009: Intestinal barrier dysfunction in inflammatory bowel diseases. Inflamm. Bowel Dis. 15, 100-113.

Mitic, L.L., Van Itallie, C.M., Anderson, J.M., 2000: Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. Am. J. Physiol. Gastrointest. Liver Physiol. 279, G250-254.

Ohno, Y., Naganuma, T., Ogawa, T., Muramoto, K., 2006: Effect of lectins on the transport of food factors in caco-2 cell monolayers. J. Agric. Food Chem. 54, 548-553.

Olthof, M.R., Hollman, P.C., Buijsman, M.N., Van Amelsvoort, J.M., Katan, M.B., 2003: Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. J. Nutr. 133, 1806-1814.

Patterson, M.K., 1979: Measurement of growth and viablity of cells in culture. In: Jacoby, W.B., Pastan, I.H. (eds.), Methods Enzymology, Vol. 58, 150-152. Academic, New York.

Perrone, D., Farah, A., Donangelo, C.M., De Paulis, T., Martin, P.R., 2008: Comprehensive analysis of major and minor chlorogenic acids and lactones in economically relevant Brazilian coffee cultivars. Food Chem. 106, 859-867.

Powell, D.W., 1981: Barrier function of epithelia. Am. J. Physiol. 241, G275-288.

Prasad, S., Mingrino, R., Kaukinen, K., Hayes, K.L., Powell, R.M., Macdonald, T.T., Collins, J.E., 2005: Inflammatory processes have differential effects on claudins 2, 3 and 4 in colonic epithelial cells. Lab. Invest. 85, 1139-1162.

Rechner, A.R., Smith, M.A., Kuhnle, G., Gibson, G.R., Debnam, E.S., Srai, S.K., Moore, K.P., Rice-Evans, C.A., 2004: Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. Free Radic. Biol. Med. 36, 212-225.

Ruhl, I., Herrmann, K., 1985: Organic acids in vegetables. I. Brassica, leaf and bulb vegetables as well as carrots and celery. Z. Lebensm. Unters. Forsch. 180, 215-220.

Sambruy, Y., Ferruzza, S., Ranaldi, G., De Angelis, I., 2001: Intestinal cell culture models: applications in toxicology and pharmacology. Cell Biol. Toxicol. 17, 301-317.

Scalbert, A., Manach, C., Morand, C., Remesy, C., Jimenez, L., 2005: Dietary polyphenols and the prevention of diseases. Crit. Rev. Food Sci. Nutr. 45, 287-306.

Scalbert, A., Williamson, G., 2000: Dietary intake and bioavailability of polyphenols. J. Nutr. 130, 2073-2085.

Schauber, J., Svanholm, C., Termen, S., Iffland, K., Menzel, T., Scheppach, W., Melcher, R., Agerberth, B., Luhrs, H., Gudmundsson, G.H., 2003: Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: relevance of signalling pathways. Gut 52, 735-741.

Scheppach, W., Dusel, G., Kuhn, T., Loges, C., Karch, H., Bartram, H.P., Richter, F., Christl, S.U., Kasper, H., 1996: Effect of L-glutamine and n-butyrate on the restitution of rat colonic mucosa after acid induced injury. Gut 38, 878-885.

Schneider, H., Blaut, M., 2000: Anaerobic degradation of flavonoids by *Eubacterium ramulus*. Arch. Microbiol. 173, 71-75.

Schoefer, L., Mohan, R., Schwiertz, A., Braune, A., Blaut, M., 2003: Anaerobic degradation of flavonoids by *Clostridium orbiscindens*. Appl. Environ. Microbiol. 69, 5849-5854.

Sefkow, M., 2001: First efficient synthesis of chlorogenic acid. Eur. J. Org. Chem., 1137-1141.

Sefkow, M., Kelling, A., Schilde, U., 2001: First efficient syntheses of 1-, 4-, and 5-caffeoylquinic acid. Eur. J. Org. Chem., 2735-2742.

Shapiro, H., Singer, P., Halpern, Z., Bruck, R., 2007: Polyphenols in the treatment of inflammatory bowel disease and acute pancreatitis. Gut 56, 426-435.

Sousa, C., Pereira, D.M., Pereira, J.A., Bento, A., Rodrigues, M.A., Dopico-Garcia, S., Valentao, P., Lopes, G., Ferreres, F., Seabra, R.M., Andrade, P.B., 2008: Multivariate analysis of tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) phenolics: influence of fertilizers. J. Agric. Food Chem. 56, 2231-2239.

Suzuki, T., Hara, H., 2009: Quercetin enhances intestinal barrier function through the assembly of zonula occludens-2, occludin, and claudin-1 and the expression of claudin-4 in Caco-2 cells. J. Nutr. 139, 965-974.

Trugo, L.C., Macrae, R., 1984: Chlorogenic Acid Composition of Instant Coffees. Analyst 109, 263-266.

Turner, A., Chen, S.N., Joike, M.K., Pendland, S.L., Pauli, G.F., Farnsworth, N.R., 2005: Inhibition of uropathogenic Escherichia coli by cranberry juice: a new antiadherence assay. J. Agric. Food Chem. 53, 8940-8947.

Wallon, C., Braaf, Y., Wolving, M., Olaison, G., Soderholm, J.D., 2005:

Endoscopic biopsies in Ussing chambers evaluated for studies of macromolecular permeability in the human colon. Scand. J. Gastroenterol. 40, 586-595.

WILL, F., ZESSNER, H., BECKER, H., DIETRICH, H., 2007: Semi-preparative isolation and physico-chemical characterization of 4-coumaroylquinic acid and phloretin-2'-xylogluco side from laccase-oxidized apple juice. LWT-Food Sci. Technol. 40, 1344-1351.

WOJDYLO, A., OSZMIANSKI, J., LASKOWSKI, P., 2008: Polyphenolic compounds and antioxidant activity of new and old apple varieties. J. Agric. Food Chem. 56, 6520-6530.

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