



## The effects of citrus flavonoids and their metabolites on immune-mediated intestinal barrier disruption using an *in vitro* co-culture model

Yala Stevens<sup>1,2\*</sup>, Tessa de Bie<sup>3</sup>, Iris Pinheiro<sup>4</sup>, Montserrat Elizalde<sup>1</sup>, Ad Masclee<sup>1</sup> and Daisy Jonkers<sup>1</sup>

<sup>1</sup>Department of Internal Medicine, Division of Gastroenterology-Hepatology, School of Nutrition & Translational Research in Metabolism (NUTRIM), Maastricht University, Maastricht, The Netherlands

<sup>2</sup>BioActor BV, Maastricht, The Netherlands

<sup>3</sup>Division of Human Nutrition and Health, Wageningen University, Wageningen, The Netherlands

<sup>4</sup>MRM Health NV, Ghent, Belgium

(Submitted 23 June 2021 – Final revision received 28 October 2021 – Accepted 30 November 2021 – First published online 28 January 2022)

### Abstract

Hesperidin and naringin are citrus flavonoids with known anti-oxidative and anti-inflammatory properties. Evidence from previous studies indicates that both these compounds and the metabolites that are formed during intestinal metabolism are able to exert beneficial effects on intestinal barrier function and inflammation. However, so far, studies investigating the relative contributions of the various compounds are lacking. Therefore, we assessed the effect of citrus flavonoids and their intestinal metabolites on immune-mediated barrier disruption in an *in vitro* co-culture model. Caco-2 cell monolayers were placed in co-culture with phorbol 12-myristate 13-acetate-stimulated THP-1-Blue™ NF-κB cells for 30 h. At baseline, the citrus flavonoids and their metabolites were added to the apical compartment (50 or 100 μM per compound). After 24 h, THP-1 cells were incubated with lipopolysaccharide (LPS) in the basolateral compartment for 6 h. Incubation with citrus flavonoids and their metabolites did not induce changes in transepithelial electrical resistance, fluorescein isothiocyanate–dextran 4 kDa permeation or gene expression of barrier-related genes for any of the compounds tested. After LPS stimulation, NF-κB activity was significantly inhibited by all compounds (100 μM) except for one metabolite (all  $P \leq 0.03$ ). LPS-induced production of the cytokines IL-8, TNF-α and IL-6 was inhibited by most compounds (all  $P < 0.05$ ). However, levels of IL-1β were increased, which may contribute to the lack of an improved barrier effect. Overall, these results suggest that citrus flavonoids may decrease intestinal inflammation via reduction of NF-κB activity and that the parent compounds and their metabolites formed during intestinal metabolism are able to exert comparable effects.

**Key words:** Citrus flavonoids: Intestinal barrier: Intestinal inflammation: Immune-mediated barrier disruption: Co-culture model

Flavonoids are polyphenolic compounds present in vegetables, tea, red wine and fruits and are widely consumed as part of a habitual diet<sup>(1)</sup>. Flavonoids that are present in citrus fruits possess diverse beneficial effects, including anti-oxidative and anti-inflammatory properties<sup>(2,3)</sup>. Several gastrointestinal diseases, such as inflammatory bowel disease and celiac disease, have been associated with increased levels of oxidative stress and inflammatory mediators, contributing to intestinal barrier disruption<sup>(4,5,6)</sup>. Certain pro-inflammatory cytokines such as TNF-α and IFN-γ are described to increase intestinal permeability<sup>(7,8)</sup>. An increased intestinal permeability may result in translocation of luminal content, further triggering the inflammatory response, thereby contributing to disease onset and progression. Via their anti-oxidative and anti-inflammatory properties, citrus flavonoids may beneficially

affect intestinal barrier function and intestinal health. Hesperidin and naringin are two important flavonoids present in citrus fruits that are largely resistant to hydrolysis in the stomach and small intestine. Once reaching the colon, they are extensively metabolised by the gut microbiota. Here, hesperidin and naringin are first converted into their aglycones hesperetin and naringenin and subsequently into various smaller phenolics<sup>(9,10,11)</sup>. A recent study using an *in vitro* digestion model of the gastrointestinal tract showed that after digestion of hesperidin, the phenolic metabolites found at the highest concentrations were dihydrocaffeic acid, isoferulic acid, 4-hydroxyphenylacetic acid and dihydroferulic acid. These metabolites were mainly present in the compartments representing the proximal parts of the colon<sup>(12)</sup>.

**Abbreviations:** LPS, lipopolysaccharide; DMSO, dimethylsulfoxide; FITC-D4, fluorescein isothiocyanate–dextran 4 kDa; PMA, phorbol 12-myristate 13-acetate; SEAP, secreted embryonic alkaline phosphatase; TEER, transepithelial electrical resistance.

\* **Corresponding author:** Dr Y. Stevens, email [yala.stevens@maastrichtuniversity.nl](mailto:yala.stevens@maastrichtuniversity.nl)

Beneficial effects of citrus flavonoids as well as their aglycones on intestinal health have been observed in various studies employing rodent colitis models<sup>(13,14,15,16)</sup>. Citrus flavonoids at dosages ranging from 10 mg/kg to 3 g/kg resulted in decreased colonic permeability, inflammation, oxidative stress and disease activity index scores<sup>(13,14,15,16)</sup>. Furthermore, *in vitro* studies using intestinal cell lines have shown beneficial effects of citrus flavonoid aglycones or phenolic metabolites on either intestinal barrier function<sup>(17,18,19,20)</sup> or inflammation<sup>(21)</sup>, indicating that both the parent compounds that are ingested and the (phenolic) metabolites formed are able to exert beneficial effects. However, as these *in vitro* models did not include the crosstalk between epithelial cells and immune cells, assessed only one or a few metabolites and/or did not include the parent compounds, the effects of the metabolites on immune-mediated barrier disruption and their relative contribution compared with the parent compounds remain unclear.

Therefore, the aim of this study was to assess the potential beneficial effects of hesperidin, naringin and several of the main metabolites formed by bacterial metabolism in the intestine on immune-mediated intestinal barrier disruption, using an *in vitro* co-culture model of intestinal inflammation and barrier dysfunction.

## Methods

### Test compounds

The citrus flavonoid extract used in this study (MicrobiomeX®, containing >85 % hesperidin and >5 % naringin) and hesperidin (Cordiart®) were kindly provided by BioActor BV. Hesperetin, naringin, naringenin, dihydrocaffeic acid, 4-hydroxyphenylacetic acid, ferulic acid, hydrocinnamic acid, phloretic acid and phloroglucinol were purchased at Sigma-Aldrich. Isoferulic acid and dihydroferulic acid were purchased at TCI Europe. All compounds were administered at a concentration of 100 µM based on previous *in vitro* studies<sup>(17,18)</sup> and because it is anticipated that both the parent compounds and metabolites can reach concentration in this order of magnitude in the intestinal lumen after oral consumption of citrus flavonoids<sup>(12,17,22)</sup>. Furthermore, to investigate whether similar effects can be expected at lower dosages, several experiments were also performed at a dose of 50 µM for the following compounds: hesperidin, naringin, hesperetin, naringenin, isoferulic acid and 4-hydroxyphenylacetic acid. These compounds were selected as they showed the most promising effects and represent different stages of intestinal metabolism (*i.e.* parent compounds, aglycones and phenolic metabolites). All test compounds were first dissolved in dimethylsulfoxide (DMSO; AppliChem) and diluted in complete Dulbecco's Modified Eagle Medium (composition as described below) resulting in a final concentration of 0.1 % DMSO.

### Cell cultures

Caco-2 cells were derived from the American Type Culture Collection and were maintained in Dulbecco's Modified Eagle Medium high glucose (Cat no. D5796, Sigma-Aldrich), supplemented with 10 % fetal bovine serum (cat no. 26140079, Invitrogen), 1 % Antibiotic-Antimycotic (cat no. 15240062,

Invitrogen) and 1 % non-essential amino acids (cat no. 11140035, Invitrogen) at 37 °C in a 5 % CO<sub>2</sub> atmosphere.

THP-1-Blue™ NF-κB cells (InvivoGen) were kindly provided by ProDigest BVBA. These cells were maintained in Roswell Park Memorial Institute medium 1640 (cat no. R8758, Sigma-Aldrich) supplemented with 10 % fetal bovine serum (Invitrogen), 1 % Antibiotic-Antimycotic (Invitrogen) and 1 % sodium pyruvate (cat no. 11360070, Invitrogen). THP-1 cells are human monocytic leukaemia cells that differentiate into macrophage-like cells after treatment with phorbol 12-myristate 13-acetate (PMA)<sup>(23)</sup>. THP-1-Blue cells include an NF-κB-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct, enabling monitoring of NF-κB activity by use of the colorimetric QUANTI-Blue™ enzyme assay (cat code rep-qb1, InvivoGen).

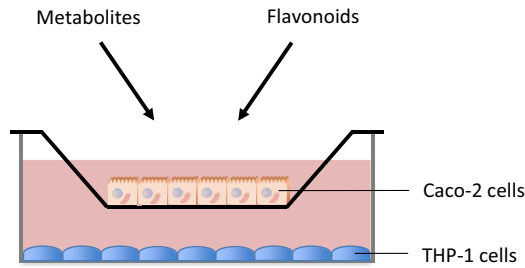
### Assessment of cytotoxicity

The cytotoxic effect of the test compounds was assessed by a fluorometric lactate dehydrogenase assay (CytoTox-ONE™ Homogeneous Membrane Integrity Assay, Promega) according to the manufacturer's protocol. Caco-2 cells in 96-well plates (Greiner CELLSTAR) were incubated with the test compounds for 24 h, after which lactate dehydrogenase release into the culture medium was determined as described previously<sup>(7)</sup>. Cytotoxicity of the test compounds was expressed as percentage of the values obtained for the 100 % cell lysis control by Triton® X-100.

### Caco-2/THP-1 co-culture experiments

Co-culture experiments were performed as described previously by Possemiers *et al.*<sup>(24)</sup>. Briefly, Caco-2 cells (passage 30–36) were seeded into 24-well Transwell inserts (cat no. 3470, Corning) at a density of 80 000 cells per insert and cultured for 14 d until a functional monolayer with a transepithelial electrical resistance (TEER) of > 330 Ω•cm<sup>2</sup> was formed<sup>(25)</sup>, before being placed in co-culture. Two days preceding the co-culture experiment, the THP-1 cells were seeded in 24-well plates at a density of 500 000 cells/well in the presence of 100 ng/mL PMA (cat no. P8139, Sigma-Aldrich) prepared in Roswell Park Memorial Institute 1640 (Sigma-Aldrich). PMA-stimulated THP-1 cells produce considerable amounts of inflammatory mediators, which when placed in co-culture with Caco-2 cells, can contribute to a disruption of the cell monolayer<sup>(25)</sup>. After 2 d of PMA treatment, the THP-1 cells were washed with PBS, placed in Caco-2 culture medium and the inserts containing the Caco-2 monolayer were placed on top of the THP-1 cells (Fig. 1). Subsequently, the test products were added apically to the Transwell inserts. Cells were also treated with culture medium containing 0.1 % DMSO as vehicle control, this condition will hereafter be referred to as the medium + DMSO control. After a treatment period of 24 h, the THP-1 cells were incubated with 500 ng/ml ultrapure lipopolysaccharide (LPS) from *E. coli* (cat code ttrl-peklps, InvivoGen) in the basolateral compartment for an additional 6 h. Binding of LPS to toll-like receptor 4 results in NF-κB activation and production of cytokines<sup>(26)</sup>. After the 6-h co-incubation period, the basolateral medium was collected and stored at -80 °C.





**Fig. 1.** Schematic overview of the co-culture model with Caco-2 cells and THP-1 cells. Caco-2 cells cultured in Transwell inserts for 14 d were placed in co-culture with PMA-stimulated THP-1-Blue™ NF- $\kappa$ B cells. After 24 h of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 h.

### Measurement of barrier function

The epithelial integrity of the Caco-2 cell monolayers was assessed by measurement of TEER, fluorescein isothiocyanate–dextran 4 kDa (FITC-D4) permeation and gene expression of different barrier-related genes. TEER of the Caco-2 monolayers was measured at baseline, after 24 h co-incubation with the test compounds and after another 6 h of co-culture with the THP-1 cells (i.e.  $t = 30$  h). TEER was measured using the EVOM<sup>2</sup> Epithelial VoltOhmmeter (World Precision Instruments). Monolayer resistance of treated wells was calculated by subtracting the TEER of empty inserts and multiplying these with the surface area of the filter membrane. Data are reported as TEER percentage of baseline values. FITC-D4 (1 mg/ml; Sigma-Aldrich) was added apically to the Caco-2 cells at  $t = 30$  h and incubated for 1 h at 37 °C, after which the basolateral medium was collected and stored at –20 °C for further analysis. FITC-D4 concentrations were measured by fluorimetry using a SpectraMax M2 microplate reader (Molecular Devices) at excitation and emission wavelengths of 485 nm and 530 nm, respectively. Gene expression of barrier-related genes (i.e. zonula occludens-1, occludin, claudin-2, claudin-3, claudin-4, E-cadherin and myosin light chain kinase) was evaluated for the following six compounds: hesperidin, naringin, hesperetin, naringenin, isoferulic acid and 4-hydroxyphenylacetic acid at  $t = 30$  h. RNA extraction, reverse transcription and qPCR were performed as described previously<sup>(7)</sup>. Briefly, RNA was extracted using the Qiagen RNeasy Mini kit (QIAGEN). After cells were lysed by adding lysis buffer to the monolayers, RNA was isolated and purified with on-column DNase digestion. All qPCR were performed with the iQ SYBR® Green Supermix (Bio-Rad Laboratories) on a thermal cycler (Bio-Rad Laboratories). The qPCR conditions used were 10 min at 95 °C, followed by 40 amplification cycles of 10 s at 95 °C and 40 s at 60 °C. Primer sequences used are listed in online Supplementary Table S1. Expressions of target genes were normalised to 18S ribosomal RNA (18S RNA) as reference gene as described previously<sup>(27)</sup>.

### Measurement of NF- $\kappa$ B activity and cytokine secretion

After stimulation with LPS, NF- $\kappa$ B activity of THP-1 cells was measured by use of the QUANTI-Blue™ enzyme assay (InvivoGen) according to the manufacturer's instructions. In brief, 20  $\mu$ l of the basolateral medium was incubated for 15 min with 200  $\mu$ l QUANTI-Blue at 37 °C. Optical density was measured at

630 nm using the Synergy HT microplate reader (BioTek Instruments). Secreted cytokine levels were quantified in basolateral medium using a cytometric bead array flex set assay (BD Biosciences) followed by measurement with a flow cytometer (BD FACS Canto II™), as described previously<sup>(28)</sup>. Levels of the pro-inflammatory cytokines IL-8, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were measured, with respective detection limits of 156.25, 39, 39 and 78 pg/ml. Data are expressed as percentage of the vehicle control to correct for limited interassay variation.

### Statistical analysis

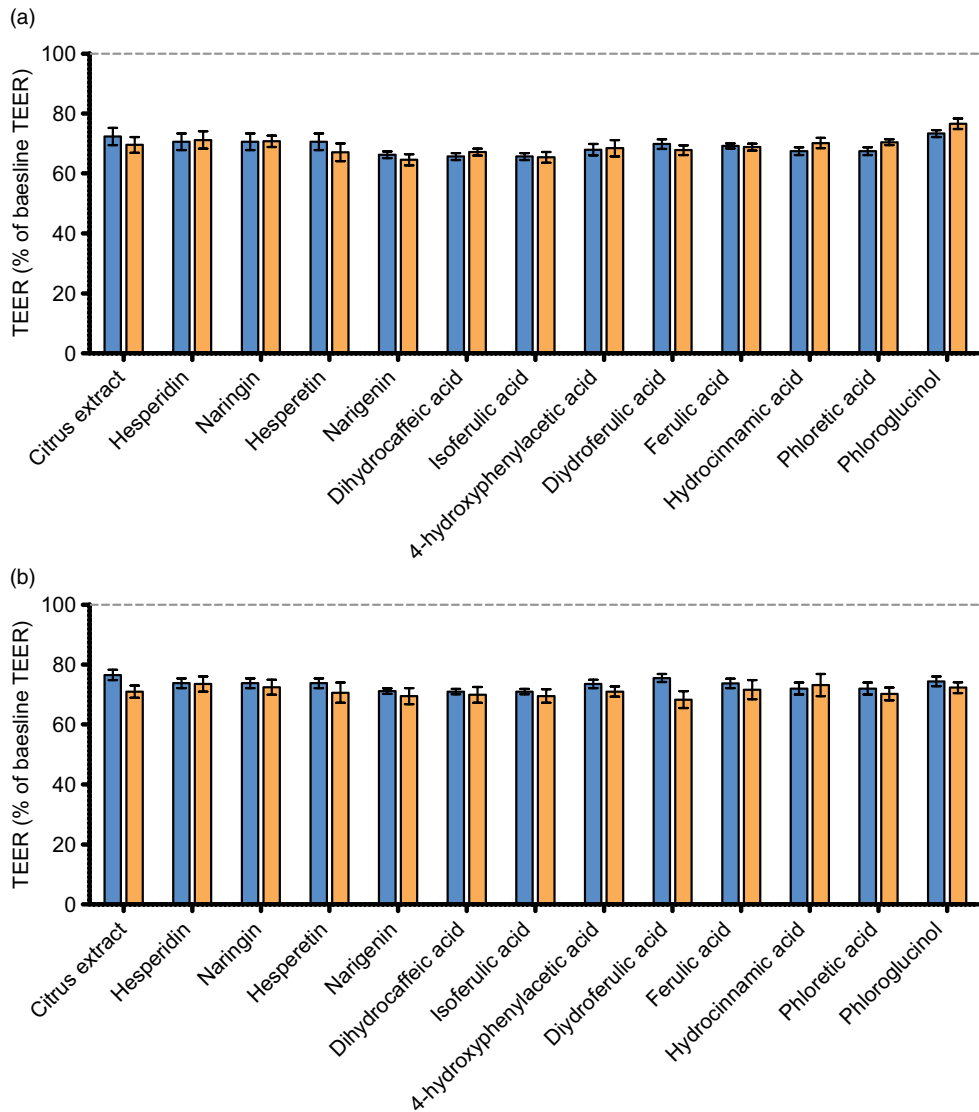
Data are presented as mean values with their standard error of the mean of three independent experiments, with three technical replicates. In the incidental case that one of the three replicates deviated more than 30 % from the median, this value was considered a technical outlier and removed from the data set. The final data were obtained from at least two independent experiments, each conducted at least in duplicate. For all cytokines, values below the detection limit were imputed with the corresponding detection limit. Statistical analyses were performed with an independent samples *t*-test using IBM SPSS Statistics version 25 (IBM Statistics) based on the individual values of each replicate. Statistical significance was set at  $P < 0.05$ . For the TEER, gene expression and cytokine results, *P*-values were corrected for multiple comparisons using the false discovery rate of Benjamini–Hochberg.

## Results

### Intestinal barrier function

As citrus flavonoids have anti-inflammatory potential, a Caco-2/THP-1 co-culture model was used<sup>(24)</sup> to assess whether these compounds may be able to exert anti-inflammatory effects and thereby attenuate cytokine-induced barrier disruption. Prior to examining these effects, the cytotoxicity of all test compounds was evaluated by measurement of lactate dehydrogenase release from cells. These results showed that there were no significant differences in percentage cytotoxicity of the test compounds at the chosen working concentration of 100  $\mu$ M as compared with the medium + DMSO control (all  $\leq 10.7$  % and  $P \leq 0.18$ ).

In this co-culture model, PMA-induced differentiation of the THP-1 cells resulted in a decrease in TEER of approximately 30 % (ranging from 23.4 to 35.4 %) compared with baseline (set at 100 %), indicating that Caco-2 monolayer integrity was compromised after 24 h of co-culture. Apical treatment with the citrus flavonoids, their aglycones and their metabolites at concentrations of 100  $\mu$ M or 50  $\mu$ M did not attenuate this decrease in TEER values (Fig. 2(a); online Supplementary Fig. S1a). After incubation for an additional 6 h in the presence of LPS, no further differences in TEER values were observed (decrease of 23.5–37.7 % compared with baseline). Treatment with the citrus flavonoid test compounds did not affect barrier function after this additional incubation period at both dosages (Fig. 2(b); online Supplementary Fig. S1b). Paracellular permeability of the monolayers was also evaluated by FITC-D4 permeation after incubation with citrus flavonoids at concentrations of 100 and 50  $\mu$ M. In



**Fig. 2.** The effects of citrus flavonoids, their aglycones and their metabolites on transepithelial electrical resistance (TEER) of Caco-2 monolayers after 24 h (a) and after 30 h (b) of co-culture with PMA-stimulated THP-1 cells. At baseline, Caco-2 cells cultured in Transwell inserts for 14 d were placed in co-culture with PMA-stimulated THP-1-Blue™ NF-κB cells. Caco-2 cells were then incubated with either the test compounds (100 μM) or culture medium containing 0.1 % DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 h of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 h. TEER was measured at baseline, after 24 h and after 30 h of incubation. TEER values are expressed as mean percentage from the baseline value ± SEM from at least two independent experiments in duplicate. No significant differences were observed between the test compounds and the medium + DMSO control. ■, control; ■, compound.

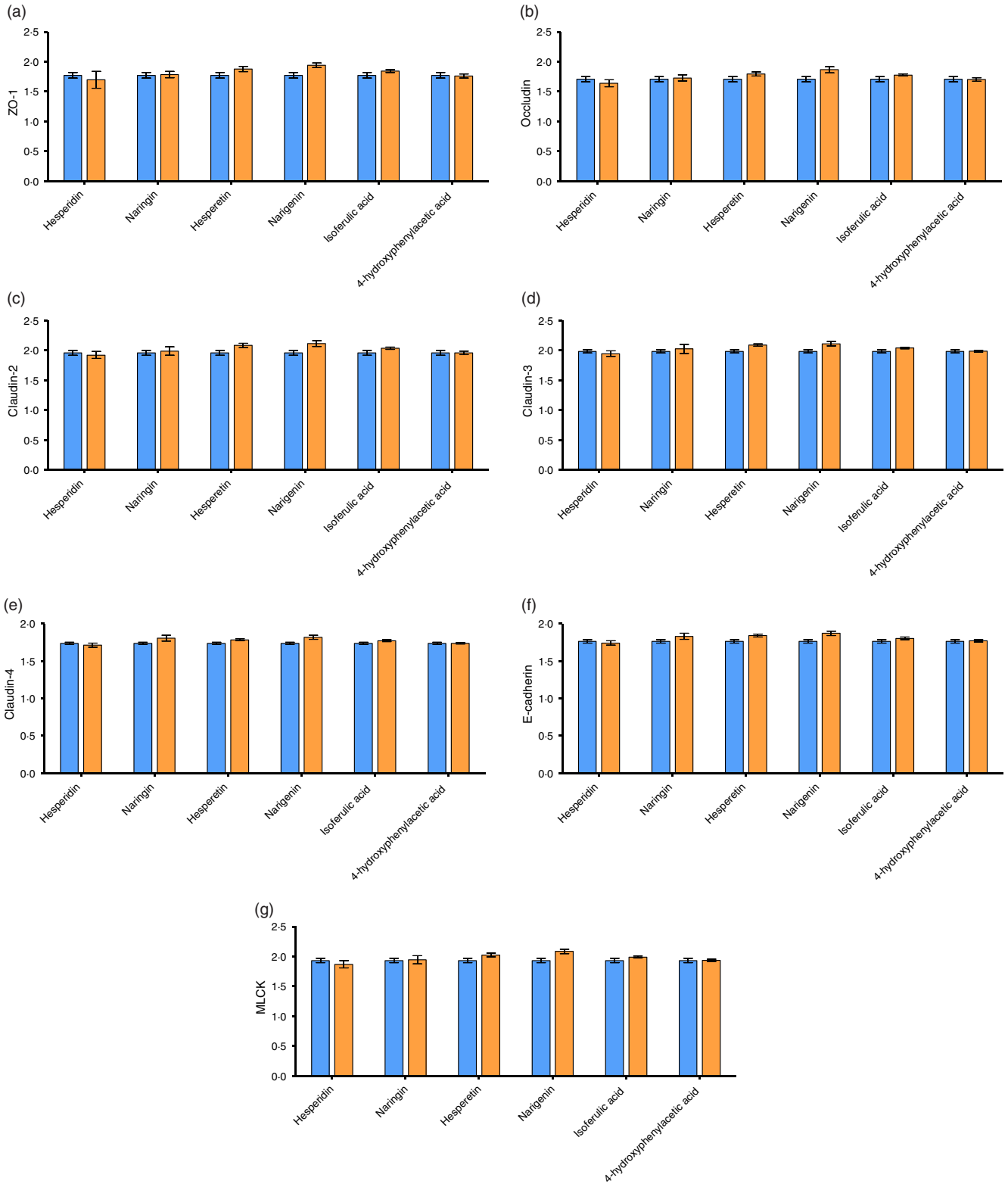
line with the TEER results, treatment with the test compounds did not affect FITC-D4 permeation (online Supplementary Fig. S2a and S2b). Furthermore, no significant effects on expression levels of tight junction or adherens junction-related genes were observed after treatment with citrus flavonoids and their key metabolites (Fig. 3).

#### NF-κB activity and cytokine secretion

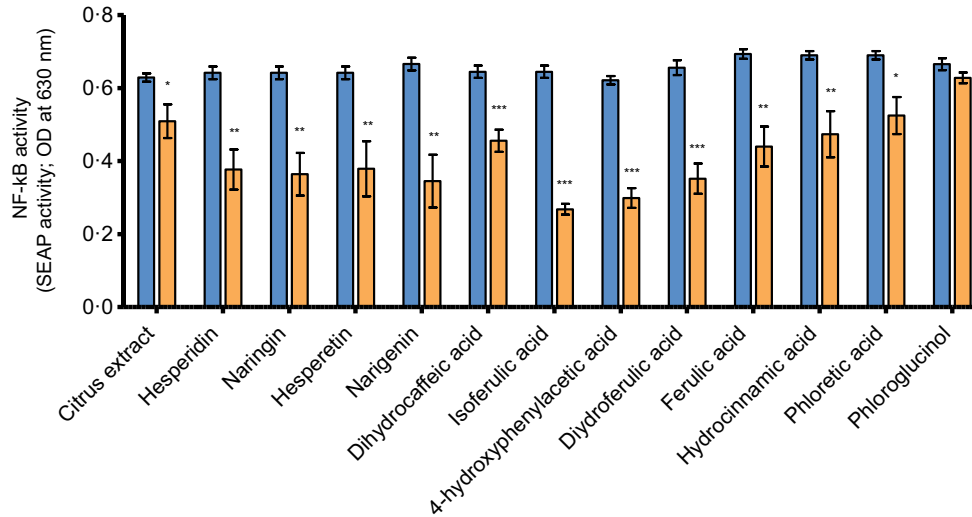
The anti-inflammatory potential of the citrus flavonoids and their metabolites was evaluated by measuring NF-κB activity and cytokine secretion in the co-culture model after the 24-h treated THP-1 cells were incubated for 6 more hours with LPS. NF-κB activity was determined by assessment of NF-κB-inducible SEAP levels

secreted into the basolateral medium. Incubation with LPS resulted in a significant increase in NF-κB-inducible SEAP levels secreted by the THP-1 cells (online Supplementary Fig. S3). The increase in NF-κB activity was significantly inhibited by all test compounds after treatment at a concentration of 100 μM except for phloroglucinol (Fig. 4; all  $P \leq 0.03$ ) and this effect seemed to be most pronounced for the phenolic metabolites isoferulic acid, 4-hydroxyphenylacetic acid and dihydroferulic acid. Except for hesperetin ( $P < 0.001$ ), no significant effects of the citrus flavonoid test compounds on NF-κB activity were observed at a lower concentration of 50 μM (online Supplementary Fig. S4).

Cytokine production was determined by measuring the levels of the pro-inflammatory cytokines IL-8, TNF-α, IL-6 and IL-1β in the basolateral medium. Stimulation with LPS during the last 6 h



**Fig. 3.** The effects of citrus flavonoids, their aglycones and their metabolites on expression levels of tight junction or adherens junction-related genes of Caco-2 mono-layers in co-culture with PMA-simulated THP-1 cells. Caco-2 cells cultured in Transwell inserts for 14 d were placed in co-culture with PMA-simulated THP-1-Blue™ NF- $\kappa$ B cells. Caco-2 cells were then incubated with either culture medium containing 0.1 % DMSO (medium + DMSO control) or test compounds (100  $\mu$ M) added to the apical compartment. RNA was extracted after 30 h of incubation. Expressions of target genes were normalised to 18S ribosomal RNA (18S RNA) as reference gene. Values are represented as mean values with their standard error of the mean. No significant differences were observed between the test compounds and the medium + DMSO control. A: ZO-1; B: occludin; C: claudin-2; D: claudin-3; E: claudin-4; F: E-cadherin; G: MLCK. ZO-1, zonula occludens-1; MLCK, myosin light chain kinase. ■, control; ■, compound.



**Fig. 4.** The effects of citrus flavonoids, their aglycones and their metabolites on basolateral secretion of NF- $\kappa$ B-inducible SEAP after LPS stimulation by PMA-stimulated THP-1 cells in co-culture with Caco-2 cells. At baseline, Caco-2 cells were placed in co-culture with PMA-stimulated THP-1-Blue™ NF- $\kappa$ B cells. Caco-2 cells were then incubated with either the test compounds (100  $\mu$ M) or culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 h of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 h. Levels of NF- $\kappa$ B-inducible SEAP were measured in basolateral medium after 30 h of incubation. Values are represented as mean values with their standard error of the mean from at least two independent experiments in duplicate. \* represents significant difference from medium + DMSO control (\*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ). ■, control + LPS; ■, compound + LPS.

of incubation resulted in significantly increased levels of these cytokines as compared with the control condition (cells treated with culture medium alone) (online Supplementary Fig. s5). Production of IL-8 was significantly inhibited by hesperidin, naringin, hesperetin, naringenin, isoferulic acid, 4-hydroxyphenylacetic acid, ferulic acid and hydrocinnamic acid (Fig. 5(a), all  $P \leq 0.02$ ). TNF- $\alpha$  levels were also significantly inhibited for all the aforementioned compounds and also for dihydroferulic acid (Fig. 5(b), all  $P \leq 0.008$ ). For IL-6, a similar pattern of inhibitory activity of the different test compounds could be observed, which was significant for isoferulic acid, dihydroferulic acid, ferulic acid and hydrocinnamic acid (Fig. 5(c), all  $P \leq 0.01$ ) and showed a trend towards significance for 4-hydroxyphenylacetic acid ( $P = 0.05$ ). Although no IL-6 levels are depicted for hesperidin, naringin, hesperetin and naringenin, as the majority of the values were below the detection limit, the results do support the previous findings indicating that these compounds are able to inhibit the production of pro-inflammatory cytokines.

In contrast, levels of IL-1 $\beta$  were increased when compared with the medium + DMSO control, being significant for hesperidin, naringin, hesperetin, naringenin, dihydrocaffeic acid, isoferulic acid, 4-hydroxyphenylacetic acid, dihydroferulic acid, ferulic acid and hydrocinnamic acid (Fig. 5(d), all  $P \leq 0.02$ ).

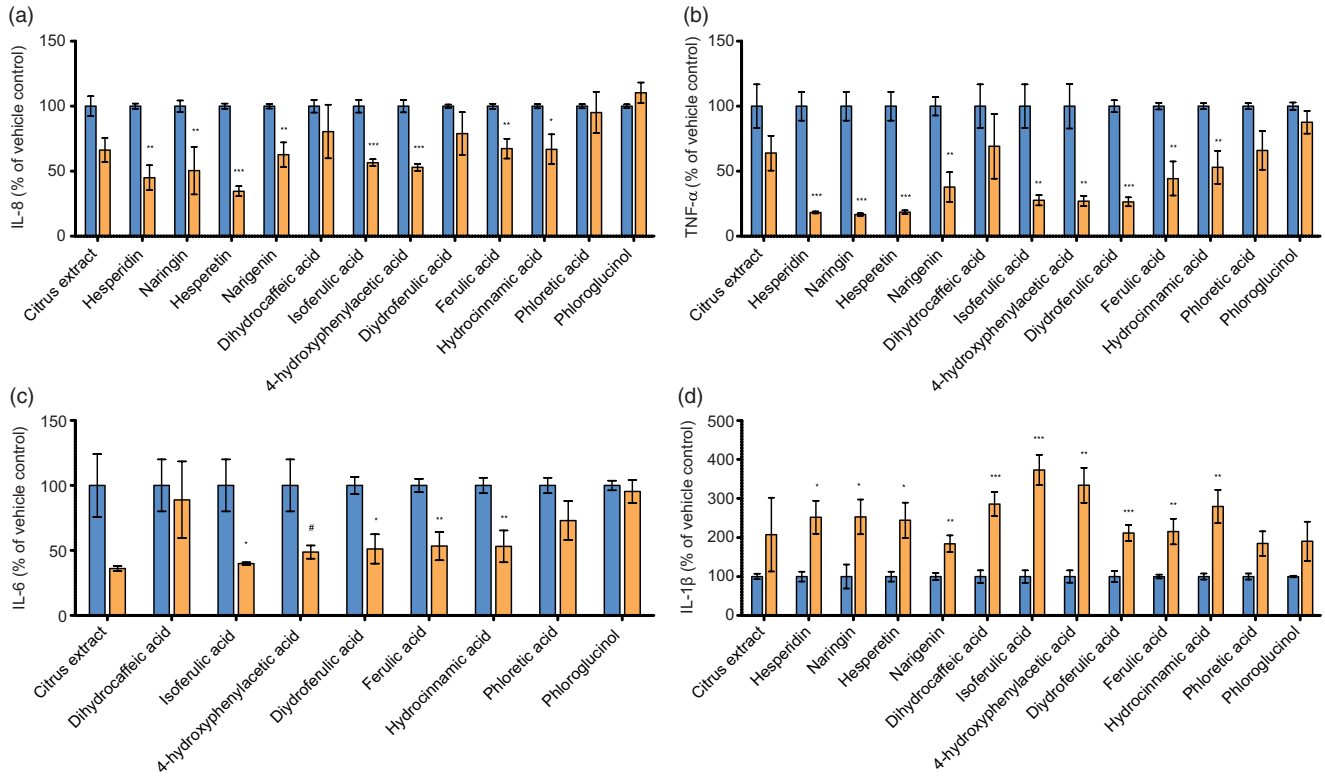
## Discussion

In this *in vitro* study, we assessed the potential beneficial effects of the citrus flavonoids hesperidin and naringin, their aglycone forms and several of their phenolic metabolites on immune-mediated barrier disruption. Incubation with the citrus flavonoids and their metabolites did not induce changes in TEER, FITC-D4 permeation or expression of barrier-related genes for

any of the compounds tested. However, LPS-induced NF- $\kappa$ B activity and production of the cytokines IL-8, TNF- $\alpha$  and IL-6 were inhibited by most compounds, whereas levels of IL-1 $\beta$  were found to be increased.

Chronic intestinal inflammation is characterised among others by an increased production of pro-inflammatory cytokines, which are important mediators that can disrupt the intestinal barrier via modulation of expression and functioning of tight junction proteins<sup>(5)</sup>. Intestinal barrier disruption, associated with local or systemic inflammation, has been observed in several gastrointestinal and metabolic diseases, where it can play a role in the onset and progression of disease<sup>(29)</sup>. Recently, we found plasma of Crohn's disease patients to induce epithelial barrier disruption in 3D Caco-2 cysts<sup>(7)</sup>.

In the present study, an *in vitro* co-culture system was used, mimicking immune-mediated barrier disruption by combining the epithelial Caco-2 cell line with activated macrophages<sup>(25)</sup>. Treatment with citrus flavonoids, their aglycone forms and their phenolic metabolites did not attenuate the decrease in barrier integrity that was observed after 24 h of co-culture. In contrast to our findings, two previous studies using Caco-2 cells did show positive effects after treatment with the aglycones hesperetin and naringenin, as shown by increased expression of several tight junction proteins and a 5–15% increase in TEER<sup>(17,18)</sup>. The aforementioned studies focused on improving barrier function in the absence of a stressor, while the present study was performed in order to assess whether citrus flavonoid treatment can attenuate an inflammation-induced disruption of barrier function, as was suggested based on animal studies. Using chemically induced mice models of colitis, it was shown that oral administration with naringenin or hesperetin was able to (partly) suppress dextran sulfate sodium-induced barrier impairment, as measured by FITC-D4 and expression of tight junction proteins<sup>(13,14)</sup>. In these



**Fig. 5.** The effects of citrus flavonoids, their aglycones and their metabolites on basolateral secretion of IL-8 (a), TNF- $\alpha$  (b), IL-6 (c) and IL-1 $\beta$  (d) after LPS stimulation by PMA-stimulated THP-1 cells in co-culture with Caco-2 cells. At baseline, Caco-2 cells were placed in co-culture with PMA-stimulated THP-1-Blue™ NF- $\kappa$ B cells. Caco-2 cells were then incubated with either the test compounds (100  $\mu$ M) or culture medium containing 0.1% DMSO as vehicle control (medium + DMSO control) added to the apical compartment. After 24 h of incubation, THP-1 cells were incubated with 500 ng/ml LPS in the basolateral compartment for an additional 6 h. Cytokine levels were measured in basolateral medium after 30 h of incubation. Values are represented as mean values with their standard error of the mean from at least two independent experiments in duplicate. Absolute levels of IL-8: 116–806 ng/ml; absolute levels of TNF- $\alpha$ : 39–959 pg/ml; absolute levels of IL-6: 39–361 pg/ml; absolute levels of IL-1 $\beta$ : 78–1182 pg/ml. \* represents significant difference from medium + DMSO control (\*:  $P < 0.05$ ; \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ ; #:  $P = 0.05$ ). ■, control + LPS; ■, compound + LPS.

mice studies, the citrus flavonoid aglycones were given at a dose of 3 g/kg per d. This dose was estimated to correspond to a daily dose of approximately 2 g in humans<sup>(13)</sup>, while more physiological dosages of approximately 50–500 mg, which are also typically used in human intervention studies, are expected to result in luminal concentrations similar to the concentration of 100  $\mu$ M that was used in the present study<sup>(12,17,22)</sup>.

Despite the lack of an effect on barrier disruption after treatment with the different test compounds, we wanted to evaluate whether they could have an effect on markers of immune function. All test compounds, except for phloroglucinol, were found to significantly attenuate the NF- $\kappa$ B activity induced by LPS after treatment at a concentration of 100  $\mu$ M. The LPS-induced secretion of IL-8, TNF- $\alpha$  and IL-6 was also reduced by most of the tested compounds. Overall, these anti-inflammatory effects seemed most pronounced for hesperidin, naringin, hesperetin, naringenin and the phenolics isoferulic acid and 4-hydroxyphenylacetic acid. Previous *in vitro* studies with various experimental setups corroborate our findings by providing evidence that citrus flavonoids and their aglycone forms exert anti-inflammatory effects, partly by inhibiting the activation of the NF- $\kappa$ B signalling pathway<sup>(30,31,32,33)</sup>. Furthermore, although these effects have not been studied extensively for all phenolic metabolites that were tested here, previous studies confirm

that several of these compounds possess anti-inflammatory properties<sup>(21,34,35,36,37,38,39,40)</sup>.

In the present study, however, citrus flavonoid treatment resulted in increased levels of the cytokine IL-1 $\beta$ . These results were consistent among the various test compounds and unexpected, as activated NF- $\kappa$ B induces the transcription of cytokine genes, including IL-1 $\beta$ . Although the increased IL-1 $\beta$  levels in combination with an inhibition of NF- $\kappa$ B activity seem contradictory, a possible explanation for these findings might be an increased pro-IL-1 $\beta$  processing. In a study by Greten *et al.*<sup>(34)</sup>, LPS treatment of IKK $\beta$ -deficient macrophages resulted in increased IL-1 $\beta$  secretion, while the expression of pro-IL-1 $\beta$  was decreased, suggesting that the increased IL-1 $\beta$  levels might be due to an increase in pro-IL-1 $\beta$  processing that was already present in the cell. Another possible explanation may be that because the NF- $\kappa$ B activity was not completely inhibited by the test compounds, in contrast to the reduction in IL-8, TNF- $\alpha$  and IL-6 secretion, some cytokines including IL-1 $\beta$  may still have been up-regulated. How our findings relate to the *in vivo* situation is however not clear. For example, a study using a mouse model of colonic inflammation showed reduced IL-1 $\beta$  levels together with a decrease in other inflammation markers such as TNF- $\alpha$  and nuclear NF- $\kappa$ B translocation after oral administration of bergamot juice, although this could also have been the effect of other compounds present in the juice<sup>(41)</sup>.

As citrus flavonoids are metabolised by the intestinal microbiota, it is also important to take the bioactivity of the large number of different metabolites that are formed and their relative contribution into account. The observed effects on NF- $\kappa$ B activity and cytokine production showed some variation between the different compounds that were tested. Phloroglucinol did not significantly affect NF- $\kappa$ B activity or cytokine production, while the citrus extract, dihydrocaffeic acid and phloretic acid significantly inhibited NF- $\kappa$ B activity but not the production of all cytokines. Although it is not entirely clear why the effects on cytokine production are not in line with the NF- $\kappa$ B activity results for these compounds, it should be noted that a slight (non-significant) inhibition could be observed in most cases. Overall, the parent compounds, aglycone forms and the phenolic metabolites isoferulic acid and 4-hydroxyphenylacetic acid showed the most potent (anti-)inflammatory effects of which the order of magnitude was similar. This shows that the parent compounds and several of the metabolites can exert comparable effects. It should be noted however that *in vivo*, the exact concentrations in the lumen after oral ingestion of citrus flavonoids will most likely be different for each compound and it is possible that not all compounds are able to reach a luminal concentration of 100  $\mu$ M. Furthermore, the anti-inflammatory effects found after treatment with a dose of 100  $\mu$ M citrus flavonoids were not observed at a lower dose of 50  $\mu$ M.

Although there is some variation in literature about the specific metabolites that are formed during intestinal metabolism of citrus flavonoids and their concentrations, evidence suggests that the highest concentrations of the aglycone forms and smaller metabolites (including isoferulic acid and 4-hydroxyphenylacetic acid) can be found in the more proximal parts of the colon, while the parent compounds are present at the highest concentrations in the small intestine<sup>(42)</sup>. Based on the results of the present study, it is therefore highly likely that citrus flavonoid ingestion will result in anti-inflammatory effects in the small intestine as well as the proximal colon and that especially in the colon effects *in vivo* will be more pronounced than *in vitro* as they are the result of a combination of the effects of the various compounds present in the intestinal lumen. Furthermore, similar to the *in vivo* situation, the parent compounds are very poorly transported across the epithelium while the aglycones and several phenolics can be converted into phase II metabolites by Caco-2 cells and are also transported to the basolateral compartment<sup>(12,43,44,45)</sup>. As the anti-inflammatory effects of the parent compounds and the most potent metabolites are of the same order of magnitude, this is likely the result of a combination of luminal and basolateral effects. Future research assessing the absorption, metabolism and transport of these compounds by intestinal epithelial cells may contribute to further insight in the underlying mechanisms.

In the present study, we used a validated co-culture system of Caco-2/THP-1 cells as model for intestinal inflammation and cytokine-induced barrier disruption<sup>(24,25)</sup>. THP-1 cells in this model are treated with PMA to induce differentiation into activated macrophages, as was supported by the secretion of inflammatory markers, such as NF- $\kappa$ B-inducible SEAP, IL-8 and IL-1 $\beta$ . At the same time, a significant decrease in TEER values of the Caco-2 monolayer was observed, indicating that the barrier

was disrupted. In order to further induce an inflammatory environment, the activated macrophages were stimulated with LPS after 24 h of co-culture, resulting in an increase in NF- $\kappa$ B activity and (enhanced) secretion of the pro-inflammatory cytokines IL-8, IL-1 $\beta$ , TNF- $\alpha$  and IL-6. Based on data from previous studies<sup>(46,47)</sup>, it is likely that the inflammatory markers measured basolaterally before and after LPS stimulation were secreted by the activated macrophages, without additional effects of the Caco-2 cells. This increased production of pro-inflammatory mediators was not associated with further disruption of the epithelial barrier, possibly because the effect of some of the cytokines was not concentration dependent and/or the maximal decrease in TEER already occurred as a result of basal cytokine release by the activated macrophages<sup>(48)</sup>. Treatment with the citrus flavonoids and their metabolites significantly inhibited NF- $\kappa$ B activity and the production of IL-8, TNF- $\alpha$  and IL-6, but this effect did not result in an improved barrier function. For some compounds, the effects on LPS-induced and basal cytokine production may not be the same<sup>(24,40)</sup> and the citrus flavonoid compounds may therefore have only reduced the release of cytokine secretion stimulated by LPS without affecting basal levels. However, we cannot exclude that the lack of an effect on TEER, FITC-D4 permeation or the expression of barrier-related genes after treatment with the test compounds may be due to the increased levels of IL-1 $\beta$  found. IL-1 $\beta$  can lead to barrier disruption, as shown by multiple *in vitro* studies using Caco-2 monolayers<sup>(48,49,50)</sup>.

The validated co-culture model<sup>(24,25)</sup> that was used provides a better reflection of the *in vivo* situation than a mono-culture, as it includes crosstalk between immune cells and epithelial cells. However, many components of the *in vivo* situation are not represented in this model. For instance, this model does not include other immune cells nor the intestinal microbial population, which varies between subjects and is affected by, for example, disease status, medication use and dietary intake<sup>(51)</sup>. The microbiota composition in particular might be important, as we know that bacteria can convert the citrus flavonoids into bioactive metabolites<sup>(9,10,11)</sup> and emerging evidence also shows that citrus flavonoids may induce alterations in the microbiota composition and activity<sup>(52,53)</sup>. Therefore, the present *in vitro* findings warrant further research and validation in human subjects.

In conclusion, we have demonstrated that treatment with citrus flavonoid compounds did result in a significant inhibition of NF- $\kappa$ B activity and production of several inflammatory cytokines for both the citrus flavonoids and several of their metabolites, but did not protect against an immune-mediated barrier disruption. Overall, these results suggest that citrus flavonoids may decrease intestinal inflammation via reduction of NF- $\kappa$ B activity and that the parent compounds and their metabolites formed during intestinal metabolism are able to exert comparable effects.

### Acknowledgements

We thank Lisa van de Ven and Monique van Erp for technical assistance.

This work was in part funded by BioActor B.V., that is, by contributing to the consumable costs and salary of Y. S. BioActor B.V. had no further role in the design, analysis or writing of this article.





Y. S.: conceptualisation, methodology, investigation, formal analysis, writing – original draft. T. D. B.: investigation. I. P.: conceptualisation, methodology, resources, writing – review and editing. M. E.: investigation. A. M.: conceptualisation, methodology, writing – review and editing, supervision. D. J.: conceptualisation, methodology, writing – review and editing, supervision.

Y. S. is an employee of BioActor B.V. D. J. is involved in research projects as part of private public partnership grants (i.e. TKI/Well on Wheat project and CCC NWO Carbokinetics) and in H2020 DISCOVERIE nr. 848228. A. M. has received a ZON MW, The Netherlands Organization for Health Research and Development, health care efficiency grant to evaluate efficacy of peppermint oil in IBS; has received an unrestricted research grant from Will Pharma SA and received research funding from Allergan and Grünenthal on IBS topics; has given scientific advice to Bayer (topic: IBS), to Kyowa Kirin (topic: constipation) and to Takeda (topic: gastroparesis); has received funding from Pentax Europe GmBH and received funding from the Dutch Cancer Society related to endoscopy and to colorectal polyps. T. D. B., I. P. and M. E. report no conflict of interest.

### Supplementary material

For supplementary materials referred to in this article, please visit <https://doi.org/10.1017/S0007114521004797>

### References

- Manach C, Scalbert A, Morand C, *et al.* (2004) Polyphenols: food sources and bioavailability. *Am J Clin Nutr* **79**, 727–747.
- Testai L & Calderone V (2017) Nutraceutical value of citrus flavanones and their implications in cardiovascular disease. *Nutrients* **9**, 502.
- Yi L, Ma S & Ren D (2017) Phytochemistry and bioactivity of citrus flavonoids: a focus on antioxidant, anti-inflammatory, anticancer and cardiovascular protection activities. *Phytochem Rev* **16**, 479–511.
- Tian T, Wang Z & Zhang J (2017) Pathomechanisms of oxidative stress in inflammatory bowel disease and potential antioxidant therapies. *Oxid Med Cell Longev* **2017**, 4535194.
- Turner JR (2009) Intestinal mucosal barrier function in health and disease. *Nat Rev Immunol* **9**, 799–809.
- Ferretti G, Bacchetti T, Masciangelo S, *et al.* (2012) Celiac disease, inflammation and oxidative damage: a nutrigenetic approach. *Nutrients* **4**, 243–257.
- Xu P, Elamin E, Elizalde M, *et al.* (2019) Modulation of intestinal epithelial permeability by plasma from patients with Crohn's disease in a three-dimensional cell culture model. *Sci Rep* **9**, 2030.
- Smyth D, Phan V, Wang A, *et al.* (2011) Interferon- $\gamma$ -induced increases in intestinal epithelial macromolecular permeability requires the Src kinase Fyn. *Lab Invest* **91**, 764–777.
- Pereira-Caro G, Borges G, Ky I, *et al.* (2015) *In vitro* colonic catabolism of orange juice (poly)phenols. *Mol Nutr Food Res* **59**, 465–475.
- Brett GM, Hollands W, Needs PW, *et al.* (2009) Absorption, metabolism and excretion of flavanones from single portions of orange fruit and juice and effects of anthropometric variables and contraceptive pill use on flavanone excretion. *Br J Nutr* **101**, 664–675.
- Nielsen ILF, Chee WSS, Poulsen L, *et al.* (2006) Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *J Nutr* **136**, 404–408.
- Van Rymenant E, Salden B, Voorspoels S, *et al.* (2018) A critical evaluation of *in vitro* hesperidin 2S Bioavailability in a model combining luminal (microbial) digestion and caco-2 cell absorption in comparison to a randomized controlled human trial. *Mol Nutr Food Res* **62**, e1700881.
- Azuma T, Shigeshiro M, Kodama M, *et al.* (2013) Supplemental naringenin prevents intestinal barrier defects and inflammation in colitic mice. *J Nutr* **143**, 827–834.
- Shigeshiro M, Tanabe S & Suzuki T (2013) Dietary polyphenols modulate intestinal barrier defects and inflammation in a murine model of colitis. *J Funct Foods* **5**, 949–955.
- Xu L, Yang ZL, Li P, *et al.* (2009) Modulating effect of hesperidin on experimental murine colitis induced by dextran sulfate sodium. *Phytotherapy* **16**, 989–995.
- Crespo ME, Galvez J, Cruz T, *et al.* (1999) Anti-inflammatory activity of diosmin and hesperidin in rat colitis induced by TNBS. *Planta Medica* **65**, 651–653.
- Noda S, Tanabe S & Suzuki T (2012) Differential effects of flavonoids on barrier integrity in human intestinal Caco-2 cells. *J Agric Food Chem* **60**, 4628–4633.
- Noda S, Tanabe S & Suzuki T (2013) Naringenin enhances intestinal barrier function through the expression and cytoskeletal association of tight junction proteins in Caco-2 cells. *Mol Nutr Food Res* **57**, 2019–2028.
- Bergmann H, Rogoll D, Scheppach W, *et al.* (2009) The using 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.
- He S, Liu F, Xu L, *et al.* (2016) Protective effects of ferulic acid against heat stress-induced intestinal epithelial barrier dysfunction *in vitro* and *in vivo*. *PLoS One* **11**, e0145236.
- Larrosa M, Luceri C, Vivoli E, *et al.* (2009) Polyphenol metabolites from colonic microbiota exert anti-inflammatory activity on different inflammation models. *Mol Nutr Food Res* **53**, 1044–1054.
- Gao K, Xu A, Krul C, *et al.* (2006) Of the major phenolic acids formed during human microbial fermentation of tea, citrus, and soy flavonoid supplements, only 3,4-dihydroxyphenylacetic acid has antiproliferative activity. *J Nutr* **136**, 52–57.
- Takashiba S, Van Dyke TE, Amar S, *et al.* (1999) Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor  $\kappa$ B. *Infect Immun* **67**, 5573–5578.
- Possemiers S, Pinheiro I, Verhelst A, *et al.* (2013) A dried yeast fermentate selectively modulates both the luminal and mucosal gut microbiota and protects against inflammation, as studied in an integrated *in vitro* approach. *J Agric Food Chem* **61**, 9380–9392.
- Satsu H, Ishimoto Y, Nakano T, *et al.* (2006) Induction by activated macrophage-like THP-1 cells of apoptotic and necrotic cell death in intestinal epithelial Caco-2 monolayers via tumor necrosis factor- $\alpha$ . *Exp Cell Res* **312**, 3909–3919.
- Sharif O, Bolshakov VN, Raines S, *et al.* (2007) Transcriptional profiling of the LPS induced NF- $\kappa$ B response in macrophages. *BMC Immunol* **8**, 1.
- Wilms E, Jonkers D, Savelkoul HFJ, *et al.* (2019) The impact of pectin supplementation on intestinal barrier function in healthy young adults and healthy elderly. *Nutrients* **11**, 1554.
- Oth T, Habets T, Germeraad WTV, *et al.* (2018) Pathogen recognition by NK cells amplifies the pro-inflammatory cytokine production of monocyte-derived DC via IFN- $\gamma$ . *BMC Immunol* **19**, 8.
- Vancamelbeke M & Vermeire S (2017) The intestinal barrier: a fundamental role in health and disease. *Expert Rev Gastroenterol Hepatol* **11**, 821–834.

30. Liu X, Wang N, Fan S, *et al.* (2016) The citrus flavonoid naringenin confers protection in a murine endotoxaemia model through AMPK-ATF3-dependent negative regulation of the TLR4 signalling pathway. *Sci Rep* **6**, 39735.
31. Hamalainen M, Nieminen R, Vuorela P, *et al.* (2007) Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF- $\kappa$ B activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF- $\kappa$ B activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. *Mediators Inflamm* **2007**, 45673.
32. Yang HL, Chen SC, Senthil Kumar KJ, *et al.* (2012) Antioxidant and anti-inflammatory potential of hesperetin metabolites obtained from hesperetin-administered rat serum: an ex vivo approach. *J Agric Food Chem* **60**, 522–532.
33. Nazari M, Ghorbani A, Hekmat-Doost A, *et al.* (2011) Inactivation of nuclear factor- $\kappa$ B by citrus flavanone hesperidin contributes to apoptosis and chemo-sensitizing effect in Ramos cells. *Eur J Pharmacol* **650**, 526–533.
34. Greten FR, Arkan MC, Bollrath J, *et al.* (2007) NF- $\kappa$ B is a negative regulator of IL-1 $\beta$  secretion as revealed by genetic and pharmacological inhibition of IKK $\beta$ . *Cell* **130**, 918–931.
35. Dilshara MG, Lee KT, Jayasooriya RG, *et al.* (2014) Downregulation of NO and PGE2 in LPS-stimulated BV2 microglial cells by trans-isoferulic acid via suppression of PI3K/Akt-dependent NF- $\kappa$ B and activation of Nrf2-mediated HO-1. *Int Immunopharmacol* **18**, 203–211.
36. Nile SH, Ko EY, Kim DH, *et al.* (2016) Screening of ferulic acid related compounds as inhibitors of xanthine oxidase and cyclooxygenase-2 with anti-inflammatory activity. *Rev Bras Farmacogn* **26**, 50–55.
37. Lampiasi N & Montana G (2018) An *in vitro* inflammation model to study the Nrf2 and NF- $\kappa$ B crosstalk in presence of ferulic acid as modulator. *Immunobiology* **223**, 349–355.
38. Koshiguchi M, Komazaki H, Hirai S, *et al.* (2017) Ferulic acid suppresses expression of tryptophan metabolic key enzyme indoleamine 2, 3-dioxygenase via NF $\kappa$ B and p38 MAPK in lipopolysaccharide-stimulated microglial cells. *Biosci Biotechnol Biochem* **81**, 966–971.
39. Navarrete S, Alarcon M & Palomo I (2015) Aqueous extract of tomato (*Solanum lycopersicum* L.) and ferulic acid reduce the expression of TNF- $\alpha$  and IL-1 $\beta$  in LPS-activated macrophages. *Molecules* **20**, 15319–15329.
40. Lampiasi N & Montana G (2016) The molecular events behind ferulic acid mediated modulation of IL-6 expression in LPS-activated Raw 264.7 cells. *Immunobiology* **221**, 486–493.
41. Impellizzeri D, Bruschetta G, Di Paola R, *et al.* (2015) The anti-inflammatory and antioxidant effects of bergamot juice extract (BJE) in an experimental model of inflammatory bowel disease. *Clin Nutr* **34**, 1146–1154.
42. Stevens Y, Rymenant EV, Grootaert C, *et al.* (2019) The intestinal fate of citrus flavanones and their effects on gastrointestinal health. *Nutrients* **11**, 1464.
43. Brand W, van der Wel PA, Rein MJ, *et al.* (2008) Metabolism and transport of the citrus flavonoid hesperetin in Caco-2 cell monolayers. *Drug Metab Dispos* **36**, 1794–1802.
44. Kobayashi S & Konishi Y (2008) Transepithelial transport of flavanone in intestinal Caco-2 cell monolayers. *Biochem Biophys Res Commun* **368**, 23–29.
45. Kobayashi S, Tanabe S, Sugiyama M, *et al.* (2008) Transepithelial transport of hesperetin and hesperidin in intestinal Caco-2 cell monolayers. *Biochim Biophys Acta* **1778**, 33–41.
46. Kampfer AAM, Urban P, Gioria S, *et al.* (2017) Development of an *in vitro* co-culture model to mimic the human intestine in healthy and diseased state. *Toxicol In Vitro* **45**, 31–43.
47. Eckmann L, Jung HC, Schürer-Maly C, *et al.* (1993) Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8. *Gastroenterology* **105**, 1689–1697.
48. Al-Sadi RM & Ma TY (2007) IL-1 $\beta$  causes an increase in intestinal epithelial tight junction permeability. *J Immunol* **178**, 4641–4649.
49. Al-Sadi R, Ye D, Said HM, *et al.* (2010) IL-1 $\beta$ -induced increase in intestinal epithelial tight junction permeability is mediated by MEKK-1 activation of canonical NF- $\kappa$ B pathway. *Am J Pathol* **177**, 2310–2322.
50. Al-Sadi R, Guo S, Ye D, *et al.* (2013) Mechanism of IL-1 $\beta$  modulation of intestinal epithelial barrier involves p38 kinase and activating transcription factor-2 activation. *J Immunol* **190**, 6596–6606.
51. Lozupone CA, Stombaugh JI, Gordon JI, *et al.* (2012) Diversity, stability and resilience of the human gut microbiota. *Nature* **489**, 220–230.
52. Brasili E, Hassimotto NMA, Del Chierico F, *et al.* (2019) Daily consumption of orange juice from citrus *Sinensis* L. Osbeck cv. Cara Cara and cv. Bahia differently affects gut microbiota profiling as unveiled by an integrated meta-omics approach. *J Agric Food Chem* **67**, 1381–1391.
53. Lima ACD, Cecatti C, Fidelix MP, *et al.* (2019) Effect of daily consumption of orange juice on the levels of blood glucose, lipids, and gut microbiota metabolites: controlled clinical trials. *J Med Food* **22**, 202–210.