**ORIGINAL ARTICLE**

WILEY | Journal of Animal Physiology and Animal Nutrition

# Influence of phytogenics on recovery of the barrier function of intestinal porcine epithelial cells after a calcium switch

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## Abstract

**Background**: The gut barrier is essential for animal health as it prevents the passage of potentially harmful foreign substances. The epithelial tight junctions support the intestinal barrier and can be disrupted by stress caused, for example, by pathogens or dietary or environmental factors, predisposing the host to disease. In animal husbandry, phytogenics (plant-derived feed additives) are used to support and maintain growth, feed efficiency and health. Therefore, several phytogenics were tested in vitro for their influence on the barrier function recovery of intestinal porcine epithelial cells (IPEC-J2) after disruption, particularly on the abundance of tight junction proteins.

**Results**: IPEC-J2 treated with 1,000 µg/ml liquorice root extract, 80 µg/ml plant powder mix, or 80 µg/ml angelica root powder showed significantly higher trans-epithelial electric resistance (TEER) 24 hr after tight junction disruption via a calcium switch assay than the control. In contrast, cells treated with 1,000 µg/ml oak bark extract showed a significantly lower TEER after 6 hr but not at later time points. The increased TEER caused by the liquorice root extract correlated with an increase in the abundance of the tight junction protein claudin-4.

**Conclusions**: This study suggests potential beneficial effects of liquorice and angelica root extracts on the gut barrier function when used as feed additives for livestock. Further studies, especially in vivo, are necessary to confirm these findings.

**KEYWORDS**
barrier function, calcium switch, IPEC-J2, liquorice, tight junctions, western blot

## 1 | BACKGROUND

The intestinal epithelium provides the main interface between the contents of the gut lumen and the blood and organs of the body. This physical barrier consists of a single layer of tightly linked cells. It is permeable to nutrients, minerals, water and selected antigens but prevents bacteria, viruses and other antigenic material from entering the underlying tissues and the bloodstream (Ivanov, 2013). To maintain and regulate the integrity of the epithelial cell barrier, the cells are connected by tight junction (TJ) complexes consisting of TJ proteins. Integral transmembrane proteins include occludin, claudins, junctional adhesion molecules and tricellulin. Intracellular

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proteins such as the zona occludens proteins (ZO-1, ZO-2 and ZO-3), cingulin and afadin are anchor proteins that connect transmembrane proteins to the actin cytoskeleton and maintain the TJ structure and function (Ivanov, 2013; Suzuki, 2013).

The barrier function can be influenced by many substances and factors. On the one hand, pathogens, pro-inflammatory agents, toxins, environmental and social stress and dietary factors can negatively influence the barrier integrity (Suzuki & Hara, 2009; Yang, Bibi, Du, Suzuki, & Zhu, 2017). In the pathogenesis of many diseases, such as inflammatory bowel syndrome, leaky gut syndrome, bacteria-induced diarrhoea and mycotoxin-induced diseases (especially deoxynivalenol), disturbances in the production and formation of TJ complexes occur (König et al., 2016; Pitt et al., 2012). Pro-inflammatory agents can initiate the expression of pro-inflammatory cytokines, which can disturb the barrier function by causing TJ disassembly. When the intestinal barrier integrity is disrupted, luminal substances, which are normally excluded by an intact barrier, can enter the body and cause an immune response such as inflammation or impair health in other ways (Ivanov, 2013).

In contrast, certain dietary factors exert protective effects on the intestinal barrier (Suzuki, 2013). Plant-derived active ingredients such as certain polyphenols have been documented to improve the barrier function, for example, via inhibition of the NF-κB, modulation of protein kinase pathways (e.g., MAPK), regulation of the activity of key enzymes, and reduction of reactive oxygen species, all resulting in beneficial effects on TJ integrity (Yang, et al., 2017).

Because of their many positive effects, plant-derived feed additives (PFAs) are used in animal husbandry and nutrition to support animals during stressful phases of life (e.g., weaning, disease, etc.), to ensure high performance and to maintain general health (Valenzuela-Grijalva, Pinelli-Saavedra, Muhlia-Almazan, Domínguez-Díaz, & González-Ríos, 2017; van der Aar, Molist, & van der Klis, 2016). PFAs are applied as plant powders (often derived from culinary or medicinal herbs or spices) or as concentrated active ingredients (e.g., as essential oils, extracts or pure phytochemicals). PFAs are also referred to as "phytogenics" (Steiner & Syed, 2015).

Many in vitro assays have been published for testing the negative influences of pathogens, toxins, dietary factors and other stressors on the integrity and morphology of a mammalian gut cell culture (Groschwitz & Hogan, 2009; König, et al., 2016; Nunes, Silva, & Chaves, 2015; Paszti-Gere et al., 2014). However, most of the stressors have a very specific mode of action, which target individual pathways or receptors. For example, during inflammatory diseases and toxin-stress, the dysregulation of the mitogen-activated protein kinase (MAPK) signalling pathways, which influence a wide range of cellular processes, can lead to the disruption of TJ expression and function (Pitt, et al., 2012; Yang, et al., 2017). Intestinal pathogens such as infectious bacteria and viruses have many different modes of action, for example adhering to the host, targeting localisation or protein expression of TJ via a number of different effector molecules, or even enzymatic protein disruption (König, et al., 2016).

In order to employ a stressor of low specificity to disrupt the TJ complex, and thus generate a general model for a leaky gut barrier in livestock, the calcium switch assay was chosen. During a calcium switch, the cells are deprived of calcium and magnesium ions, which disrupts the TJ protein complex (Cereijido, Robbins, Dolan, Rotunno, & Sabatini, 1978; Springler, Hessenberger, Schatzmayr, & Mayer, 2016; Tobey, Argote, Hosseini, & Orlando, 2004). The TJ protein complex is naturally rebuilt after the challenge. However, there is a risk of uncontrolled passage of luminal content across the epithelial barrier before complete recovery of barrier integrity has been achieved (Stewart, Pratt-Phillips, & Gonzalez, 2017). The rapid recovery of full barrier function could prevent further health threats.

Therefore, this study provides an evaluation of the influence of several PFAs on the recovery of the barrier function of intestinal porcine epithelial cells (IPEC-J2) after a calcium switch. Furthermore, elucidation of the mode of action was attempted by determining the abundance of seven TJ proteins.

## 2 | METHODS

### 2.1 | Cell culture

Intestinal porcine epithelial cells (IPEC-J2) (ACC701; Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were grown in complete cultivation medium consisting of Dulbecco's modified Eagle medium (DMEM)/ Ham's 12 (1:1) (Biochrom AG, Berlin, Germany) supplemented with 5% foetal bovine serum, 1% insulin-transferrin-selenium, 5 ng/ml epidermal growth factor, 2.5 mM Glutamax (all Gibco™, Life Technologies, Carlsbad, CA, USA) and 16 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma-Aldrich, St. Louis, MO, USA) and cultivated at 39°C and 5% $CO_2$ in humidified air. The cells were seeded at a density of $1 \times 10^6$ cells/ml in 150 $cm^2$ tissue culture flasks (Eppendorf, Hamburg, Germany) containing 28 ml complete cultivation medium and were subcultured every three to four days for a maximum of 15 passages. Polymerase chain reaction (PCR) was used every other month to exclude mycoplasma contamination (Venor® GeM Mycoplasma Detection Kit; Minerva Biolabs, Berlin, Germany).

### 2.2 | Test substances

Oak bark extract (*Quercus robur* bark, extracted with ethanol [60% v/v]; 4.9% [w/w] tannins, calculated as pyrogallol), liquorice root extract (*Glycyrrhiza glabra* roots, extracted with water; 6.5% [w/w] glycyrrhizin), and milk thistle fruit extract (*Silybum marianum* fruits, extracted with ethanol [60% v/v]; 14% [w/w] silymarin) were solubilised in ethanol (70% v/v) (Merck, Kenilworth, NJ, USA) at a concentration of 100 mg/ml. These stock solutions were shaken for 1 hr, aseptically filtered, and stored at −20°C for a maximum of three weeks. Stock solutions of a plant powder mix (PP) and its components—ground gentian root (*Gentiana lutea*; 44.2% [w/w] extractable content), ground angelica root (*Angelica archangelica*; 0.31% [v/m] volatile oil content) and ground cinnamon bark (*Cinnamomum*

*verum;* 0.99% [w/w] *trans*-cinnamaldehyde)—were prepared as described above but at a concentration of 8 mg/ml. Final test solutions were created by diluting the stock solutions in complete cultivation medium. All test substances were provided by BIOMIN Phytogenics GmbH (Stadtoldendorf, Germany). As a positive control for the assay, MAPK inhibitor U0126 monoethanolate (Sigma-Aldrich) was used as previously described (Springler, et al., 2016).

## 2.3 | Calcium switch assay

For the calcium switch assay, IPEC-J2 were seeded in complete cultivation medium in the apical compartment of 1.12 cm$^2$ Transwell® polyester membrane inserts with 0.4 µm pores (Corning Inc., New York, NY, USA) at a density of 1 × 10$^5$ cells/insert and differentiated for 8 days. Apical and basolateral medium was exchanged every other day.

All of the differentiated IPEC-J2 were washed with Ca$^{2+}$- and Mg$^{2+}$-free Hank's balanced salt solution (HBSS) (Gibco, Life Technologies) and were deprived of calcium by exposure to 2 mM ethylene glycol-bis(2-aminoethylether)N,N,N',N'-tetraacetic acid (EGTA) (Sigma-Aldrich) in Ca$^{2+}$- and Mg$^{2+}$-free HBSS in the apical compartment for 20 min. After being washed with Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline (PBS) (Sigma-Aldrich), the cells were allowed to recover in either complete cultivation medium (cell control, CC), in complete cultivation medium containing 10 µM MAPK inhibitor U0126 monoethanolate (positive control, PC), or in complete cultivation medium containing the diluted test substances (three wells per treatment). The basolateral compartments only contained complete cultivation medium. No control for EGTA-untreated cells was included, as the TEER of untreated cells is stable between day 7 and 11 according to internal performance standards, as previously published by our group (Springler, et al., 2016).

TEER recovery was monitored using a Millicell Electrical Resistance System (Merck Millipore, Billerica, MA, USA) at 0, 6, 24 and 48 hr after the calcium switch, measuring the same wells repeatedly at the indicated time points. An increase in TEER indicated TJ reassembly and restoration of the barrier function.

To exclude effects mediated by changes in cell viability, the neutral red cytotoxicity assay (Aniara, West Chester, OH, USA) was performed after the final TEER measurement according to the manufacturer's instructions.

## 2.4 | Cell protein extraction

For the determination of the TJ protein abundance, IPEC-J2 were seeded in 24 mm Transwell® polyester membrane inserts with 0.4 µm pores (Corning Inc.) at a density of 4 × 10$^5$ cells/insert. After the cells had been differentiated for 8 days, a calcium switch was performed and cultures were incubated with or without test substances as described above. One well per test group was extracted.

After 24 hr, the cells were washed twice with ice-cold PBS (Sigma-Aldrich) and then incubated for 5 min with 250 µl radioimmunoprecipitation assay buffer (RIPA) (Sigma-Aldrich) supplemented with

1× complete™ mini Protease Inhibitor Cocktail (Roche, Rotkreuz, Switzerland) and 1 M dithiothreitol (Sigma-Aldrich). The cells were harvested by scraping, incubated for 30 min on ice, and centrifuged at 10,000× *g* and 4°C for 30 min. The protein concentrations of the supernatants were determined in duplicate via the bicinchoninic acid assay according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). The supernatants were diluted with RIPA buffer and 4× sodium dodecyl sulphate (SDS) buffer consisting of β-mercaptoethanol, glycerol, SDS and 0.25 M Tris (all Sigma-Aldrich) to a final concentration of 1× SDS and 625 µg/ml protein.

## 2.5 | SDS-PAGE and western blotting

The protein samples were denatured by heating to 95°C for 5 min. After the addition of bromophenol blue, the samples (8 µg of protein per well) and a pre-stained ladder (SM1811, 10–250 kD; Fermentas, Darmstadt, Germany) were loaded on SDS polyacrylamide gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) and electrophoresis was performed at 100 V for 80 min.

Subsequently, the bands were transferred onto polyvinylidene fluoride membranes via semi-dry electroblotting using a Pierce Power Blotter (Thermo Fisher Scientific). The membranes were blocked with 5% w/v skimmed milk powder in 1× Tris-buffered saline (TBS) with 0.1% Tween®20 (all Sigma-Aldrich) for 1.5 hr at room temperature.

For the TJ analysis, the membranes were incubated with the primary antibodies in 1× TBS with 5% w/v bovine serum albumin (BSA) and 0.1% Tween®20 (all Sigma-Aldrich), overnight at 4°C with gentle shaking. The following primary antibodies were used: anti-ß-actin (13E5) rabbit monoclonal antibody (1:2,000), rabbit anti-ZO-1 (D7D12) (1:2,000), rabbit anti-ZO-2 (1:2,000), rabbit anti ZO 3 (D57G7) XP™ (1:2,000), rabbit anti-claudin-1 (D5H1D) XP® (1:1,000) (all Cell Signaling, Danvers, MA, USA), rabbit anti-claudin-3 (1:1,000), mouse anti-claudin-4 (1:1,000) and mouse anti-occludin Novex™ (1:1,000) (all Thermo Fisher Scientific). In all experiments, detection of the house-keeping gene ß-actin was used as an internal control. Following incubation with the primary antibodies, the membranes were washed and incubated with the secondary antibodies for 1.5 hr whilst being gently shaken at room temperature. The secondary antibody alkaline phosphatase-labelled goat anti-rabbit IgG (Sigma-Aldrich) was used for the detection of all of the ZO proteins (claudin-1, claudin-3 and ß-actin). The secondary antibody alkaline phosphatase-labelled goat anti-mouse IgG (Sigma-Aldrich) was used for the detection of claudin-4 and occludin. After washing, the blots were developed in substrate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM MgCl2 [all Sigma-Aldrich]) supplemented with 5-bromo-4-chloro-3-indolyl phosphate disodium salt and nitro-blue tetrazolium chloride (both Thermo Fisher Scientific).

The protein bands were analysed using ImageJ 1.50i software (Wayne Rasband, National Institutes of Health, USA, 2016). The results were normalised to β-actin and expressed as relative values to the CC values.

## 2.6 | Statistical analysis

Statistical analysis was done using data of five independent experiments (means of triplicates) for the calcium switch assay, and using data of three individual experiments (means of triplicates) for the neutral red cytotoxicity assay. For statistical analysis of protein content, the data of four individual extractions (means of duplicates) were used, and for the western blot trials, the data of four individual experiments (one blot per extraction) were used. Statistical analysis was performed with IBM® SPSS Statistics (Version 19.0, IBM corp., New York, NY, USA, 2010). Data were analysed for normality (Shapiro–Wilk) as well as for homogeneity of variance (Levene statistics). Normally distributed data with a homogenous variance were analysed by ANOVA and Dunnett's t test, and were compared to the cell control. If data were normally distributed but not homogenous, ANOVA and Dunnett's T3-test were used. Data that were not normally distributed were analysed by ANOVA according to Kruskal–Wallis. For ANOVA, treatment was used as main factor.

## 3 | RESULTS

### 3.1 | Influence of phytogenics on the recovery of the TEER of IPEC-J2 after the calcium switch

Before treatment, the TEER of IPEC-J2 ranged between 8 and 10 kOhm/cm$^2$, which was monitored in three randomly chosen wells per transwell plate (detailed data not shown). Directly after the calcium switch, the TEER was greatly reduced in all wells, showing no significant difference between groups (Figure 1). After 48 hr, the TEER of the challenged CC had recovered to the value of the unchallenged cells before treatment. TEER data at 6 hr showed normal distribution, homogeneity of variances and significant differences between treatments ($p < 0.001$, ANOVA), whilst data at 24 hr showed normal distribution, no homogeneity of variances, but significant differences between treatments ($p < 0.001$, ANOVA). TEER data at 48 hr were not normally distributed and ANOVA according to Kruskal–Wallis showed significant difference between treatments ($p = 0.011$). However, pair-wise comparisons of treatments did not result in further specification of differences.

Testing the three plant extracts revealed that liquorice root extract (1,000 µg/ml) significantly ($p = 0.010$, post hoc Dunnett's T3) increased the TEER 24 hr after the calcium switch, numerically comparable to the TEER of the CC after 48 hr. Oak bark extract (1,000 µg/ml) significantly ($p = 0.003$, post hoc Dunnett's t test) decreased TEER recovery after 6 hr but did not influence the TEER at later time points. Milk thistle fruit extract (250 µg/ml) did not influence TEER recovery. The use of MAPK inhibitor U0126 as a PC significantly increased the TEER at all time points (Figure 1).

The plant powder mix ($p = 0.018$, post hoc Dunnett's T3) and its component angelica root powder ($p = 0.015$, post hoc Dunnett's T3) (both 80 µg/ml) significantly increased the TEER 24 hr after the calcium switch, numerically comparable to the TEER of the CC after 48 hr. Cinnamon bark powder and gentian root powder (both 80 µg/ml) showed no influence on TEER recovery (Figure 2).

None of the test substances influenced the viability of the cells as indicated by the NR assay (Figure 3).
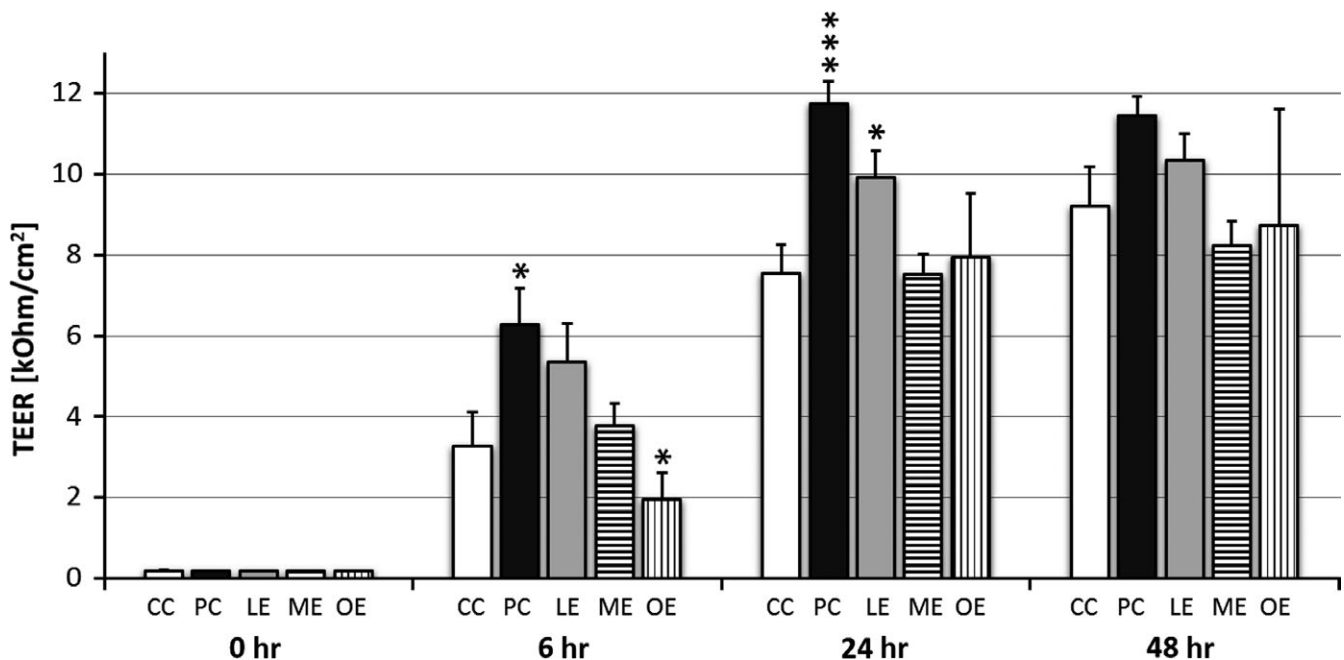


**FIGURE 1** TEER increase in IPEC-J2, measured 0, 6, 24 and 48 hr after calcium switch. Cells were allowed to recover in complete cultivation medium without additive (cell control, CC), with 10 µM MAPK inhibitor U0126 (positive control, PC), with 1,000 µg/ml Liquorice root extract (LE), with 250 µg/ml milk thistle fruit extract (ME), or with 1,000 µg/ml oak bark extract (OE). Values are expressed as means ± SD, n = 5. Stars indicate significant difference compared to the CC (*p < 0.05; ***p < 0.001; h6: post hoc Dunnett's t test; h24: post hoc Dunnett's T3). TEER: trans-epithelial electrical resistance

## 3.2 | Influence of plant extracts and the plant powder mix on the TJ protein abundance in IPEC-J2 after calcium switch

The total protein content of the cell extracts was not significantly influenced by the PC or the test substances in comparison to the CC (Figure 4).

Data on TJ proteins showed normal distribution, no homogeneity of variances, but significant differences between treatments ($p$ = 0.044, ANOVA). Of the transmembrane TJ proteins, only claudin-4 was significantly increased by the PC ($p$ = 0.011, post hoc Dunnett's T3) and liquorice root extract at 1,000 µg/ml ($p$ = 0.031, post hoc Dunnett's T3). Claudin-1 and claudin-3 were not significantly influenced by the PC or the test substances (Figure 5).

The intracellular TJ proteins ZO-1, ZO-2 and ZO-3 were not significantly influenced by the PC or the phytogenic test substances (Figure 6).
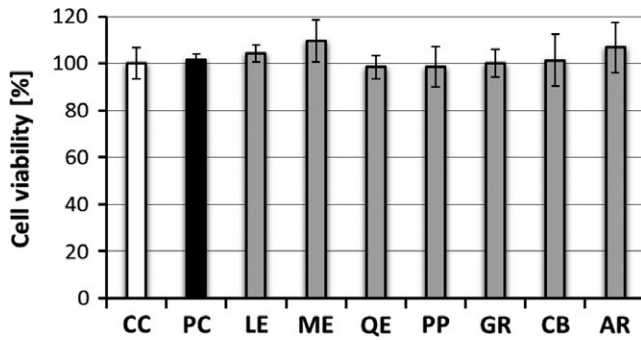
## 4 | DISCUSSION

In the present study, we have shown that liquorice root extract at 1,000 µg/ml positively influenced TEER recovery in IPEC-J2 24 hr after a calcium switch assay. The positive effect of the liquorice extract on barrier function recovery was correlated with a significant increase in the claudin-4 abundance in the cells, providing a potential explanation for the underlying mode of action.

Liquorice root has a long history of use in traditional medicines and folk remedies. Together with its active ingredients, for example, glycyrrhizin (GL) and its metabolite glycyrrhetinic acid (GA), liquorice root also plays a role in modern medicine (Hosseinzadeh & Nassiri-Asl, 2015). Moreover, liquorice has shown beneficial effects when used as a PFA. For example, a commercial product containing liquorice extract has been shown to positively influence calf performance and reduce the need for medical treatment for fever and diarrhoea (Schieder, Steiner, & Friedrichkeit, 2014). High doses of GL increased the transcript abundance of claudin-31 and tricellulin in rainbow trout gills but decreased the mRNA abundances of claudin-23a and claudin-27b. GA also increased the transcript abundance of claudin-31 but decreased that of tricellulin (Chen, Kolosov, & Kelly, 2015). A protective in vitro effect of GA on lipopolysaccharide (LPS)-induced injury in intestinal epithelial cells 6 has been documented. The TEER, which was strongly reduced by LPS, was partially recovered after GA treatment. The abundance of the TJ protein ZO-1 was also lowered by LPS and restored by GA (Hu et al., 2013). In contrast, it has been shown that GA, but not GL, decreased the TEER and increased the permeation of sodium fluorescein in Caco-2 cell monolayers in vitro and increased the intestinal absorption of drugs in an in vivo absorption study in rats (Imai, Sakai, Ohtake, Azuma, & Otagiri, 1999). These partly conflicting results might be explained by the use of different cell lines. The abundance of certain TJ proteins varies considerably across different tissues and sections of the gut (Ivanov, 2013). Different results of in vivo and in vitro studies might also
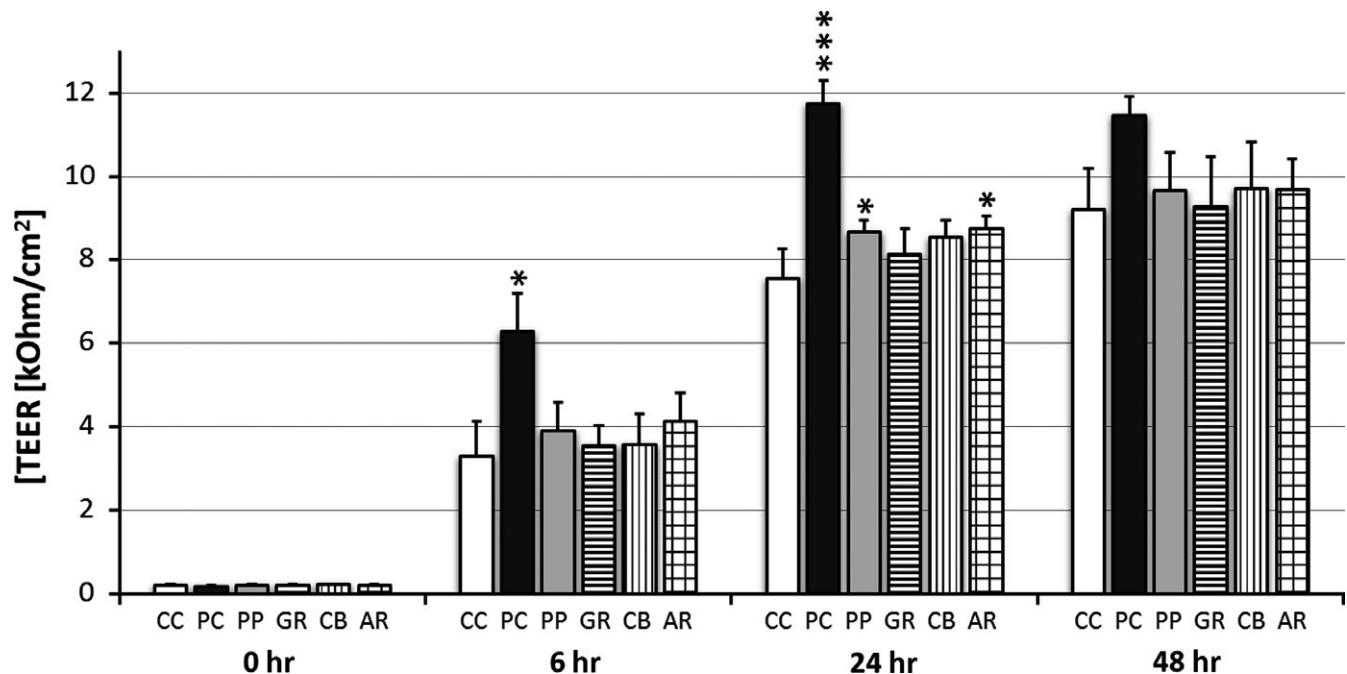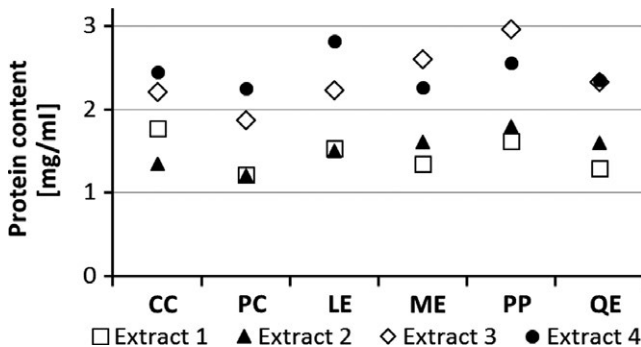


**FIGURE 2** TEER increase in IPEC-J2, measured 0, 6, 24 and 48 hr after calcium switch. Cells were allowed to recover in complete cultivation medium without additive (cell control, CC), with 10 µM MAPK inhibitor U0126 (positive control, PC), with 80 µg/ml plant powder mix (PP), with 80 µg/ml ground gentian root (GR), with 80 µg/ml ground cinnamon bark (CB), or with 80 µg/ml ground angelica root (AR). Values are expressed as means ± *SD*, *n* = 5. Stars indicate significant difference compared to the CC (* $p$ < 0.05; *** $p$ < 0.001; h6: post hoc Dunnett's *t* test; h24: post hoc Dunnett's T3). TEER: trans-epithelial electrical resistance

**FIGURE 3** Cell viability of IPEC-J2, measured via neutral red cytotoxicity assay 48 hr after calcium switch. Cells were allowed to recover in complete cultivation medium without additive (cell control, CC), with 10 µM MAPK inhibitor U0126 (positive control, PC), with 1,000 µg/ml Liquorice root extract (LE), with 250 µg/ml milk thistle fruit extract (ME), with 1,000 µg/ml oak bark extract (OE), with 80 µg/ml plant powder mix (PP), with 80 µg/ml ground gentian root (GR), with 80 µg/ml ground cinnamon bark (CB), or with 80 µg/ml ground angelica root (AR). Values are expressed as means ± *SD*, *n* = 3. No significant differences were observed
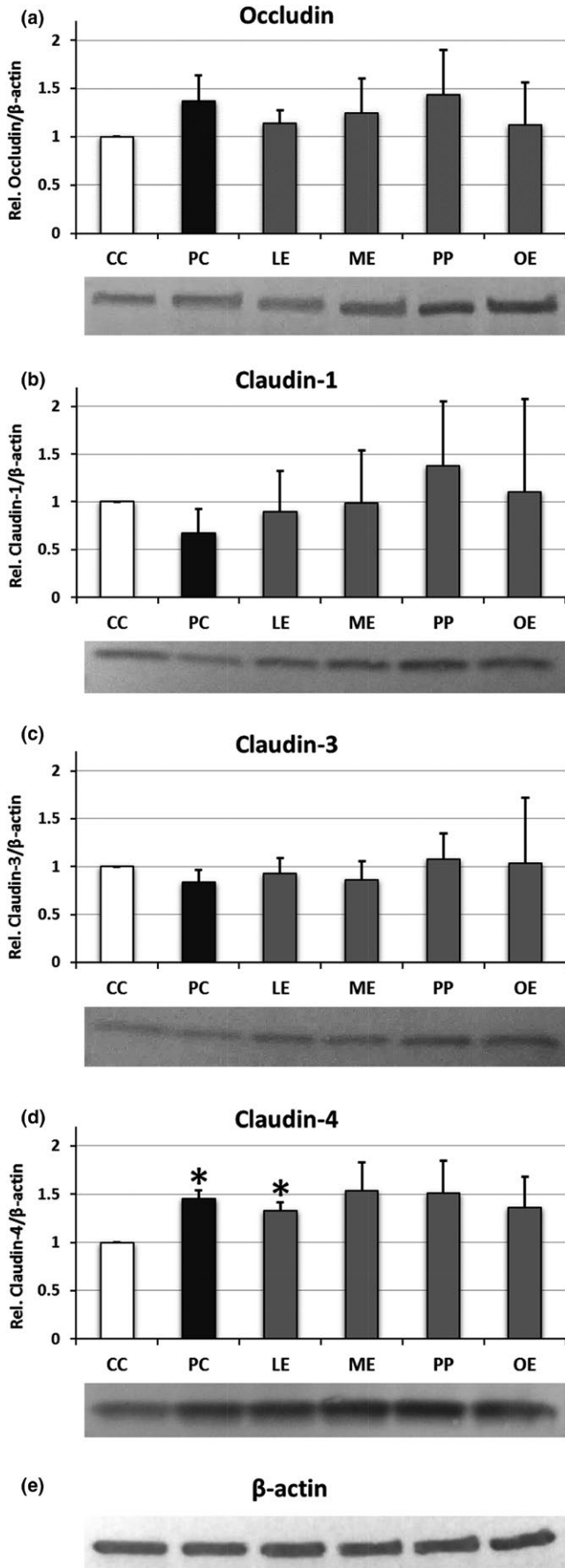


**FIGURE 4** Total protein content of IPEC-J2 extracted 24 hr after calcium switch. Cells were allowed to recover in complete cultivation medium without additive (cell control, CC), with 10 µM MAPK inhibitor U0126 (positive control, PC), with 1,000 µg/ml Liquorice root extract (LE), with 250 µg/ml milk thistle fruit extract (ME), with 80 µg/ml plant powder mix (PP), or with 1,000 µg/ml oak bark extract (OE), Values are shown for individual extractions, *n* = 4. No significant differences were observed

be explained by the fact that metabolism of GL into GA is not expected to occur in cell culture as GL is usually metabolised in vivo by intestinal flora or liver enzymes (Kim, Jang, Lee, Jung, & Lee, 1996). Furthermore, ingredients other than GL and GA might influence the barrier function since even deglycyrrhizinated liquorice could successfully be used against gastrointestinal ulcers in rats (Bennett, Clark-Wibberley, Stamford, & Wright, 1980) and human patients (Larkworthy & Holgate, 1975). These other ingredients might include flavones or isoflavones, which have been reported to affect the barrier function (Yang, et al., 2017). To our knowledge, the present study is the first to relate an increase in TEER recovery in the presence of liquorice extract after a calcium switch to an increase in claudin-4 abundance. Deeper insight into the mode of action of liquorice related to strengthening the barrier

function after stress is provided as well as additional evidence in favour of using liquorice as a PFA.

The positive control, MAPK inhibitor U0126 monoethanolate, induced an increase in TEER recovery over 24 hr, as was previously published by our group (Springler, et al., 2016). The substance U0126 was chosen as positive control as it protects the intestinal barrier, for example also against the harmful influence of mycotoxins in vitro (Springler, et al., 2016). The suggested mode of action for the observed TEER increase is the inhibition of different extracellular-signal-regulated kinases, which can prevent disruption of tight junctions in some monolayers (Aggarwal, Suzuki, Taylor, Bhargava, & Rao, 2011; Springler, et al., 2016). A significant increase of claudin-4 abundance 24 hr after the calcium switch was apparent in cell cultures treated with U0126 in the present study, which has not been previously published to our knowledge. However, positive effects on stressed cells have been documented before, as U0126 inhibited the carbachol-induced serine phosphorylation of claudin-4 (Cong et al., 2015) and prevented TEER-decrease caused by C-terminal fragment of *Clostridium perfringens* enterotoxin 194, which is known to disrupt the TJ barrier by binding claudin-4 (Kono et al., 2015).

Oak bark extract at 1,000 µg/ml caused a delayed recovery of the barrier function of IPEC-J2, as the TEER was lower than in the CC 6 hr after a calcium switch assay in the present study. However, no effect on the TEER was observed at later time points. The TJ protein abundance measured after 24 hr was not significantly different from that in the control group. On the one hand, the ingredients of the oak bark extract might negatively affect the intracellular anchor proteins, possibly because of the chelating potential of tannins contained in oak, which can bind the $Ca^{2+}$ ions (Das, Bhattacharyya, & Ghosh, 2015) and therefore reduce the number of ions that can be used for TJ reassembly. In particular, ZO-1 has been documented to be susceptible to a reduced ion concentration after a calcium switch (Rajasekaran et al., 2001). On the other hand, certain active ingredients of oak bark, such as polyphenols, might promote transmembrane proteins (Yang, et al., 2017). A positive effect of quercetin—a flavonoid abundant in plants and found in oak bark—on the TEER and the transcriptional expression of claudin-4 in the Caco-2 cell line has been described (Amasheh et al., 2008), albeit without using the calcium switch assay. Similar results were found in another study, along with increases in ZO-2, occludin and claudin-1 due to the inhibition of protein kinase Cd (Suzuki & Hara, 2009). Quercetin had a TEER-increasing effect in LLC-PK1 renal epithelial cell layers (derived from the outer cortex of the porcine kidney) at a concentration of 400 µM, which was again related to an increase in claudin-4 abundance. Positive effects were also documented for claudin-5 and claudin-7, but negative effects were observed for claudin-3. No significant changes were seen regarding the abundance of occludin or claudin-1 (Mercado et al., 2013), which is in agreement with the findings of the present study. In another study, quercetin protected the TJ ZO-1 and occludin in ECV304 monolayers from disruption by hydrogen peroxide. The authors suggested that the protective effect might involve its anti-oxidative activity and/or the inhibition of phosphorylated p38 MAP activity (Chuenkitiyanon, Pengsuparp, &
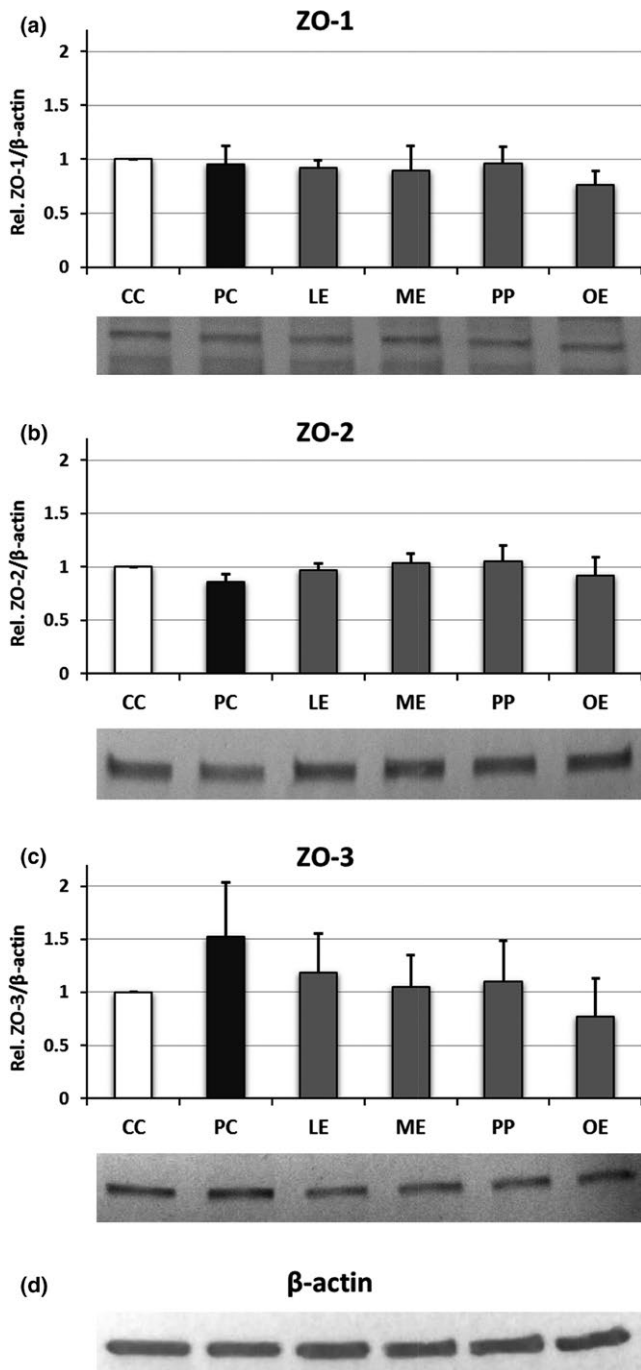
**FIGURE 5** Protein abundance for (a) claudin-1, (b) claudin-3, (c) claudin-4, (d) occludin and (e) β-actin extracted from IPEC-J2 24 hr after calcium switch. Cells were allowed to recover in complete cultivation medium without additive (cell control, CC), with 10 µM MAPK inhibitor U0126 (positive control, PC), with 1,000 µg/ml Liquorice root extract (LE), with 250 µg/ml milk thistle fruit extract (ME), with 80 µg/ml plant powder mix (PP) or with 1,000 µg/ml oak bark extract (OE). Values are expressed as means ± *SD*, *n* = 4. Stars indicate significant difference compared to the CC (\**p* < 0.05; post hoc Dunnett's T3)

Jianmongkol, 2010; Chuenkitiyanon, Vardhanabhuti, & Jianmongkol, 2012). Our own preliminary experiments showed that an oak wood extract enhanced the TEER both in unchallenged cells and in cells treated with a stressor other than the calcium switch assay (Novak, Springler, Stelzer, Mayer, & Schatzmayr, 2016). Considering the results of the present study in light of previously published data, it might be suggested that oak bark extract and its active ingredient quercetin can positively influence the barrier function but that a calcium switch might not be the best model for testing oak bark extract or other polyphenol-rich samples with chelating properties.

Milk thistle extract at 250 µg/ml showed no effect on TEER recovery or TJ abundance in the present study. *Silybum marianum* is a medicinal herb which is used mostly as hepatoprotective agent, but also shows other beneficial properties, for example, anti-inflammatory and anti-oxidative effects (Kumar, Larokar, Iyer, Kumar, & Tripathi, 2011). Silymarin, the main active ingredient of *Silybum marianum*, was found to be effective in reducing the severity indicators of gastrointestinal mucositis in mice (Sasu et al., 2015). However, since mucositis is a disorder of the gastrointestinal tract resulting from chemotherapy, and no data on TEER or TJ were shown, a comparison to the present study is not possible. In an in vivo study with broiler chicks intoxicated with a mix of aflatoxins, silymarin at the level of 1,000 ppm increased villi height and height-to-crypt depth ratio (Jahanian, Mahdavi, Asgary, & Jahanian, 2017). This indicates that the active ingredient of *Silybum marianum* might positively affect the gut by enhancing absorptive surface instead of influencing the barrier function (TEER or TJ).

The plant powder mix at 80 µg/ml increased the TEER of IPEC-J2 24 hr after the calcium switch assay. Therefore, its three components ground gentian root, ground angelica root and ground cinnamon bark were also tested. Only one of its components, angelica root powder (also tested at 80 µg/ml), increased the TEER of IPEC-J2 24 hr after a calcium switch assay.

Studies on *Angelica archangelica* and its use as PFA are rather rare. *A. archangelica* is used primarily to improve digestion and to counter mild gastrointestinal disturbances (Wedge et al., 2009). Anti-ulcerogenic effects were documented in rats and were related to a reduced acid output, increased secretion of mucin, increased prostaglandin E2 release and decreased leukotrienes. The authors suggested that the flavonoid content might be a reason for the effect of *A. archangelica* (Khayyal, et al., 2001). In rat epidermis, an ethanolic extract of *A. archangelica* enhanced the permeation

**FIGURE 6** Protein abundance for (a) ZO-1, (b) ZO-2, (c) ZO-3 and (d) β-actin extracted from IPEC-J2 24 hr after calcium switch. Cells were allowed to recover in complete cultivation medium without additive (cell control, CC), with 10 μM MAPK inhibitor U0126 (positive control, PC), with 1,000 μg/ml Liquorice root extract (LE), with 250 μg/ml milk thistle fruit extract (ME), with 80 μg/ml plant powder mix (PP) or with 1,000 μg/ml oak bark extract (OE). Values are expressed as means ± SD, n = 4. No significant differences were observed. ZO: Zona occludens

of repaglinide and increased the intercellular space. Additionally, treatment of a HaCaT cell line with the extract (0.16% w/v) for 6 hr decreased ZO-1 (Kaushal, Naz, & Tiwary, 2011). These results are in contrast to those of the present study but might be explained

by the use of different cell types and much higher concentrations of the *A. archangelica* extract (Kaushal, et al., 2011). To our knowledge, the present study is the first to document an increase in TEER recovery after a calcium switch by *A. archangelica*. We suggest that at least a part of its beneficial effects as a medicinal herb may be related to an increase in the epithelial barrier function recovery after stress.

Gentian root powder at 80 μg/ml showed no effect on TEER recovery or TJ abundance in the present study. To our knowledge, no effects of *Gentiana lutea* on intestinal barrier function have been published so far. Its root is used as digestive aid and for treatment of diarrhoea, but the major mode of action seems to be based on stimulation of gustatory receptors in the taste buds, promoting saliva, gastric juice and bile secretion (Franz et al., 2007). In a recent study, *Gentiana lutea* enhanced lipid synthesis in human keratinocytes, which are essential for building an intact epidermal barrier. It induced the expression of epidermal ceramide synthase 3, which also involves a MAPK signalling pathway (Wölfle et al., 2017). However, whilst *Gentiana lutea* can be used as treatment in some gastric disorders and might also influence the construction of specific tissue, no effect on the intestinal barrier function recovery after stress could be shown in the present study.

Ground cinnamon bark at 80 μg/ml showed no effect on TEER recovery or TJ abundance in the present study. An increase of the TEER in IPEC-1 mediated by cinnamaldehyde—the main constituent of the essential oil of cinnamon bark—was recently published, and an increase in claudin-1 and claudin-3 abundance was indicated as a possible mode of action (Sun, Lei, Wang, Wu, & Wu, 2017). Differences in the experimental setup and in the concentrations of cinnamaldehyde applied might explain the difference between the results of that study and the present study. Physiological differences between IPEC-1 and IPEC-J2 might also be a cause for the different results. IPEC-J2 is functionally and morphologically more differentiated than IPEC-1 and there are differences in the regulation of several pathways (Nossol et al., 2015). In an in vivo model with mice with inflammatory bowel syndrome, cinnamaldehyde decreased inflammatory symptoms and the expression of inflammatory markers. No influence on the TJ proteins was seen (Hagenlocher, Hösel, Bischoff, & Lorentz, 2016). In the Caco-2 cell line, cinnamaldehyde alone had a negative effect on TJ integrity, but when combined with thymol, a beneficial effect was observed (Putaala, Nurminen, & Tiihonen, 2017). This finding suggests that cinnamaldehyde in combination with other active ingredients can have different effects than when applied alone and that a combination of cinnamon bark with other plant powders in a PFA might provide superior effects on the barrier function compared to cinnamon bark alone.

Although TEER measurement after calcium switch can be used to gather information on the recovery of barrier function of the IPEC-J2 monolayer, it does not provide detailed information on cellular level by its own (Paszti-Gere, et al., 2014; Tobey, et al., 2004). TEER is a crucial, but not the only indicator of epithelial barrier integrity. Non-pore pathways are known to allow

charge- and size-independent transport of molecules which can be regulated without influencing the pore pathways (Anderson & Van Itallie, 2009). However, TEER has been shown to be a highly suitable readout for the calcium switch assay before (Cereijido, et al., 1978) and in the present study. Since viability of the cells was not affected by the test substances as indicated by the neutral red cytotoxicity assay, the TEER recovery increasing effect of the active phytogenics could not be related to a stimulation of cell viability in this study. Also, no significant effect on total protein content could be observed, suggesting that similar amounts of cells were still attached to the well membrane in all treatments, since the total protein content can be used for rough estimation of cell mass (Milo, 2013). Therefore, no relation between TEER recovery and amount of attached/detached cells could be established for any test substance in this study. The analysis of TJ abundance after 24 hr of recovery time provided greater insight into the mode of action of the test substances, but more specific data are needed to support the current findings. For example, it is not yet known how the test substances in this study affected the TJ distribution within the cell, which has been suggested as a critical factor for gut barrier function (König, et al., 2016). Effects of phytogenic substances on TJ distribution have been shown before, for example for quercetin (Suzuki & Hara, 2009) and cinnamaldehyde (Sun et al., 2017), and might therefore also be expected of the test substances used in the current study.

## 5 | CONCLUSIONS

Studies on barrier function recovery and TJ abundance in IPEC-J2 after a calcium switch assay have shown that certain plant-derived active ingredients can influence the epithelial gut barrier. Liquorice root extract, a plant powder mix (gentian root, angelica root, cinnamon bark), and one of its components, angelica root powder, increased the speed of recovery of the barrier function after stress. Oak bark extract slowed the recovery of the barrier function early after the calcium switch but had no influence at later time points, indicating an assay-specific effect. The activity of liquorice root extract was related to an increase in claudin-4 abundance. This study suggests that beneficial effects of liquorice and angelica roots on the gut barrier function may be expected when they are used as a feed additive for livestock.

### CONFLICT OF INTEREST

BIOMIN Holding GmbH operates the BIOMIN Research Center and is a producer and trader of animal feed additives. This, however, did not influence the design of the experimental study or bias the presentation and interpretation of results.

### AUTHOR CONTRIBUTIONS

KT, EM, TK, CS and DB designed the experiments; DB conducted the experiments; EM performed the statistical analysis; DB wrote the paper; KT, EM and JK gave input to interpretation of results; all authors read and approved the final manuscript.

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