Eugenol attenuates inflammatory response and enhances barrier function during lipopolysaccharide (LPS)-induced inflammation in the porcine intestinal epithelial (IPEC-J2) cells

Qianru Hui,[*,1] Emily Ammeter,[*,1] Shangxi Liu,[*] Runqiang Yang,[*,†] Peng Lu, [*] Ludovic Lahaye,[‡] Chengbo Yang[*,2]

* Department of Animal Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada

† College of Food Science and Technology, Nanjing Agricultural University, Nanjing 210095, China

‡ Jefo Nutrition Inc., Saint-Hyacinthe, Quebec J2S 7B6, Canada

[1] These authors contributed equally to this work.
[2] Corresponding author: Chengbo.Yang@umanitoba.ca

ABSTRACT: Eugenol (4-allyl-2-methoxyphenol) is an essential oil component, possessing anti-microbial, anti-inflammatory and anti-oxidative properties, however the effect of eugenol on porcine gut inflammation has not yet been investigated. In this study, an *in vitro* lipopolysaccharide (**LPS**)-induced inflammation model in porcine intestinal epithelial cells (**IPEC-J2**) has been set up. Cells were pre-treated with 100 μM (16.42 mg/L) eugenol for 2 h followed by 10 μg/mL LPS stimulation for 6 h. Pro-inflammatory cytokine secretion, reactive oxygen species, gene expression of pro-inflammatory cytokines, tight junction proteins and nutrient transporters, the expression and distribution of zonula occludens-1 (**ZO-1**), trans-epithelial electrical resistance (**TEER**) and cell permeability were measured to investigate the effect of eugenol on inflammatory responses and gut barrier function. The results showed that eugenol pre-treatment significantly suppressed the LPS-stimulated interleukin 8 (**IL-8**) level and the mRNA abundance of tumor necrosis factor α (**TNF-α**); restored the LPS-stimulated decrease of mRNA abundance of tight junction proteins *zonula occludens-1 (**ZO-1**), occludin (**OCLN**)* and the mRNA abundance of nutrient transporters *B^0-system neutral amino acid co-transporter (**B^0AT1**), system ASC sodium-dependent neutral amino acid exchanger 2 (**ASCT2**), apical sodium-dependent glucose transporter 1 (**SGLT1**), excitatory amino acid transporter 1 (**EAAC1**) and peptide transporter 1 (**PepT1**)*. In addition, eugenol improved the expression and even redistribution of ZO-1 and tended to increase TEER value and maintained the barrier integrity. In conclusion, a low dose of eugenol attenuated inflammatory responses and enhanced selectively permeable barrier function during LPS-induced inflammation in the IPE-J2 cell line.

Key words: eugenol, LPS-induced inflammation, inflammatory responses, barrier function, IPEC-J2 cells

Abbreviations

Intestinal epithelial cell	IEC
Gastrointestinal tract	GIT
B^0 system neutral amino acid co-transporter	B^0AT
System ASC sodium-dependent neutral amino acid exchanger	ASCT
Apical sodium-dependent glucose transporter	SGLT
Excitatory amino acid transporter	EAAC
Peptide transporter	PepT
Antibiotic growth promoter	AGP
Lipopolysaccharide	LPS
Water-soluble tetrazolium salt	WST
Four KDa fluorescein isothiocyanate-dextran	FITC-dextran 4
Paraformaldehyde	PFA
Dithiothreitol	DTT
Transepithelial electrical resistance	TEER
Interleukin	IL
Tumor necrosis factor	TNF
Transepithelial electrical resistance	TEER
Enzyme-linked immunosorbent assay	ELISA
Phosphate buffered saline	PBS
Reactive oxygen species	*ROS*
2', 7'dichlorofluorescein diacetate	DCF-DA
Real-time quantitative polymerase chain reaction	RT-qPCR
Zonula occludens	ZO
Occludin	OCLN
Claudin	CLDN
Generally recognized as safe	GRAS
Transient receptor potential vanilloid	TRPV
Minimal inhibitory concentration	MIC

Introduction

Weaning is one of the most stressful events during the life of a pig because they have to encounter various stressors including environmental, biological, social and psychological changes. These stressors may trigger a compromised immune system and a disrupted intestinal structure and barrier function, resulting in compromised pig health, growth performance, and a series of enteric diseases with symptoms such as diarrhea and gut inflammation present (Campbell et al., 2013), which if not properly treated, can lead to greater mortality and economic loss for commercial swine production (Lallès et al., 2007).

Intestinal epithelial cells (**IECs**) line the gastrointestinal tract (**GIT**) and form a physical monolayer barrier to serve as the first line of defense, which plays a key role in immunity (Kong et al., 2019). The gut barrier formed by circumferential intercellular junctions (including tight junctions, adherens junctions and desmosomes) connecting with neighboring cells, can prevent the invasion of microbial pathogens and the passage of other harmful molecules (Förster, 2008). The gut barrier also allows transcellular and paracellular movements of nutrients and molecules to be regulated in both passive and active ways (Pitman and Blumberg, 2000). Tight junctions create a passive paracellular transport pathway between cells, while the transcellular pathway is both active and passive, and primarily operates by nutrient transporters located on the basal, lateral and apical sides of the cell (Anderson, 2001). Furthermore, IECs are also involved in the absorption process. Nutrient transporters are essential for gut homeostasis and proper absorption of nutrients. B^0 system neutral amino acid co-transporter 1 (**B^0AT1**), system ASC sodium-dependent neutral amino acid exchanger 2 (**ASCT2**), apical sodium-dependent glucose transporter 1 (**SGLT1**), excitatory amino acid transporter 1 (**EAAC1**) and peptide transporter 1 (**PepT1**) are main nutrient transporters and chemosensing systems which are located on the apical side of IECs, and they play important roles in the transport of glucose, amino acids and peptides (Omonijo et al., 2019). Paracellular permeability is mainly regulated by tight junctions localized at the apical-lateral membranes to prevent leakage of macromolecules from the gut lumen (Laukoetter et al., 2008). However, when piglets are experiencing a weaning period, they are prone to be subjected to disrupted gut barrier, which cannot

prevent piglets from the harmful microorganism, toxins and antigens in the gut (Campbell et al., 2013). Inflammation is a common immune response to invasive pathogens, and it is one of the consequences of the "leaky gut" and compromised gut barrier function (Groschwitz and Hogan, 2009; Moeser et al., 2017). This causes malfunctioning epithelial cells and breaks tight junctions, leading to barrier malfunction and damaged GIT health.

Antibiotics have been used at both low and high levels in the swine industry for over 50 years. They have been proven to be an effective growth promoter when they are used at low levels (subtherapeutic) in swine diets and at high levels (therapeutic) to treat swine diseases (Cromwell, 2002). Antibiotic growth promoter (**AGP**) can improve growth performance, feed utilization efficiency, and decrease morbidity and mortality. Despite these benefits, there is an increasing concern that the widespread use of antibiotics in feed may lead to antibiotic resistance in both animals and humans (Barton, 2000). Thus, many countries have banned or placed strict restrictions on antibiotic use in animals (Lagha et al., 2017). Consequently, AGP alternatives are required and urgently needed.

Eugenol (4-allyl-2-methoxyphenol) is a major phenolic constituent of essential oils extracted from clove, nutmeg and cinnamon. It exhibits anti-microbial, anti-inflammatory, anti-oxidative properties (Rathinam et al., 2017). It has also been widely used in flavoring, as well in the cosmetic, pharmaceutical, and dentistry industries (Mohammadi Nejad et al., 2017). Nowadays, the application of eugenol in the agricultural industry, such as in animal feeds, is increasing. Previous studies have reported that the administration of eugenol improved the health status and growth performance in beef cattle (Compiani et al., 2013). Additionally, eugenol showed anti-microbial activity against pig gut flora (Michiels et al., 2007). Moreover, eugenol has been found to possess anti-inflammatory properties by decreasing the production of pro-inflammatory cytokines through regulating inflammation and redox status in lipopolysaccharide (**LPS**)-induced inflammation in acute lung injury of mice (Huang et al., 2015). Eugenol suppressed cyclooxygenase-2 expression in LPS-stimulated mouse macrophage RAW264.7 cells (Kim et al., 2003). Therefore, eugenol has the potential to be an alternative to AGP, by preventing and alleviating the inflammatory gut diseases caused by weaning stress. Additionally, the flavor of eugenol in feeds positively affects postweaning performance and

feeding behavior of piglets, which helped piglets adapt to a novel solid food from liquid (Blavi et al., 2016).

To our knowledge, no one has investigated the anti-inflammatory effect of eugenol on porcine intestinal epithelial cells. We hypothesized that a low dose of eugenol can suppress LPS-induced inflammation and improve barrier and absorption function in porcine intestinal epithelial cells. Therefore, this study was to investigate whether eugenol has anti-inflammatory effects and if eugenol can improve barrier and absorption function using an *in vitro* lipopolysaccharide (**LPS**)-induced inflammation model in porcine intestinal epithelial cells (**IPEC-J2**).

Materials and methods

Animal care and use committee approval was not needed because no animal trial was involved in this study. All the experiments were conducted using a commercial cell line (IPEC-J2).

Materials and reagents

Eugenol (99%), LPS from *Salmonella enterica* serotype *typhimurium* (L6143), water-soluble tetrazolium salts-1 (**WST-1**) cell proliferation reagent, 2', 7'-dichlorofluorescein diacetate (**DCF-DA),** four KDa fluorescein isothiocyanate-dextran (**FITC-dextran 4**), RIPA buffer, paraformaldehyde (**PFA**) and dithiothreitol (**DTT**) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRIzol™ reagent, Halt™ protease, phosphatase inhibitor cocktail, and Pierce™ BCA protein assay kit were purchased from Thermo Scientific (Waltham, MA, USA). 4 × Laemmli sample buffer was purchased from Bio-Rad (Hercules, CA, USA).

Cell culture and treatments

Porcine intestinal epithelial cells (IPEC-J2, ACC 701, RRID: CVCL_2246) were purchased from DSMZ-German Collection of Microorganisms and Cell Cultures and maintained in the Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (Gibco/Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (**FBS**, Gibco/Life Technologies) and 50 U/ mL penicillin/streptomycin (Gibco/Life Technologies) at 37 °C with a 5% CO_2 atmosphere. Fresh medium was replaced every 2 to 3 days. Cells were seeded into different types of multi-well plates

depending on different experimental requirements. Eugenol solution was prepared in ethanol as a 100 mM stock solution and diluted with the culture medium right before using. For optimizing eugenol concentration, cells were treated with eugenol at different concentrations (0, 10, 20, 50, 100, 200, 500, 1000, 1500, 2000 and 2500 μM) for 24 h, and cell viability was measured (Figure 1). According to the result, 100 μM was set as a working concentration for formal experiments. There were three treatment groups: Control: 0 μM eugenol dilution pre-treatment for 2 h and complete medium culture for 6 h (without LPS stimulation); LPS: cells were pre-treated with 0 μM eugenol for 2 h and then stimulated with 10 μg/mL LPS stimulation for 6 h (Omonijo et al., 2019); LPS + Eugenol: cells were pre-treated with 100 μM (16.42 mg/L) eugenol for 2 h and then stimulated with LPS (10 μg/mL) for 1 h [for reactive oxygen species assay and transepithelial electrical resistance (**TEER**)] or 6 h (for other assays).

Cell viability

WST-1 cell proliferation reagent was used to measure cell viability according to the manufacturer's protocol. IPEC-J2 cells were seeded into a 96-well plate (Corning Costar) at a density of 5×10^4 cells/mL and cultured for at least one week. After different treatments, WST-1 with 20 times dilution ratio in 5% FBS medium was added to cells followed by washing with PBS. The absorbance was measured at 450 nm using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek) after 1 h incubation at 37 °C. Cell viability was presented as a percentage of untreated control cells.

Pro-inflammatory cytokine assay

After different treatments, the cell supernatants were collected for interleukin 8 (**IL-8**) and tumor necrosis factor-alpha (**TNF-α**) measurement using a porcine IL-8 and TNF-α enzyme-linked immunosorbent assay (**ELISA**) kit (Thermo Scientific) according to the manufacturer's protocol. The absorbance was read at the endpoint at 450 nm using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek). The IL-8 concentrations were calculated from the IL-8 standard calibration curve, The IL-8 level was expressed as pg/mL.

Reactive oxygen species (ROS) assay

Cellular ROS was measured using a fluorescent dye, DCF-DA according to the method of Omonijo et al. (2019) with some modifications. Cells were cultured in a 96-well plate for one week. After a eugenol pre-treatment, cells were stimulated with LPS for 1 h. DCFDA dissolved in phosphate buffered saline (**PBS**) was added into wells at 10 µM in PBS. Cells were incubated for 30 min and then the fluorescence of DCF (Ex/Em = 485/528 nm) was detected by a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek).

RNA extraction and gene expression analysis by real-time quantitative polymerase chain reaction (RT-qPCR)

Cells were cultured in a 6-well plate. After treatments, total RNA extraction using TRIzol™ reagent was conducted as described by the manufacturer's instructions. The quality and integrity of RNA was checked by Nanodrop-2000 spectrophotometer (Thermo Scientific) and RNA electrophoresis in agarose gel. Next, the first-strand cDNA was synthesized using the iScript™ cDNA Synthesis kit (Bio-Rad) according to the corresponding manufacturer's protocol. The relative mRNA abundances of *TNF-α, IL-8, zonula occludens-1 (**ZO-1**), occludin (**OCLN**), claudin (**CLDN**)-1, CLDN-3, PepT1, B^0AT1, ASCT2, SGLT1* and *EAAC1* were measured using SYBR Green Supermix (Bio-Rad) by a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). PCR amplification was performed at a setting of three min at 95 °C, then 40 cycles of 20 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. At the end of each cycle, the fluorescence was monitored for 10 s. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. Primers used in this study are listed in Table 1 (Hyland et al., 2006; Omonijo et al., 2019). Relative gene expression was calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Cyclophilin A (**CypA**) was used as the reference gene (Hyland et al., 2006).

Western blot analysis

The expression of *ZO-1* was determined by Western blot analysis. Cells were cultured in a 6-well plate. After treatments, cells were washed with PBS twice and lysed using pre-cooled RIPA buffer containing Halt™ protease and phosphatase inhibitor cocktail on ice. Cell lysates were vortexed for 40 s and placed on ice for a 5 min period. This cycle was repeated five times, and then the cell lysates were centrifuged at 12,500 ×g for 15 min. Total protein concentration was determined using the Pierce™ BCA protein assay kit as described by the manufacturer's instructions. Protein was then denatured using 4 x Laemmli sample buffer with 0.1 M DTT at 95 ℃ for 9 min, the denatured protein samples (30 µg) were loaded and separated by 4–15% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (Bio-Rad), and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were blocked using 5% skim milk powder dissolved in tris-buffered saline, 0.1% Tween™ 20, and incubated with the ZO-1 polyclonal primary antibody (Cat# 61-7300, 1:2000 dilution, Thermo Scientific) and beta actin monoclonal primary antibody (Cat# AM4302, 1:5000 dilution, Thermo Scientific) at 4 °C overnight. Secondary antibody incubation used horseradish peroxidase-conjugated goat anti-rabbit IgG (Cat# 324300, 1:10000 dilution, Thermo Scientific) and goat anti-mouse IgG (Cat# 31430, 1:1000 dilution, Thermo Scientific). After enhanced chemiluminescent reaction using Clarity Western ECL Substrate (Bio-Rad), the images were detected by ChemiDoc™ Imaging Systems (Bio-Rad). The protein determination was normalized to β-actin and the quantitative results were presented relative to the control.

Immunofluorescent staining

Immunofluorescent staining was conducted as described previously Omonijo et al. (2018). Briefly, cells were cultured onto coverslips (Thermo Scientific) in 12-well plates for at least one week, and then fixed with 4% PFA. The cells were blocked with 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and then incubated with a ZO-1 polyclonal antibody (Cat# 61-7300, 1:100 dilution, Thermo Scientific), followed by an Alexa fluor 488 goat anti-rabbit antibody (Cat# A-11034, 1:1000 dilution, Thermo Scientific) incubation. Rinsed cells were

mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). For F-actin staining, fixed cells were incubated with Phalloidin, CF^{TM}488A (Cat# 00042-T, 1:100 dilution, Biotium, Fremont, CA, USA) and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories). Images were captured by a Zeiss Fluorescence Microscope (Zeiss group, Oberkochen, Germany).

TEER and cell permeability measurements

The integrity of the IPEC-J2 cells monolayer seeded onto Millicell membrane cell inserts (24 wells, Corning Costar) was measured by monitoring the TEER across the monolayer using a Millicell ERS-2 Voltohmmeter (Millipore-Sigma). TEER value was measured referring to the method of Omonijo et al. (2019). The TEER was monitored and measured every two days until a monolayer of cells was completely differentiated and the TEER values were over 4000 Ω, and then the cells can be pre-treated with eugenol for 2 h. TEER was measured before and after 1 h LPS treatments. The data were presented as a percentage of initial values. The paracellular permeability of cell monolayers was measured using 1 mg/mL of FITC-Dextran 4. FITC-Dextran 4 was added to the apical side of Millicell membrane cell inserts (24 wells, Corning Costar). The fluorescence intensity (Ex/Em = 485/528 nm) of basolateral medium aliquots was measured after 6 h and 16 h incubation by a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek).

Statistical analysis

Each well from a multi-well plate was used as the experimental unit for all analyses. The data from one of the three independent experiments were expressed as mean ± SEM with at least three biological repeats and at least two technical repeats. Statistical significance was determined by *t*-test or one-way ANOVA followed by Tukey's multiple-comparison test using GraphPad Prism 6 software (San Diego, CA, USA). *P* value < 0.05 was considered statistically significant, and *P* value between 0.05 and 0.1 was recognized a tendency.

Results

Dose-effect of eugenol on the viability of IPEC-J2 cells

As shown in Figure 1, concentrations of eugenol lower than 100 μM did not have significant differences in the cell viability of IPEC-J2 cells, but concentrations above 200 μM significantly decreased the cell viability compared to 0 μM ($P < 0.05$). Based on the results, 100 μM was chosen as the working concentration of eugenol throughout all experiments.

Effect of eugenol on cell viability and IL-8 secretion

As shown in Figure 2A, the LPS treatment and LPS + Eugenol treatment had no significant effects on cell viability compared with the control. LPS stimulation significantly increased IL-8 secretion indicating that it was a successful LPS-induced inflammation model ($P < 0.05$), and eugenol pre-treatment has a significant effect on returning IL-8 secretion back to similar levels as the control ($P < 0.05$, Figure 2B). However, TNF-α concentration was under the detection limit (data not shown).

Effect of eugenol on ROS production

ROS production has been shown to be involved in LPS-induced inflammatory responses. As shown in Figure 3, cells with LPS-induced inflammation significantly increased the fluorescent intensity of ROS compared to the control ($P < 0.05$). Eugenol pre-treatment did not significantly suppress the ROS production caused by LPS ($P > 0.1$).

Effect of eugenol on the gene expression of pro-inflammatory cytokines

Cells treated with LPS significantly up-regulated the relative mRNA abundance of TNF-α compared to the control ($P < 0.05$) and eugenol pre-treatment significantly suppressed this effect (Figure 4A, $P < 0.05$). Both LPS and eugenol treatment had no significant effect on the relative mRNA abundance of *IL-8* ($P > 0.05$) compared to the control, though LPS tended to up-regulate the relative mRNA abundance of *IL-8* ($P = 0.0889$, Figure 4B).

Effect of eugenol on the gene expression of tight junction proteins

Tight junction proteins, such as *ZO-1*, *OCLN*, *CLDN-1* and *CLDN-3*, are very important and help maintain barrier function. As shown in Figure 5, LPS treatment significantly down-regulated the relative mRNA abundance of *ZO-1* ($P < 0.05$, Figure 5A) and *OCLN* ($P < 0.05$, Figure 5B), but had no effect on *CLDN-1* (Figure 5C) and *CLDN-3* (Figure 5D) compared with the control ($P > 0.1$). Cells pre-treated with eugenol significantly recovered the down-regulation of *OCLN* mRNA abundance by LPS stimulation (Figure 5B, $P < 0.05$). Eugenol pre-treatment also significantly up-regulated the relative mRNA abundance of *CLDN-3* compared to the control or the LPS treatment (Figure 5D, $P < 0.05$).

Effect of eugenol on the gene expression of nutrient transporters

The LPS treatment significantly down-regulated the relative mRNA abundance of *PepT1* (Figure 6A) and *ASCT2* (Figure 6C) compared to the control ($P < 0.05$) and eugenol significantly reversed the effects caused by LPS stimulation ($P < 0.05$). Meanwhile, LPS treatment tended to down-regulate the relative mRNA abundance of B^0AT1 ($P = 0.0606$, Figure 6B), *SGLT1* ($P = 0.0558$, Figure 6D) compared to the control, respectively. However, the LPS + eugenol treatment significantly up-regulated these gene expressions compared to the LPS treatment ($P < 0.05$, Figure 6B and 6D). As shown in Figure 6E, the relative mRNA abundance of *EAAC1* in the LPS treatment group did not significantly differ compared to the control ($P > 0.1$), but eugenol significantly up-regulated the mRNA abundance of *EAAC1* compared to the LPS treatment ($P < 0.05$).

Effect of eugenol on tight junction ZO-1 expression

The result of western blotting shows that the protein expression of ZO-1 in IPEC-J2 cells stimulated with LPS was decreased compared to the control, but this effect was recovered by eugenol pre-treatment (Figure 7A). The relative quantitative result further consolidated the above conclusion (Figure 7B).

Effect of eugenol on the morphological changes of tight junction protein

Morphology of tight junction was visualized by ZO-1 immunofluorescent staining. As shown in Figure 8, in control cells, the paracellular distribution of ZO-1 was continuous, and the staining delineated the points of cell-cell contact around the periphery of the cells at the apical surface. LPS stimulation resulted in some loss of continuity of staining, suggesting the damage of the tight junction. However, eugenol pre-treatment can offset the negative changes to some extent. ZO-1 staining was more intense, indicating the even redistribution of ZO-1 was facilitated. The cytoskeletal structure of the actin fiber in IPEC-J2 cells was disorganized due to LPS stimulation compared to the control, but eugenol pre-treatment restored the morphology of actin fiber.

Effect of eugenol on TEER and cell permeability

As shown in Figure 9A, IPEC-J2 cells pre-treated with eugenol tended to promote the TEER value compared to the control (P = 0.0874). Figure 9B showed that there were no significant differences of relative TEER value among the control, LPS treatment and LPS + Eugenol treatment (P > 0.1). The leakage of FITC-dextran from the apical side to the basolateral side represents the cell permeability of the barrier formed by the IPEC-J2 cells. As shown in Figure 9C, the relative fluorescent intensity did not significantly differ between LPS and LPS + Eugenol after 6 h incubation, whereas 16 h eugenol pre-treatment significantly decreased the relative fluorescent intensity compared to the LPS treatment (P < 0.05).

Discussion

Weaning is a stressful period for piglets, where both physiological and physical changes might cause disturbances of the intestinal barrier, resulting in the translocation of luminal harmful bacteria, toxins, and antigens across intestinal epithelial cells. The imbalanced GI homeostasis is linked to gut disorders such as inflammatory bowel diseases and infectious diarrhea (Smith et al., 2009; Campbell et al., 2013). Gut inflammation is known as an immune response to noxious stimuli such as endotoxins and bacterial pathogens, which can cause tissue and cell damage (Andrade et al., 2014). This damage can result in compromised mucosal integrity and barrier function of the gut, thereby

negatively affecting the growth performance and leading to high morbidity in young piglets, especially weaned piglets Omonijo et al. (2019). The IPEC-J2 cell line is isolated from the jejunum of neonatal unsuckled piglets. These cells are ideal to study, because they are neither transformed nor tumorigenic in nature (Vergauwen et al., 2015). Eugenol has been recognized as a functional major constituent of clove essential oil and possess anti-microbial, anti-oxidative, anti-inflammatory, anti-fungal, anti-cancer, antipyretic and analgesic properties (Rathinam et al., 2017). The US Food and Drug Administration has stated that eugenol is generally recognized as safe (**GRAS**) when consumed orally (Sung et al., 2012).

In this study, 10 μg/mL LPS derived from *Salmonella enterica* serotype Enteritidis was used to induce inflammation in porcine intestinal epithelial cells, in order to explore the potential protective effects of eugenol on LPS-induced inflammatory reactions in porcine intestinal epithelial cells and its underlying mechanisms. According to our results, eugenol at concentrations above 100 μM (16.42 mg/L) significantly inhibited the viability of IPEC-J2 cells. Therefore, the critical point 100 μM was chosen to pre-treat cells in the following experiments.

Generally, the occurrence of acute inflammation is accompanied by oxidative damage. ROS are signaling molecules that can cause oxidative stress if found in elevated levels and ultimately produce tissue damage and inflammation (Mittal et al., 2014). After LPS treatment, ROS production increased immediately during 1 h, but the 2 h eugenol pre-treatment did not help IPEC-J2 cells scavenge the ROS or prevent the production of ROS. A possible explanation for this might be that the LPS stimulation time was not long enough to detect the changes, or that eugenol had not been involved in mitochondrial electron transport to limit the generation of ROS (Kowaltowski et al., 2009).

Cytokine production is induced by invading pathogen-associated molecular patterns such as bacterial endotoxin or LPS, which activate toll-like receptors on the cell surface, initiating a series of intracellular downstream signaling cascades and lead to the secretion of pro-inflammatory cytokines, such as TNF-α and IL-8 (Burkey et al., 2007). The secretion of IL-8, a typical pro-inflammatory biomarker, increased accordingly in IPEC-J2 cells treated with LPS for 6 h, and eugenol did exhibit

the protective effect on release of this cytokine associated to LPS-induced inflammation; meanwhile, the treatment did not have a negative effect on cell viability. These results indicated that this *in vitro* inflammation model was successful, which can be used to study the anti-inflammatory effects of eugenol in porcine intestinal epithelial cells. Similar results can be found in the relative mRNA abundance of *TNF-α*, where it was up-regulated in LPS treatment and down-regulated in LPS + Eugenol treatment. However, the variation trend of IL-8 mRNA expression was not completely the same as its protein expression (eugenol significantly decreased the IL-8 production compared to the LPS treatment group, but eugenol just tended to downregulate the mRNA abundance of IL-8). A similar result was found in a study performed by Omonijo et al. (2019). There might be two reasons: 1) the transcription and translation occur at different time, thus they reached to the highest level of expression at different time after stimulation. For example, in the IPEC-J2 cells, after *Salmonella enterica* serovars *Typhimurium* invasion, the IL-8 protein expression pattern was different from the IL-8 gene expression pattern, and the IL-8 gene expression pattern was different from the TNF-α gene expression pattern as reported by Skjolaas et al. (2007); 2) post-translational modification may affect the innate inflammatory responses and the expression of pro-inflammatory cytokines (Liu et al., 2016). Many studies reported changes in the expression of inflammatory cytokines in the intestine of humans and animals during enteric infection and inflammatory bowel diseases (Turner, 2009). Both *in vitro* and *in vivo* investigations show that uncontrolled synthesis of pro-inflammatory cytokines can have a strong influence on gut integrity and epithelial barrier functions, including the permeability to macromolecules and transport of nutrients and ions (Pié et al., 2004).

The IPEC-J2 cells are non-transformed and non-tumorigenic intestinal porcine enterocytes originally derived from the jejunum isolated from a neonatal unsuckled piglet, and the IPEC-J2 cell line has been characterized as a good model to study gut barrier function and nutrient absorption (Schierack et al., 2006; Vergauwen, 2015). IPEC-J2 cells were first used to investigate transepithelial ion transport and enterocyte differentiation (Berschneider, 1989). Numerous studies used IPEC-J2 cells to investigate feedstuffs and functional phytochemicals related to inflammation, oxidative stress, and intestinal permeability (Geens and Niewold, 2010; Hermes et al., 2011; Pan et al., 2013; Farkas et

al., 2015; Vergauwen et al., 2015; Palócz et al., 2016; Gao et al., 2017; Tang et al., 2018; Xiao et al., 2018; Omonijo et al., 2019). The intestinal mucosal barrier provides a physical separation between the luminal contents and the body (Laukoetter et al., 2008), and is selectively permeable in order for cells to adequately absorb nutrients while still remaining protected from invasive molecules, such as LPS (Turner, 2009). Tight junction proteins are one of the most important protein complexes to regulate the selectively permeable barrier, because they are involved in both paracellular transport and transcellular transport pathways (Turner, 2009). The claudin family is a very important group of transmembrane proteins, which are involved in several aspects of tight junction permeability (Turner, 2009). Occludin can directly interact with claudins and actin, and it was the first transmembrane tight junction protein to be identified (Turner, 2009). Peripheral membrane proteins like ZO-1 are crucial to tight junction assembly and maintenance, due to their multiple domains linked with other tight junctions and protein including claudins, occludin and actin (Turner, 2009). The relative mRNA abundance of both *CLDN-1* and *CLDN-3* did not down-regulate after LPS stimulation, however, significant down-regulations were found in that of *OCLN* and *ZO-1*, which illustrated that the gene expression of different tight junction proteins had different responses to LPS stimulation, and the injury in tight junctions was induced selectively. The expressions of *OCLN* and *ZO-1* were most-likely regulated by post-transcriptional and post-translational mechanisms (Robinson et al., 2015). Therefore, causing them to function differently during LPS-induced inflammation. A similar finding was reported by Chen et al. (2014) that after LPS treatment, the mRNA abundance of *OCLN* and *ZO-1* significantly down-regulated, but that of *CLDN-3* did not decline. Moreover, the increased pro-inflammatory cytokine secretion might contribute to the declined expression of ZO-1 in turn after LPS stimulation (Figure 2 and 4), because the increased permeability has been associated with damage to tight junction proteins.

Eugenol pre-treatment up-regulated the relative mRNA abundance of *CLDN-1*, *CLDN-3*, *OCLN* and *ZO-1* to varying degrees, suggesting that eugenol is beneficial at maintaining barrier function. In this study, the measurements of relative gene expression, protein expression and immunofluorescent staining indicated that ZO-1 played an important role in regulating LPS induced gut inflammation,

because the eugenol pre-treatment did enhance the expression of ZO-1 and its even redistribution, which has also been demonstrated by many other studies (Purohit et al., 2008; Vivinus-Nébot et al., 2014; Wang et al., 2015). Moreover, the gut integrity, tightness and permeability can be reflected by TEER value and the FITC-Dextran 4 flux result. The cell permeability was increased significantly with time. Especially at 16 h, eugenol pre-treatment significantly decreased the post-effect caused by LPS, and it was consistent with the TEER value result that eugenol pre-treatment tended to increase TEER value and maintained the barrier function accordingly. Nutrient transporters are essential for gut homeostasis and proper absorption of nutrients. The transcellular pathway primarily operates by nutrient transporters located on the basal, lateral and apical sides of the cell (Anderson, 2001). Based on the results of mRNA abundance of five transporters, we found that *B^0AT1*, *ASCT2*, *SGLT1*, *EAAC1* and *PepT1* were all up-regulated after eugenol pre-treatment, which means that absorption efficiency of energy-based nutrients, carbohydrates, amino acids, and proteins (in the form of glucose, amino acids, di- and tripeptides) might be increased (Pan et al., 2004; Omonijo et al., 2019).

In this study, results indicated that eugenol maintained the barrier function and selective permeability of IPEC-J2 cells and had an excellent effect on attenuating inflammatory response caused by LPS. Similarly, studies using other essential oil components such as cinnamaldehyde to treat porcine intestinal epithelial cells, showed a positive effect on enhancing intestinal integrity and nutrient transport and absorption (Sun et al., 2017). There are many possible explanations associated with the positive effects of eugenol. Firstly, eugenol possesses a high absorption rate in the stomach and the proximal small intestine (Michiels et al., 2008), thereby allowing eugenol to play a role in the epithelial cells and affect the expression and distribution of tight junction proteins effectively, and further maintain the function of selectively permeable barrier. Secondly, eugenol is a typical phenolic compound that possesses anti-oxidative and anti-inflammatory effects (Pramod et al., 2010). Thirdly, eugenol might be a potential ligand or factor which directly affects the inflammatory signaling pathways or indirectly modulates other receptors to alleviate inflammatory responses. For example, it is recognized that eugenol can suppress NF-κB and thus inhibit the production of TNF-α (Aggarwal et al., 2005). Our result of the mRNA abundance of *TNF-α* is also consistent with the Aggarwal et al.

study. In another study, eugenol was reported to be involved in lipometabolism by modulating a transient receptor potential vanilloid-1 (**TRPV1**) receptor and by increasing antioxidant enzymes (Harb et al., 2019). Overall, the underlying mechanism of eugenol is still worth being explored.

Furthermore, many studies have recognized that eugenol essential oil exhibits an active anti-microbial property against a wide range of gram-negative, gram-positive bacteria and fungi (Marchese et al., 2017). The concentration of eugenol against the number of total anaerobic bacteria with a probability of 99.7% in small intestine simulations of pig gut flora was 223 mg/L (Michiels et al., 2007). 1,000 mg/kg eugenol significantly decreased fecal *E. coli* concentration in growing pigs (Yan and Kim, 2012). The minimal inhibitory concentration (**MIC**) of eugenol against enteroinvasive *E. coli* strain EcK 262/75 was 2.5 mM when incubated for 24 h *in vitro* (Dušan et al., 2006). Overall, previous studies indicate that the anti-microbial effect of eugenol is in a dose-dependent manner and that only a high dose of eugenol is effective (Marchese et al., 2017). No one has previously reported that a low dose of eugenol, like 100 μM (16.42 mg/L) has shown an anti-microbial effect. However, in this study, a 100 μM dose of eugenol exhibited anti-inflammatory activity, which indicated that different levels of eugenol might serve in different roles. The low dose of eugenol could be a promising AGP alternative to prevent gut inflammation and maintain a heathy intestinal barrier function. In addition, 100 μM is far lower than the toxic dosage. The LD50 of eugenol is more than 2000 mg/kg in a rat (shown in the safety data sheet of eugenol). According to previous MIC studies and in *vivo* intake studies of eugenol (Michiels et al., 2008; Marchese et al., 2017), the dose range of eugenol between 30 mg/L and 230 mg/L might be reasonable to investigate the anti-inflammatory effect of eugenol in piglets. In addition, encapsulation is an effective way to transport eugenol, as it has volatile characteristic and it is easily degraded in the stomach and proximal small intestine (Michiels et al., 2008).

In conclusion, 100 μM (16.42 mg/L) eugenol pre-treatment can attenuate inflammatory responses and enhance the selectively permeable barrier function in IPEC-J2 cells during LPS-induced inflammation by reducing the mRNA abundance and production of pro-inflammatory cytokines; increasing the expression and distribution of tight junctions; up-regulating the gene expression of five

nutrient transporters involving glucose, amino acids, and protein transport. Eugenol has a promising potential to be used widely as an AGP alternative to prevent gut inflammation, to decrease the stress caused by weaning, and to boost the immune system of piglets. More *in vivo* studies still need to be conducted to further investigate the effect of eugenol on IPEC-J2 cells. This study, using a low dose of eugenol, can provide support for practical applications in the swine industry.

Acknowledgements

This work was financially supported by the Natural Sciences and Engineering Council of Canada (NSERC) CRD Grant (C. Yang, CRDPJ 503580-16), Manitoba Pork Council (C. Yang, 47370), Jefo Nutrition Inc. (C. Yang, 47369), the Start-Up Grant (C. Yang, 46561) from the University of Manitoba, and Canada Foundation for Innovation (CFI) and Research Manitoba.

Conflict of interest statement

The authors declare no real or perceived conflicts of interest.

Literature cited

Aggarwal, B., S. Shishodia, Y. Takada, D. Jackson-Bernitsas, K. Ahn, G. Sethi, and H. Ichikawa. 2005. TNF blockade: an inflammatory issue, Cytokines as potential therapeutic targets for inflammatory skin diseases. Springer, Berlin, Heidelberg. p. 161-186. doi:10.1007/3-540-37673-9_10.

Anderson, J. M. 2001. Molecular structure of tight junctions and their role in epithelial transport. Physiology. **16**:126-130. doi:10.1152/physiologyonline.2001.16.3.126.

Andrade, L., R. dos Reis Barreto de Oliveira, and D. de Sousa. 2014. A review on anti-inflammatory activity of phenylpropanoids found in essential oils. Molecules. **19**:1459-1480. doi:10.3390/molecules19021459.

Barton, M. D. 2000. Antibiotic use in animal feed and its impact on human healt. Nutr. Res. Rev. **13**:279-299. doi:10.1079/095442200108729106.

Blavi, L., D. Solà-Oriol, J. Mallo, and J. Pérez. 2016. Anethol, cinnamaldehyde, and eugenol inclusion in feed affects postweaning performance and feeding behavior of piglets. J. Anim. Sci. **94**:5262-5271. doi:10.2527/jas.2016-0760.

Berschneider, H. 1989. Development of normal cultured small intestinal epithelial cell lines which transport Na and Cl. Abstract of the Annual Meeting of the American Gastroenterological Association. **96**(Suppl. Pt. 2):A41.

Burkey, T. E., K. Skjolaas, S. Dritz, and J. Minton. 2007. Expression of Toll-like receptors, interleukin 8, macrophage migration inhibitory factor, and osteopontin in tissues from pigs challenged with Salmonella enterica serovar Typhimurium or serovar Choleraesuis. Vet. Immunol. Immunopathol. **115**:309-319. doi:10.1016/j.vetimm.2006.11.012.

Campbell, J. M., J. D. Crenshaw, and J. Polo. 2013. The biological stress of early weaned piglets. J. Anim. Sci. Biotechnol. **4**:19. doi:10.1186/2049-1891-4-19.

Chen, J., R. Zhang, J. Wang, P. Yu, Q. Liu, D. Zeng, H. Song, and Z. Kuang. 2014. Protective effects of baicalin on LPS-induced injury in intestinal epithelial cells and intercellular tight junctions. Can. J. Physiol. Pharmacol. **93**:233-237. doi:10.1139/cjpp-2014-0262.

Compiani, R., C. S. Rossi, A. Pizzi, and V. Dell'Orto. 2013. Administration of Essential Oils Cinnamaldehyde, Eugenol, and Capsicum to Beef Cattle: Effects on Health Status and Growth Performance, Trends in Veterinary Sciences. Springer. Berlin, Heidelberg. p. 177-180.

Cromwell, G. L. 2002. Why and how antibiotics are used in swine production. Anim. Biotechnol. **13**:7-27. doi:10.1081/ABIO-120005767.

Dušan, F., S. Marián, D. Katarína, and B. Dobroslava. 2006. Essential oils—their antimicrobial activity against Escherichia coli and effect on intestinal cell viability. Toxicol. Vitro. **20**:1435-1445. doi:10.1016/j.tiv.2006.06.012.

Farkas, O., O. Palócz, E. Pászti-Gere, and P. Gálfi. 2015. Polymethoxyflavone apigenin-trimethylether suppresses LPS-induced inflammatory response in nontransformed porcine intestinal cell line IPEC-J2. Oxidative Med. Cell. Longev. **2015**: 673847. doi:10.1155/2015/673847.

Förster, C. 2008. Tight junctions and the modulation of barrier function in disease. Histochem. Cell Biol. **130**:55-70. doi:10.1007/s00418-008-0424-9.

Gao, K., C. Wang, L. Liu, X. Dou, J. Liu, L. Yuan, W. Zhang, and H. Wang. 2017. Immunomodulation and signaling mechanism of Lactobacillus rhamnosus GG and its components on porcine intestinal epithelial cells stimulated by lipopolysaccharide. J. Microbiol. Immunol. Infect. **50**:700-713. doi:10.1016/j.jmii.2015.05.002.

Geens, M. M., and T. A. Niewold. 2010. Preliminary characterization of the transcriptional response of the porcine intestinal cell line IPEC-J2 to enterotoxigenic *Escherichia*

coli, *Escherichia coli*, and *E. coli* lipopolysaccharide. Int. J. Genomics. **2010**:469583. doi:org/10.1155/2010/469583.

Groschwitz, K. R., and S. P. Hogan. 2009. Intestinal barrier function: molecular regulation and disease pathogenesis. J. Allergy Clin. Immunol. **124**:3-20. doi:10.1016/j.jaci.2009.05.038.

Harb, A. A., Y. K. Bustanji, I. M. Almasri, and S. S. Abdalla. 2019. Eugenol Reduces LDL Cholesterol and Hepatic Steatosis in Hypercholesterolemic Rats by Modulating TRPV1 Receptor. Sci. Rep. **9**:1-10. doi:10.1038/s41598-019-50352-4.

Hermes, R. G., E. G. Manzanilla, S. M. Martín-Orúe, J. F. Pérez, and K. C. Klasing. 2011. Influence of dietary ingredients on *in vitro* inflammatory response of intestinal porcine epithelial cells challenged by an enterotoxigenic *Escherichia coli* (K88). Comp. Immunol. Microbiol. Infect. Dis. **34**:479-488. doi:org/10.1016/j.cimid.2011.08.006.

Huang, X., Y. Liu, Y. Lu, and C. Ma. 2015. Anti-inflammatory effects of eugenol on lipopolysaccharide-induced inflammatory reaction in acute lung injury via regulating inflammation and redox status. Int. Immunopharmacol. **26**:265-271. doi:10.1016/j.intimp.2015.03.026.

Hyland, K. A., D. R. Brown, and M. P. Murtaugh. 2006. Salmonella enterica serovar Choleraesuis infection of the porcine jejunal Peyer's patch rapidly induces IL-1β and IL-8 expression. Vet. Immunol. Immunopathol. **109**:1-11. doi: 10.1016/j.vetimm.2005.06.016.

Kim, S. S., O.-J. Oh, H.-Y. Min, E.-J. Park, Y. Kim, H. J. Park, Y. N. Han, and S. K. Lee. 2003. Eugenol suppresses cyclooxygenase-2 expression in lipopolysaccharide-stimulated mouse macrophage RAW264. 7 cells. Life Sci. **73**:337-348. doi: 10.1016/S0024-3205(03)00288-1.

Kong, S., Y. H. Zhang, and W. Zhang. 2018. Regulation of intestinal epithelial cells properties and functions by amino acids. Biomed Res. Int. **2018:**1-10. doi: 10.1155/2018/2819154.

Kowaltowski, A. J., N. C. de Souza-Pinto, R. F. Castilho, and A. E. Vercesi. 2009. Mitochondria and reactive oxygen species. Biomed Res. Int. **47**:333-343. doi:10.1016/j.freeradbiomed.2009.05.004.

Lagha, A. B., Haas, B., Gottschalk, M., & Grenier, D. 2017. Antimicrobial potential of bacteriocins in poultry and swine production. Vet. Res. 48:22. doi: 10.1186/s13567-017-0425-6.

Lallès, J.-P., P. Bosi, H. Smidt, and C. R. Stokes. 2007. Nutritional management of gut health in pigs around weaning. Proc. Nutr. Soc. **66**:260-268. doi: 10.1017/S0029665107005484.

Laukoetter, M. G., P. Nava, and A. Nusrat. 2008. Role of the intestinal barrier in inflammatory bowel disease. World J. Gastroenterol. **14**:401. doi: 10.3748/wjg.14.401.

Liu, J., C. Qian, and X. Cao. 2016. Post-translational modification control of innate immunity. Immunity. 45:15-30. doi:10.1016/j.immuni.2016.06.020.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. **25**:402-408. doi: 10.1006/meth.2001.1262.

Marchese, A., R. Barbieri, E. Coppo, I. E. Orhan, M. Daglia, S. F. Nabavi, M. Izadi, M. Abdollahi, S. M. Nabavi, and M. Ajami. 2017. Antimicrobial activity of eugenol and essential oils containing eugenol: A mechanistic viewpoint. Crit. Rev. Microbiol. **43**:668-689. doi: 10.1080/1040841X.2017.1295225.

Michiels, J., J. Missotten, N. Dierick, D. Fremaut, P. Maene, and S. De Smet. 2008. In vitro degradation and *in vivo* passage kinetics of carvacrol, thymol, eugenol and trans-cinnamaldehyde along the gastrointestinal tract of piglets. J. Sci. Food Agric. **88**:2371-2381. doi: 10.1002/jsfa.3358.

Michiels, J., J. Missotten, D. Fremaut, S. De Smet, and N. Dierick. 2007. In vitro dose–response of carvacrol, thymol, eugenol and trans-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. Livest. Sci. **109**:157-160. doi: 10.1016/j.livsci.2007.01.132.

Mittal, M., M. R. Siddiqui, K. Tran, S. P. Reddy, and A. B. Malik. 2014. Reactive oxygen species in inflammation and tissue injury. Antioxid. Redox Signal. **20**:1126-1167. doi:10.1089/ars.2012.5149

Moeser, A. J., C. S. Pohl, and M. Rajput. 2017. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. Anim. Nutri. **3**:313-321. doi:10.1016/j.aninu.2017.06.003.

Nejad, S. M., Ozgunes, H. and Basaran, N. 2017. Pharmacological and toxicological properties of eugenol. Turk. J. Pharm. Sci. **14**:201-206. doi:10.4274/tjps.62207.

Omonijo, F. A., S. Liu, Q. Hui, H. Zhang, L. Lahaye, J.-C. Bodin, J. Gong, M. Nyachoti, and C. Yang. 2019. Thymol improves barrier function and attenuates inflammatory responses in porcine intestinal epithelial cells during lipopolysaccharide (LPS)-induced inflammation. J. Agric. Food Chem. **67**:615-624. doi:10.1021/acs.jafc.8b05480.

Palócz, O., E. Pászti-Gere, P. Gálfi, and O. Farkas. 2016. Chlorogenic acid combined with *Lactobacillus plantarum* 2142 reduced LPS-induced intestinal inflammation and oxidative stress in IPEC-J2 cells. PloS one. **11**:e0166642. doi:10.1371/journal.pone.0166642.

Pan, L., G. Qin, Y. Zhao, J. Wang, F. Liu, and D. Che. 2013. Effects of soybean agglutinin on mechanical barrier function and tight junction protein expression in intestinal epithelial cells from piglets. Int. J. Mol. Sci. **14**:21689-21704. doi:10.3390/ijms141121689.

Pan, X., T. Terada, M. Okuda, and K.-I. Inui. 2004. The diurnal rhythm of the intestinal transporters SGLT1 and PEPT1 is regulated by the feeding conditions in rats. J. Nutri. **134**:2211-2215. doi:10.1093/jn/134.9.2211.

Pié, S., J. Lallès, F. Blazy, J. Laffitte, B. Sève, and I. Oswald. 2004. Weaning is associated with an upregulation of expression of inflammatory cytokines in the intestine of piglets. J. Nutri. **134**:641-647. doi:10.1093/jn/134.3.641.

Pitman, R. S., and R. S. Blumberg. 2000. First line of defense: the role of the intestinal epithelium as an active component of the mucosal immune system. J. Gastroenterol. **35**:805-814. doi:10.1007/s005350070017.

Pramod, K., S. H. Ansari, and J. J. N. p. c. Ali. 2010. Eugenol: a natural compound with versatile pharmacological actions. Nat. Prod. Commun. **5**:1999-2006. doi:10.1177/1934578X1000501236.

Purohit, V., J. C. Bode, C. Bode, D. A. Brenner, M. A. Choudhry, F. Hamilton, Y. J. Kang, A. Keshavarzian, R. Rao, and R. B. Sartor. 2008. Alcohol, intestinal bacterial growth, intestinal permeability to endotoxin, and medical consequences: summary of a symposium. Alcohol. **42**:349-361. doi:10.1016/j.alcohol.2008.03.131.

Rathinam, P., H. Vijay Kumar, and P. Viswanathan. 2017. Eugenol exhibits anti-virulence properties by competitively binding to quorum sensing receptors. Biofouling. **33**:624-639. doi:10.1080/08927014.2017.1350655.

Robinson, K., Z. Deng, Y. Hou, and G. Zhang. 2015. Regulation of the intestinal barrier function by host defense peptides. Front. Vet. Sci. **2**:57. doi:10.3389/fvets.2015.00057.

Schierack, P., M. Nordhoff, M. Pollmann, K. D. Weyrauch, S. Amasheh, U. Lodemann, J. Jores, B. Tachu, S. Kleta, and A. Blikslager. 2006. Characterization of a porcine intestinal epithelial cell line for *in vitro* studies of microbial pathogenesis in swine. Histochemistry and cell biology **125**:293-305. doi:10.1007/s00418-005-0067-z.

Skjolaas, K., T. Burkey, S. Dritz, and J. Minton. 2007. Effects of *Salmonella enterica* serovar *Typhimurium*, or serovar *Choleraesuis*, *Lactobacillus reuteri* and *Bacillus licheniformis* on chemokine and cytokine expression in the swine jejunal epithelial cell line, IPEC-J2. Vet. Immunol. Immunopathol. 115:299-308. doi:10.1016/j.vetimm.2006.10.012.

Smith, F., J. E. Clark, B. L. Overman, C. C. Tozel, J. H. Huang, J. E. Rivier, A. T. Blisklager, and A. J. Moeser. 2009. Early weaning stress impairs development of mucosal barrier function in the porcine intestine. Am. J. Physiol.-Gastroint. Liver Physiol. **298**:G352-G363. doi:10.1152/ajpgi.00081.2009.

Sun, K., Y. Lei, R. Wang, Z. Wu, and G. Wu. 2017. Cinnamicaldehyde regulates the expression of tight junction proteins and amino acid transporters in intestinal porcine epithelial cells. J. Anim. Sci. Biotechnol. **8**:66. doi:10.1186/s40104-017-0186-0.

Sung, B., S. Prasad, S. C. Gupta, S. Patchva, and B. B. Aggarwal. 2012. Chapter 3 - Regulation of Inflammation-Mediated Chronic Diseases by Botanicals. In: L.-F. Shyur and A. S. Y. Lau, editors, Advances in Botanical Research No. 62. Academic Press. San Diego, CA. p. 57-132.

Tang, X., B. Liu, X. Wang, Q. Yu, and R. Fang. 2018. Epidermal growth factor, through alleviating oxidative stress, protect IPEC-J2 cells from lipopolysaccharides-induced apoptosis. Int. J. Mol. Sci. **19**:848. doi:10.3390/ijms19030848.

Turner, J. R. 2009. Intestinal mucosal barrier function in health and disease. Nat. Rev. Immunol. **9**:799. doi:10.1038/nri2653

Vergauwen, H., B. Tambuyzer, K. Jennes, J. Degroote, W. Wang, S. De Smet, J. Michiels, and C. Van Ginneken. 2015. Trolox and ascorbic acid reduce direct and indirect oxidative stress in the IPEC-J2 cells, an *in vitro* model for the porcine gastrointestinal tract. PLoS One. **10**:e0120485. doi:10.1371/journal.pone.0120485.

Vivinus-Nébot, M., G. Frin-Mathy, H. Bzioueche, R. Dainese, G. Bernard, R. Anty, J. Filippi, M.-C. Saint-Paul, M. K. Tulic, and V. Verhasselt. 2014. Functional bowel symptoms in quiescent inflammatory bowel diseases: role of epithelial barrier disruption and low-grade inflammation. Gut. **63**:744-752. doi: 10.1136/gutjnl-2012-304066.

Xiao, Z., L. Liu, W. Tao, X. Pei, G. Wang, and M. Wang. 2018. *Clostridium tyrobutyricum* protect intestinal barrier function from LPS-induced apoptosis via p38/JNK signaling pathway in IPEC-J2 cells. Cell. Physiol. Biochem. 46(5):1779-1792. doi:10.1159/000489364.

Wang, W., T. Xia, and X. Yu. 2015. Wogonin suppresses inflammatory response and maintains intestinal barrier function via TLR4-MyD88-TAK1-mediated NF-κB pathway *in vitro*. Inflamm. Res. **64**:423-431. doi:10.1007/s00011-015-0822-0.

Yan, L., and I. Kim. 2012. Effect of eugenol and cinnamaldehyde on the growth performance, nutrient digestibility, blood characteristics, fecal microbial shedding and fecal noxious gas content in growing pigs. Asian Australas. J. Anim. Sci. **25**:1178. doi: 10.5713/ajas.2012.12111.

Table 1. Primers used in this study

Genes	Accession No.	Primer sequences
TNF-α	NM_214022.1	5'-ATGGATGGGTGGATGAGAAA-3'
		5'-TGGAAACTGTTGGGGAGAAG-3'
IL-8	NM_213867.1	5'-CACCTGTCTGTCCACGTTGT-3'
		5'-AGAGGTCTGCCTGGACCCCA-3'
SGLT1	NM_001012297.1	5'-GGCTGGACGAAGTATGGTGT-3'
		5'-GAGCTGGATGAGGTTCCAAA-3'
ASCT2	XM_003355984.4	5'-GCCAGCAAGATTGTGGAGAT-3'
		5'-GAGCTGGATGAGGTTCCAAA-3'
EAAC1	NM_001164649.1	5'-CCAAGGTCCAGGTTTTGGGT-3'
		5'-GGGCAGCAACACCTGTAATC-3'
B^0AT1	XM_003359855.4	5'-AAGGCCCAGTACATGCTCAC-3'
		5'-CATAAATGCCCCTCCACCGT-3'
PepT1	NM_214347.1	5'-CATCGCCATACCCTTCTG-3'
		5'-TTCCCATCCATCGTGACATT-3'
ZO-1	XM003353439.2	5'-GATCCTGACCCGGTGTCTGA-3'
		5'-TTGGTGGGTTTGGTGGGTT-3'
OCLN	NM_001163647.2	5'-GAGAGAGTGGACAGCCCCAT-3'
		5'-TGCTGCTGTAATGAGGCTGC-3'
CLDN-1	NM_001244539.1	5'-CTGTGGATGTCCTGCGTGT-3'

		5’-GGTTGCTTGCAAAGTGGTGTT-3’
CLDN-3	NM_001160075.1	5’-CTACGACCGCAAGGACTACG-3’
		5’-TAGCATCTGGGTGGACTGGT-3’
CypA (reference)	JX523419.1	5’-GCGTCTCCTTCGAGCTGTT-3’
		5’-CCATTATGGCGTGTGAAGTC-3’

Note: TNF-α: tumor necrosis factor α; IL-8: interleukin 8; SGLT1: apical sodium-dependent glucose transporter 1; ASCT2: system ASC sodium-dependent neutral amino acid exchanger 2; EAAC1: excitatory amino acid transporter 1; B^0AT1: B^0-system neutral amino acid co-transporter; PepT1: H^+/peptide transporter 1; ZO-1: zonula occludens-1; OCLN: occludin; CLDN-1: claudin 1; CLDN-3: claudin 3; CypA: cyclophilin-A.

Figure legends:

Figure 1. Dose-effect of eugenol on the viability of IPEC-J2 cells. Cells were treated with eugenol at the indicated concentrations for 24 h. The data were presented as mean ± SEM, n = 6. * represents a significant difference ($P < 0.05$) compared with 0 μM eugenol.

Figure 2. Effect of eugenol on the cell viability and IL-8 secretion during LPS-induced inflammation. IPEC-J2 cells were treated with eugenol (100 μM) for 2 h prior to LPS (10 μg/mL) stimulation for 6 h. The data were presented as mean ± SEM, n = 6. Different letters represent a significant difference ($P < 0.05$).

Figure 3. Effect of eugenol on ROS production. IPEC-J2 cells were treated with eugenol (100 μM) for 2 h prior to LPS (10 μg/mL) stimulation for 1 h. The data were presented as mean ± SEM, n = 3. Different letters represent significant differences ($P < 0.05$).

Figure 4. Effect of eugenol on the gene expression of pro-inflammatory cytokines TNF-α (A) and IL-8 (B) during LPS-induced inflammation. IPEC-J2 cells were treated with eugenol (100 μM) for 2 h prior to LPS (10 μg/mL) stimulation for 6 h. Data were presented as mean ± SEM, n = 3. Different letters a and b represent a significant difference ($P < 0.05$), and x and y mean a trend ($0.05 < P < 0.1$).

Figure 5. Effect of eugenol on the gene expression of tight junction protein *ZO-1* (A), *OCLN* (B), *CLDN-1* (C) and *CLDN-3* (D) during LPS-induced inflammation. IPEC-J2 cells were treated with eugenol (100 μM) for 2 h prior to LPS (10 μg/mL) stimulation for 6 h. Data were presented as mean ± SEM, n = 3. Different letters represent a significant difference ($P < 0.05$).

Figure 6. Effect of eugenol on the gene expression of nutrient transporters PepT1 (A), B^0AT1 (B), ASCT2 (C), SGLT1 (D) and EAAC1 (E) during LPS-induced inflammation. IPEC-J2 cells were treated with eugenol (100 μM) for 2 h prior to LPS (10 μg/mL) stimulation for 6 h. Data were presented as mean ± SEM, n = 3. Different letters represent a significant difference ($P < 0.05$).

Figure 7. Effect of eugenol on tight junction ZO-1 expression during LPS-induced inflammation. Representative Western blot images (A) and relative expression level (B) of ZO-1 protein are shown.

IPEC-J2 cells were treated with eugenol (100 μM) for 2 h prior to LPS (10 μg/mL) stimulation for 6 h. Data were presented as mean ± SEM, n = 3. Different letters represent a significant difference ($P < 0.05$).

Figure 8. Effect of eugenol on the morphological changes of tight junction protein ZO-1 and actin fiber during LPS-induced inflammation. IPEC-J2 cells were treated with eugenol (100 μM) for 2 h prior to LPS (10 μg/mL) stimulation for 6 h.

Figure 9. Effect of eugenol on trans-epithelial electrical resistance (TEER) and permeability during LPS-induced inflammation. The relative TEER value measured after 2 h eugenol pre-treatment in IPEC-J2 cells are shown (A); The relative TEER value for 2 h eugenol pre-treatment, followed by 1 h LPS (10 μg/mL) stimulation in IPEC-J2 cells was measured (B); Permeability was tested by FITC-Dextran 4, and the relative fluorescence intensity is shown (C). Data for TEER value were presented as a percentage of initial TEER value and given as means ± SEM, n = 4. Different letters a and b represent a significant difference ($P < 0.05$), and x and y mean a trend ($0.05 < P < 0.1$).

Figure 1

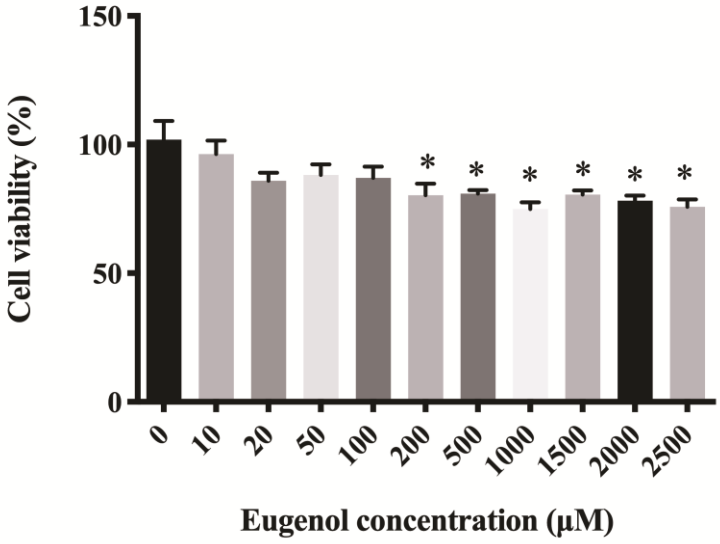

Figure 2

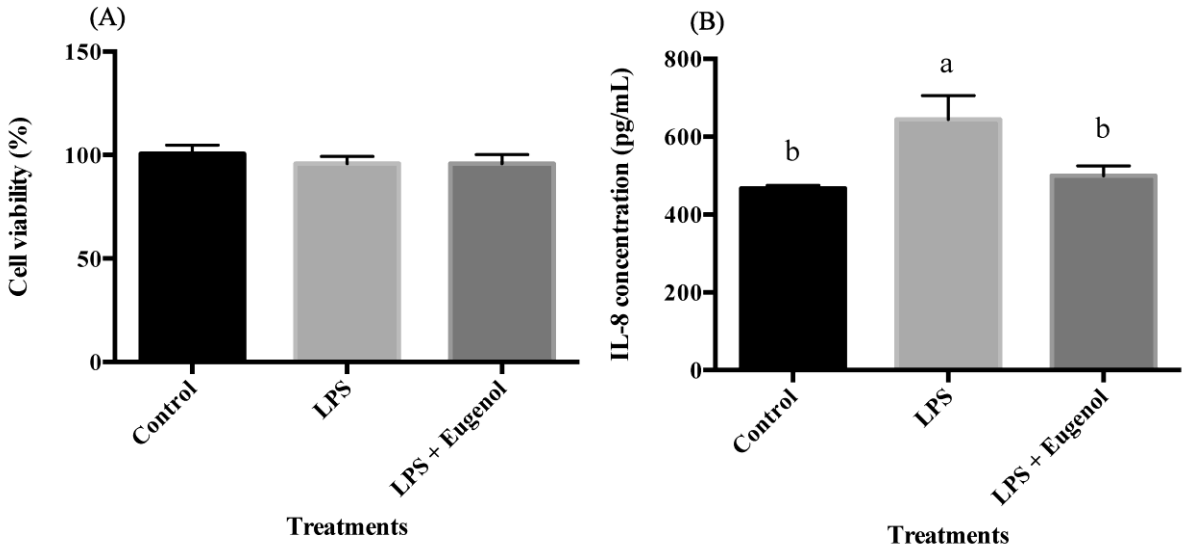

Figure 3

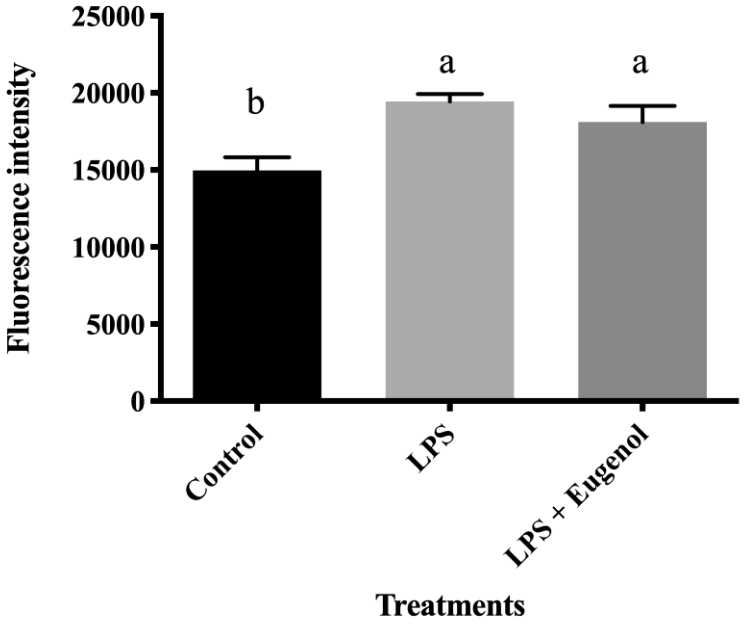

Figure 4

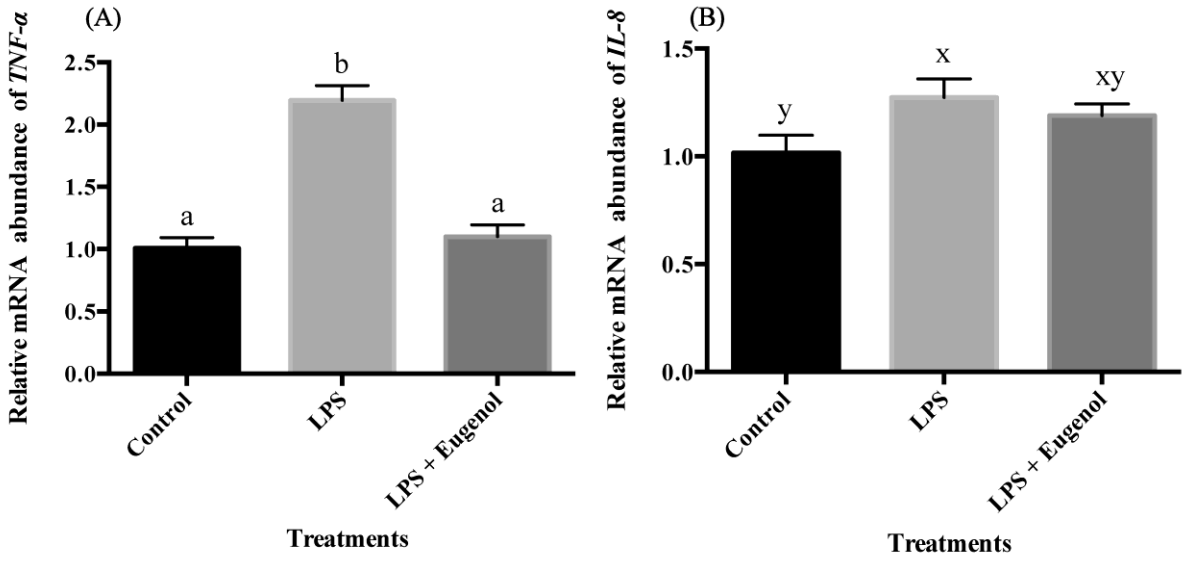

Figure 5

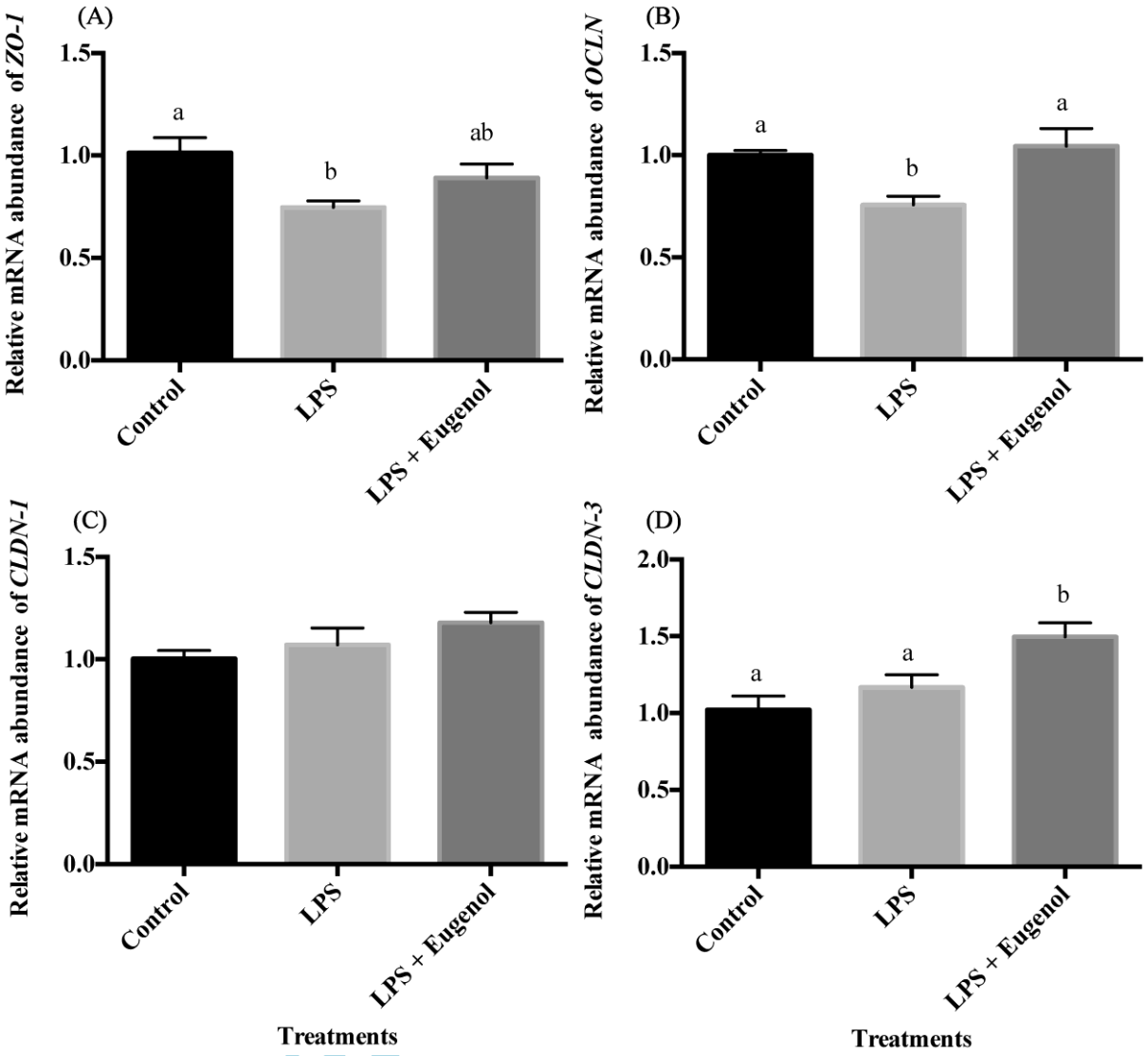

Figure 6

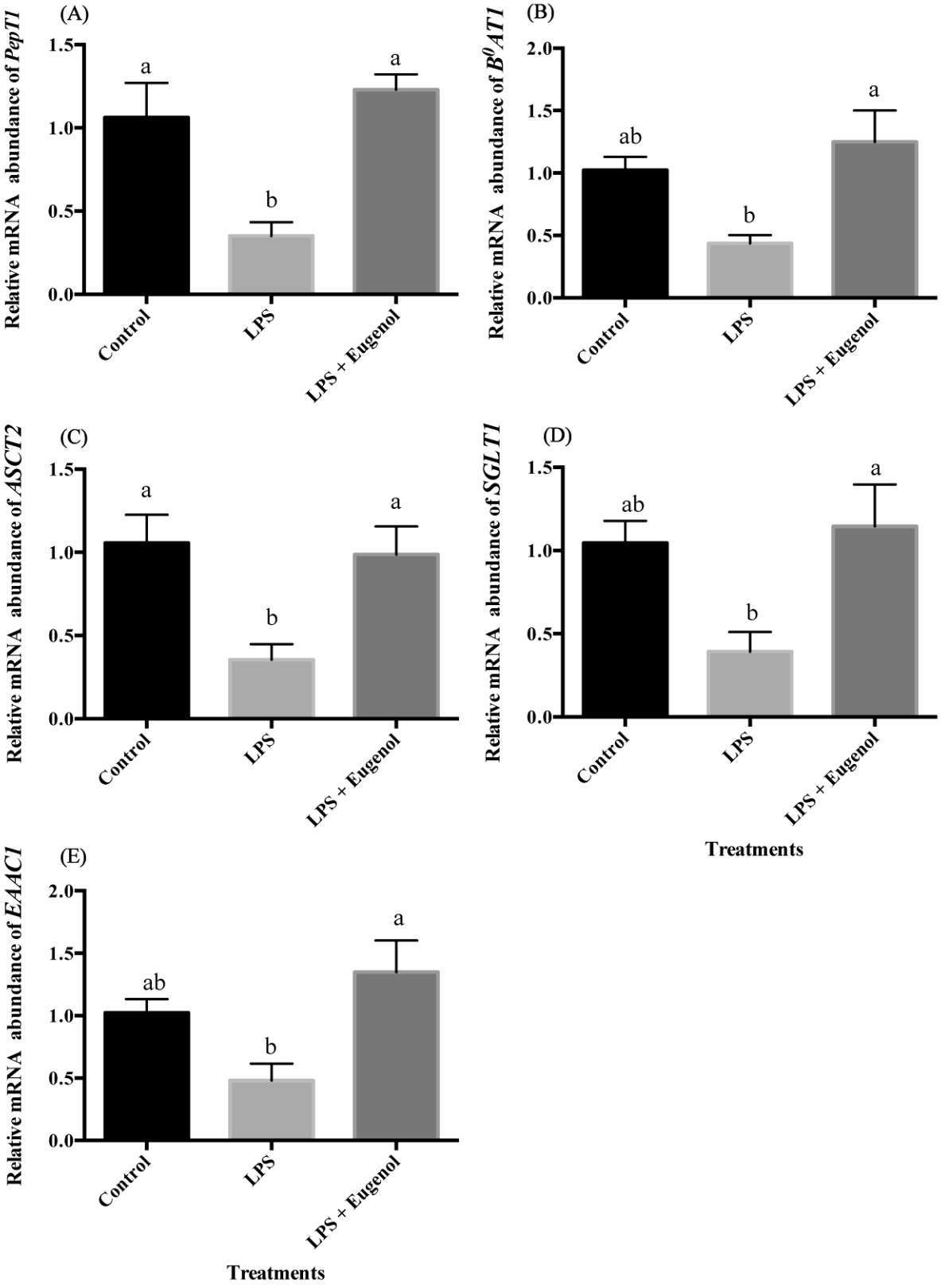

figure 7

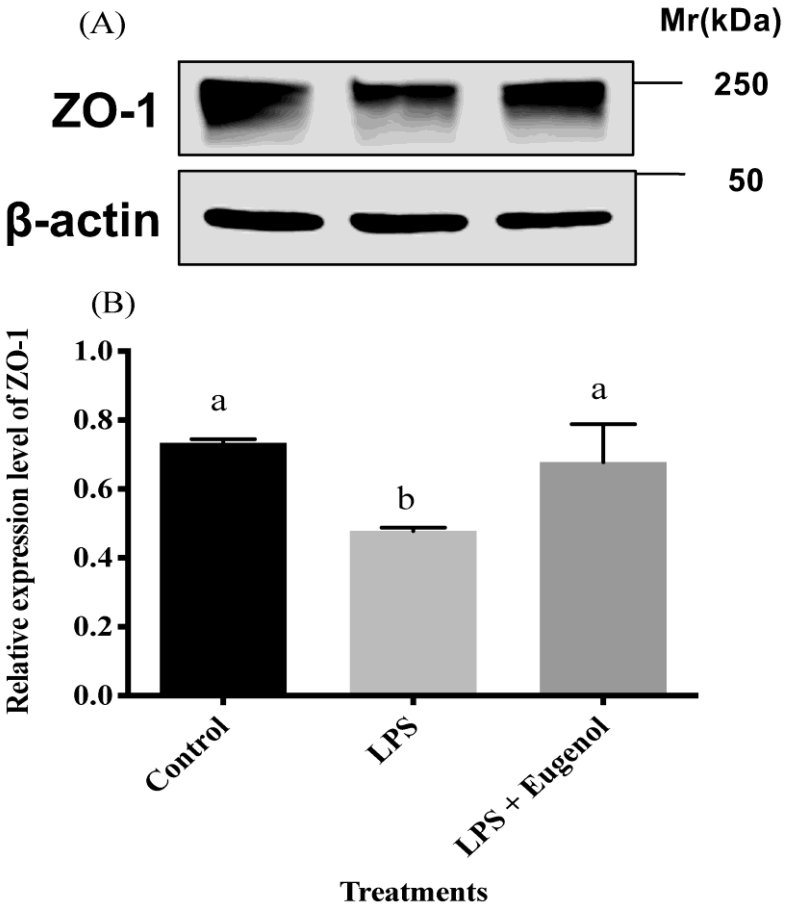

Figure 8

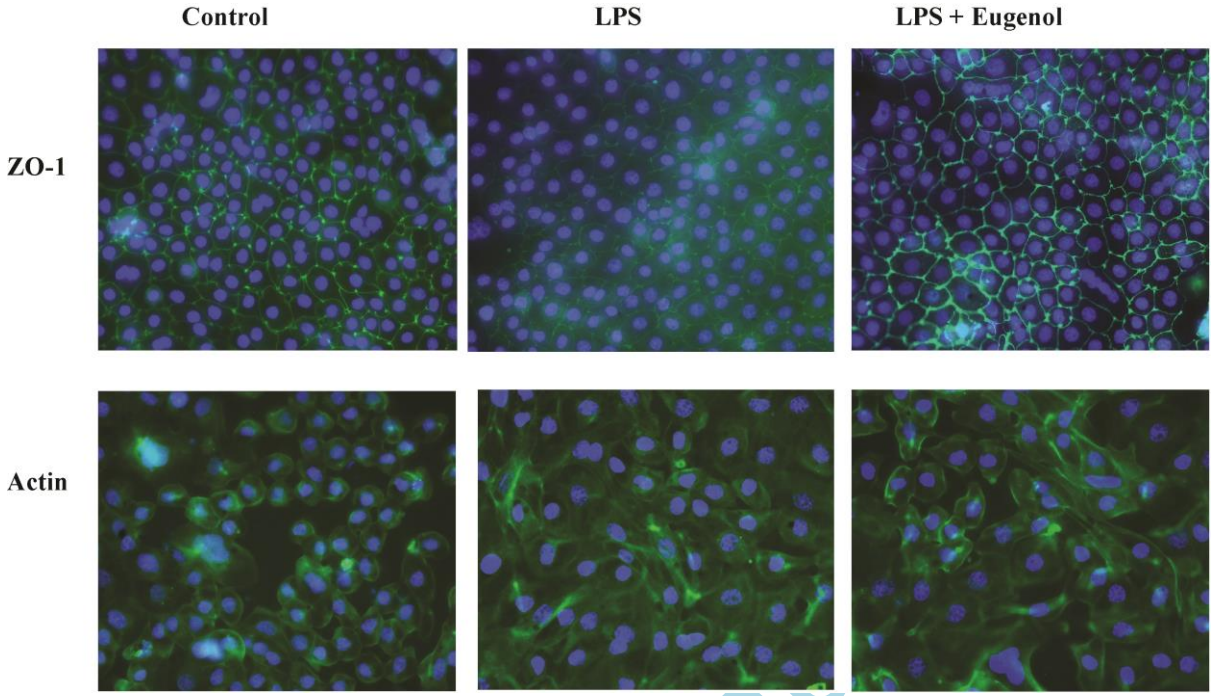

Figure 9