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Thymol Improves Barrier Function and Attenuates Inflammatory Responses in Porcine Intestinal Epithelial Cells during Lipopolysaccharide (LPS)-Induced Inflammation.

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Bioactive Constituents, Metabolites, and Functions

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Thymol improves barrier function and attenuates inflammatory responses in porcine intestinal epithelial cells during lipopolysaccharide (LPS)-induced inflammation

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ABSTRACT: It is well known that essential oil thymol exhibits antibacterial activity. The protective effects of thymol on pig intestine during inflammation is yet to be investigated. In this study, an *in vitro* lipopolysaccharide (LPS)-induced inflammation model using IPEC-J2 cells was established. Cells were pre-treated with thymol for 1 h and then exposed to LPS for various assays. Interleukin 8 (IL-8) secretion, the mRNA abundance of cytokines, reactive oxygen species (ROS), nutrient transporters, and tight junction proteins was measured. The results showed that LPS stimulation increased IL-8 secretion, ROS production, and tumor necrosis factor alpha (TNF-α) mRNA abundance ($P < 0.05$), but the mRNA abundance of sodium-dependent glucose transporter 1 (SGLT1), excitatory amino acid transporter 1 (EAAC1) and H^+/peptide cotransporter 1 (PepT1) were decreased ($P < 0.05$). Thymol blocked ROS production ($P < 0.05$) and tended to decrease the production of LPS-induced IL-8 secretion ($P = 0.0766$). The mRNA abundance of IL-8 and TNF-α was reduced by thymol pre-treatment ($P < 0.05$), but thymol did not improve the gene expression of nutrient transporters ($P > 0.05$). The transepithelial electrical resistance (TEER) was reduced and cell permeability increased by LPS treatment ($P < 0.05$), but these effects were attenuated by thymol ($P < 0.05$). Moreover, thymol increased zonula occludens-1 (ZO-1) and actin staining in the cells. However, the mRNA abundance of ZO-1 and occludin-3 was not affected by either LPS or thymol treatments. These results indicated that thymol enhances barrier function and reduce ROS production and pro-inflammatory cytokine gene expression in the epithelial cells during inflammation. The regulation of barrier function by thymol and LPS may be at post-transcriptional or post-translational levels.

KEYWORDS: barrier function, IPEC-J2 cells, inflammatory responses, lipopolysaccharide (LPS)-induced inflammation, thymol

INTRODUCTION

Intestinal epithelial cells (IECs) are continuously lined monolayer cells, which play important roles in the animal's physical defense. Normally, IECs function as the first line of defense against the invasion of pathogenic agents in the external environment of gut lumen.[1] The maintenance of the barrier function of IECs contributes to the gut homeostasis and health of animals. Gut disorder and dysfunction might be harmful to the growth performance of food-producing animals, and may induce gut diseases such as inflammatory bowel diseases and diarrhea, possibly due to complex interactions among immunologic, genetic, microbial and environmental factors.[2] For instance, diarrhea is a common gut disorders, which causes almost 5% mortality per year in weaned piglets.[3] Therefore, it is necessary to prevent gut disorder and diseases by maintaining proper barrier function of IECs in animals.

In addition to the physical barrier, IECs also function as an extrinsic barrier. Under certain circumstances, IECs secrete signaling molecules like mucins, cytokines, and chemokines to prevent the invasion of harmful microorganisms in the gut.[4] Also, a series of immune responses within IECs and the interaction among IECs, leukocytes, and adjacent cells is initiated by the invasion of pathogenic bacteria. When toll-like receptors (TLRs) of IECs are activated by invading pathogen-associated molecular patterns (PAMs) like lipopolysaccharide (LPS), a signaling cascade in TLR-activated pathway leads to the activation of transcription factor nuclear factor kappa B (NF-κB) and the secretion of pro-inflammatory cytokines,[5] such as tumor necrosis factor α (TNF-α), interleukin 6 and 8 (IL-6 and IL-8). Therefore, it is necessary to improve gut health through suppressing unnecessary inflammatory responses.

Tight junctions are one of the junctional multiprotein complexes which seal the paracellular space among adjacent epithelial cells. These tight junctions lie at the apical side of the lateral

membrane of epithelial cells and function as a barrier to keep the diffusion of solutes through the intercellular space.[6] Tight junctions regulate ion transport, water, and solutes via the paracellular pathway and blockage of immunogenic macromolecules, and only controlled and selective movements are allowed during the passive permeability process.[1] Disruption of tight junctions would increase the paracellular permeability, which enables harmful substances like pathogens and endotoxins to translocate, and subsequently resulting in tissue damage and inflammation. Tight junctions consist of over 30 structural or functional proteins.[6] Occludin, zonula occludens-1 (ZO-1) and claudin-1 are three crucial proteins to maintain the physiological functions of tight junctions. Previous studies demonstrated that the upregulation of ZO-1 and occludin could suppress the increase of intestinal permeability caused by the disruption of tight junctions in weaned piglets.[7-8] Therefore, maintaining a proper expression level of occludin and ZO-1 is widely considered as an effective target for the therapies of intestinal diseases.

Oxidative stress is also responsible for gut inflammation in animals. Generally, it results from the excessive accumulation of reactive oxygen species (ROS) and the imbalance between ROS and antioxidant agents. Oxidative stress could cause biomacromolecule damage, which leads to inflammation and other diseases.[9] Meanwhile, oxidative stress dysregulates the proliferation, differentiation, and apoptosis of IECs and is detrimental to the maintenance of physiological functions of intestinal epithelia in animals.[10]

Many reports have elucidated that essential oils have anti-oxidative and anti-inflammatory properties[11] and they have been successfully applied to animal feeds.[12-13] Among them, thymol is a natural monoterpene phenolic compound, which exhibits antimicrobial,[14] anti-oxidative[15] and anti-inflammatory[16] properties. It has been certified by the U.S. Food and Drug Administration (FDA), as having Generally Recognized as Safe (GRAS) status.[17] Moreover, thymol has been

identified as having therapeutic potential of fighting against pathogenic bacteria and boosting the immune system.[18] However, little is known about the protective effect of thymol on porcine intestinal epithelial cells during inflammation. Understanding the mechanisms underlying the protective effects of thymol is critical to its effective application in enhancing gut health and function in swine.

Thus, the objective of this study was to explore how thymol regulates the barrier function and inflammatory responses in porcine intestinal epithelial cells. We assumed that thymol might maintain barrier functions by regulating inflammatory signaling pathways and the expression of crucial proteins of tight junctions, and eventually attenuating LPS-induced inflammatory responses in the IPEC-J2 cell line. The underlying molecular mechanism needed to be revealed further.

MATERIALS AND METHODS

Materials. Thymol (≥ 98.5%), fluorescein isothiocyanate-dextran (FITC-dextran, 4 kDa), dimethyl sulfoxide (DMSO) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

Cell culture. The non-transformed neonatal jejunal epithelial cell line IPEC-J2 was grown in DMEM–Ham's F-12 (1:1) (Invitrogen, Fisher Scientific, Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Canadian Origin; Fisher Scientific, Ottawa, ON, Canada), penicillin (100 IU/mL), streptomycin (100 μg/mL) and 0.25 μg/mL of amphotericin B (Fisher Scientific, Ottawa, ON, Canada) and maintained in an atmosphere of 5% CO_2 at 37°C for cultures and assays. Culture medium was replaced every 2-3 d.

Inflammation was induced by LPS that was derived from *Salmonella Enterica* ser. Typhimurium (Sigma-Aldrich). IPEC-J2 cells cultured into a 12-well plate or Millicell membrane

cell inserts (24 wells, Corning Costar, New York City, NY) were first washed with plain medium and then were treated with 10 μg/mL of LPS[4] for different time periods. After treatment, cells cultured into a 12-well plate (Corning Costar) were used for RNA extraction and gene expression assays, and cells cultured in Millicell membrane cell inserts (Corning) were used for transepithelial electrical resistant (TEER) and permeability assays.

For thymol treatment, 100 mM stock solution dissolved in DMSO was freshly prepared and diluted in complete medium at appropriate concentrations (10-100 μM). To eliminate the influence of DMSO, equal levels of DMSO was added to all the experimental groups. For viability assays, cells were treated with thymol at different concentrations (10 -100 μM) for 24 h and for rest of experiments, cells were pre-treated with thymol (50 μM) for 1 h and then continuously treated with the same concentration during the LPS stimulation.

Cytokine measurement by ELISA. The IL-8 and TNF-α concentrations of culture supernatants were measured by ELISA kits (Invitrogen, Fisher Scientific), following the manufacturer's instructions. Briefly, 100 μL of culture supernatant was used for both IL-8 and TNF-α assays. At the end of the reaction process, the plates were read at 450 nm using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). Cytokine concentrations were calculated from a standard curve that had been created using seven 2-fold dilutions of porcine recombinant IL-8 and TNFα. The IL-8 level was expressed as pg/mL. TNF-α level was under the detection limit.

Reactive oxygen species (ROS) assay. Cellular ROS was measured using a fluorescent dye, 2',7'dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich), which is a nonpolar compound that is readily diffusible into cells. It is hydrolyzed to the non-fluorescent polar derivative DCFH and thereby trapped within cells. In the presence of oxidants, DCFH is converted into the highly

fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, cells were cultured in a 96-well plate. After different treatments, cells were washed two times with PBS, and DCFDA at 10 μM in PBS was added to the wells. The cells were then incubated for 30 min and the fluorescence of DCF was detected by a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek) with a maximum excitation and emission spectra of 485 nm and 528 nm.[19]

Cell viability assay. Cell viability was measured using the water-soluble tetrazolium salts (WST-1) Cell Proliferation Reagent (Sigma Aldrich) according to the manufacturer's instructions. Briefly, IPEC-J2 cells were seeded into a 96-well plate (Corning Costar) at a density of 1×10^4 cells/mL and cultured in a medium for two weeks. After different treatments, the cells were washed once with PBS, then 100 μL fresh culture medium containing 10% WTS-1 was added. The cells were then incubated for 1 h. The absorbance at 450 nm was measured using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek). Cell viability was presented as a percentage of untreated control cells.

RNA extraction and real-time PCR. Total RNA was extracted from IPEC-J2 cells using Trizol reagents, (Invitrogen) following the manufacturer's protocol. RNA concentration, OD260/OD280, and OD260/OD230 were measured by Nanodrop-2000 spectrophotometer (Thermo Scientific, Ottawa, ON, Canada). The integrity of RNA was verified by visualization in an agarose gel. Two μg of total RNA was reverse transcribed into cDNA using the iScriptTM cDNA Synthesis kit (Bio-Rad, Mississauga, ON, Canada), following the manufacturer's instructions. Quantitative RT-PCR was performed using SYBR Green Supermix (Bio-Rad) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The Primers for real-time PCR analysis were designed with Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the published mRNA sequence in the Genbank. All the primers spin at least two exons

each. Sequences of primers are listed in Table1. The thermal profile for all reactions was 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. RT-PCR data were analyzed using the $2^{-\Delta\Delta}$CT method[20] to calculate the relative fold change of target gene, using hypoxanthine phosphoribosyl transferase (HPRT) and Cyclophilin-A (CycA) as the reference genes.[21]

TEER measurement. The TEER of cell monolayers was measured using a Millicell Electrical Resistance System (ESR-2) (Millipore-Sigma). IPEC-J2 cells were seeded into Millicell membrane cell inserts (24 wells, Corning Costar) at a density of 7×10^4 cells/cm^2. The TEER was monitored every other day. When a monolayer of cells was completely differentiated, cells were treated with LPS, and TEER was measured before and after treatments. The data were presented as a percentage of initial values before treatments.

Measurement of cell permeability. To quantify the paracellular permeability of cell monolayers, 1 mg/mL of 4 kDa FITC-dextran (Sigma-Aldrich) was added to the apical side of the inserts. The basolateral medium aliquots were taken after 6 h of incubation. The diffused fluorescent tracer was then measured by fluorometry (excitation, 485 nm; emission, 528 nm) using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek).

Immunofluorescent staining. Cells were cultured onto coverslips (Fisher Scientific) and fixed with 4% paraformaldehyde (PFA) (Sigma). The cells were blocked with 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour and then incubated with an anti-rabbit ZO-1 polyclonal antibody (1:100 dilution, Thermal Scientific) at 4℃ overnight. The cells were then washed 3 times with PBS and incubated with an Alexa fluor 488 goat anti-rabbit antibody (Thermal Scientific, Cat # A-11034) for 1 h at room temperature. Rinsed cells were

mounted with Vectashield Mounting Medium with DAPI (**Vector Laboratories, Inc.** Burlingame, CA, USA). For actin staining, fixed cells were washed 3 times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 20 min and incubated with Phalloidin, CF^{TM}488A (1:100 dilution in PBS, Biotium, Inc, Fremont, CA 94538, USA) at room temperature for 1 h. The cells were then washed 3 time with PBS and mounted with Vectashield Mounting Medium with DAPI (**Vector Laboratories, Inc.).** Images were taken by a Zeiss Fluorescence Microscope (Car-Zeiss Ltd, Toronto ON, Canada).

Statistical analysis. Data were presented as means ± standard deviations. The statistical analyses were performed with the GraphPad Prism 7 (GraphPad Software, La Jolla, USA). Differences between means were evaluated by one-way ANOVA. Multiple comparisons were done using Tukey's multiple comparisons test. Level of significance was set at $P < 0.05$.

RESULTS

Dose-effect of thymol on the viability of IPEC-J2 cells. As shown in Figure 1, thymol concentrations less than 100 μM did not significantly affect cell viability ($P > 0.05$), but the viability of IPEC-J2 significantly decreased at the concentrations above 100 μM compared with control ($P < 0.05$). Therefore, 50 μM was set as the working concentration of thymol for further experiments.

Effect of thymol on IL-8 secretion, ROS production, and cell viability in IPEC-J2 cells. As shown in Figure 2A and 2B, the levels of IL-8 and ROS in the LPS treatment were significantly higher than that in the control ($P < 0.05$). Although thymol pre-treatment significantly inhibited LPS-induced production of ROS ($P < 0.05$), thymol tended to decrease IL-8 secretion ($P = 0.0766$). The effect of LPS and thymol treatment on cell viability was shown in Figure 2C. LPS and thymol treatment had no significant effect on the cell viability ($P > 0.05$).

Effect of thymol on cytokine gene expression in IPEC-J2 cells. As shown in Figure 3A, there was no significant effect on the relative gene expression of IL-8 in the LPS treatment compared with the control ($P > 0.05$), whereas thymol treatment before LPS stimulation significantly down-regulated the gene expression of IL-8 compared with LPS treatment group ($P < 0.05$). The relative gene expression of TNF-α was significantly up-regulated in cells stimulated with LPS compared with the control ($P < 0.05$), but thymol showed a significant suppressive effect on gene expression of TNF-α in cells treated with thymol + LPS ($P < 0.05$) (Figure 3B). The relative gene expression of IL-6 did not respond to LPS stimulation in IPEC-J2 cells compared with the control ($P > 0.05$), and also LPS + Thymol treatment had no effect ($P > 0.05$) (Figure 3C).

Effect of thymol on transporter gene expression in IPEC-J2 cells. Figure 4A and 4C showed that LPS significantly downregulated the expression of intestinal Na^+/glucose cotransporter 1 (SGLT-1) ($P < 0.05$) and excitatory amino-acid carrier 1 (EAAC-1) ($P < 0.05$) compared with the control. However, thymol pre-treatment of cells did not significantly ($P > 0.05$) upregulate the expression of these two transporter genes compared with only LPS-stimulated cells. LPS did not show a significant effect on ASC amino-acid transporter 2 (ASCT2) transporter gene expression ($P > 0.05$) (Figure 4B). Figure 4D shows that LPS downregulated the expression of H^+/peptide cotransporter 1 (PepT1) significantly compared with the control ($P < 0.05$), but pre-treatment of cells with thymol had no effects on the expression of this gene ($P > 0.05$). As shown in Figure 4E, LPS significantly upregulated the gene expression of B^0-type amino acid transporter 1 (B^0AT1) in IPEC-J2 cells compared with the control ($P < 0.05$), but the pre-treatment of cells with thymol did not show a significantly positive effect on LPS-induced B^0AT1 transporter gene expression ($P > 0.05$).

Effect of thymol on relative gene expression of tight junction proteins in IPEC-J2 cells. There were no significant differences in the gene expression of ZO-1 and CLDN3 in both LPS and LPS + thymol treatment groups compared with the control ($P > 0.05$) (Figure 5).

Effect of thymol on TEER and permeability of IPEC-J2 cells. As shown in Figure 6A, the percentage of relative TEER value was the lowest for 1 h LPS stimulation at 0 h, 2h and 3h ($P < 0.05$), whereas after 1 h LPS stimulation, there was no significant difference ($P > 0.05$) at 2h and 3 h. This indicated that TEER adapted to LPS stimulation after 1 h, and thus 1 h LPS stimulation was chosen to test the effect of thymol on TEER values in LPS-induced IPEC-J2 cells. As shown in Figure 6B, the percentage of relative TEER value of cells stimulated with LPS for 1 h was significantly lower compared with the control ($P < 0.05$), whereas pre-treatment of cells with thymol restored the value to the same level as the control ($P > 0.05$). The protective effect of thymol on LPS-induced damage in IPEC-J2 was also apparent in the FITC-dextran flux experiment (Figure 6C). Pre-treatment with thymol significantly decreased the leakage of FITC-dextran caused by LPS stimulation compared with the LPS treatment group ($P < 0.05$), and the measurements were similar to the control ($P > 0.05$).

Effect of thymol on LPS-induced morphological changes in tight junction and actin fiber in IPEC-J2 cells. As shown in Figure 7, the distribution of ZO-1 protein within the tight junction was broken after LPS stimulation compared with the control, whereas pre-treatment of cells with thymol promoted the even redistribution of ZO-1 protein within the tight junction. The cytoskeletal structure of the actin fiber in IPEC-J2 cells was disorganized due to LPS stimulation compared with the control, whereas the pre-treatment of cells with thymol alleviated the severity.

DISCUSSION

Thymol has been known as a functional phytochemical isolated from plant essential oils and possesses many pharmacological properties.[18] Thymol as one of popular essential oil components derived from thyme has been used in animal feeds for several years due to its antioxidant, antimicrobial, and anti-inflammatory properties.[22] Many studies have shown that thymol has a positive effect on attenuating LPS-induced inflammation *in vitro* and *in vivo* via regulating immune responses. [23-24] The effectiveness of thymol is mainly attributed to the phenolic hydroxyl group in its chemical structure.[25] Gut inflammation can be caused by infection, diet allergy and weaning in piglets.[26-27] Although gut inflammation may not cause the full-blown clinical symptoms in weaned piglets, it could lead to increased mucosal permeability and reduced growth performance. The dietary supplementation of thymol can prevent the intestinal injury by improving the intestinal integrity and modulating immune responses in the *Clostridium perfringens*–challenged broiler chickens.[28] However, the potential anti-inflammatory effects of thymol on porcine intestinal cells remain to be elucidated. Therefore, an *in vitro* inflammation model using IPEC-J2 cells was established in the present study to evaluate the protective effects of thymol on LPS-induced inflammatory responses in epithelial cells.

Our results showed that LPS significantly increased IL-8 secretion (Figure 2A) and TNF-α mRNA abundance (Figure 3B) in cells when compared with control cells without LPS treatment, indicating inflammation was successfully induced by LPS. The thymol treatment was able to attenuate the inflammatory responses induced by LPS evidenced by reduced IL-8 and TNF-α mRNA abundance (Figure 3A and 3B) as well as the tendency to decrease the production of LPS-induced IL-8 secretion (Figure 2A). Interestingly, IL-6 mRNA abundance (Figure 3C) was not affected by LPS or thymol. The possible explanation was that different LPS sources and different treatment time might cause different inflammatory responses in the IPEC-J2 cells. It has been

reported that IPEC-J2 cells treated with 1 μg/mL LPS (*E. coli* 055:B5) with different treatment times showed a varied effect on IL-6 secretion.[29] Previous studies indicated that oxidative stress affected IL-8 and TNF-α expression, but IL-6 expression changed insignificantly in IPEC-J2 cells.[30] Farkas et al.[21] found that modified apigenin did not influence IL-6 expression as apigenin did in IPEC-J2 cells during LPS-induced inflammation. Many reports have indicated that the signaling pathways like NF-κB and mitogen-activated protein kinase (MAPK) are involved in the transcriptional regulation of cytokine genes. Thymol could inhibit LPS-induced inflammation by suppressing NF-κB and MAPK signaling pathways,[24, 31] and activating Nrf2 pathways.[32] The underlying mechanisms might be that the anti-inflammatory effects of thymol are related to the regulation of these signaling pathways. Therefore, supplementation with thymol might be a potential approach to reduce inflammatory responses in weaned piglets.

Generally, during LPS-induced inflammation, phagocytes might be stimulated to generate excessive ROS and thereby leading to the imbalance between ROS and antioxidants.[33] The imbalanced redox state could induce oxidative stress, which causes the dysfunction of porcine IECs by damaging the structure of biomacromolecules like DNA, proteins, and lipids.[9] However, the ROS production induced by LPS was significantly blocked by thymol (Figure 2B). The results were consistent with a recent study indicating that thymol attenuated the ROS production and showed myeloperoxidase inhibitory activity in isolated human neutrophils.[34] Thymol as a phenolic compound is an effective antioxidant, which could scavenge ROS and free radicals to maintain the normal cellular functions and signaling pathways. The decrease of ROS intensity might be that the barrier functions of IPEC-J2 cells was enhanced. Thymol also has hydrophobicity which allows it to permeabilize cellular or organelle membranes and can be cytotoxic but the cytotoxicity is dose-dependent.[35] Thymol does not alter cell membrane permeabilization up to 50 mg/L (about 300

μM).[36] A study of ultrastructural morphological examination by electron microscopy indicated that Caco-2 cell death is induced when treated with 250 μM thymol for 24 and 48 h.[37] In our study, the dose of thymol addition (50 μM) did not show any harmful effects on cell viability (Figure 2C). The minimum inhibitory concentrations of thymol against several pathogens are from 80-640 mg/L.[38] So a low dose of thymol addition (50 μM) in the present study unlikely has antimicrobial activities but can effectively reduce inflammation in the porcine intestinal epithelial cells through scavenging ROS. Small intestine is the major organ for the digestion and absorption of dietary carbohydrates and proteins. Hence, nutrient transporters SGLT1, ASCT2, EAAC1, PepT1, and B^0AT1 play important roles in the absorption of carbohydrates, amino acids, and proteins (in the form of glucose, amino acids, di- and tripeptides).[39] Previous studies demonstrated that the dietary supplementation of essential oils could enhance carbohydrate and protein absorption by improving the gene expression of nutrient transporters in animals.[40-42] In the present study, LPS reduced the mRNA abundance of these nutrient transporters except ASCT2 and B^0AT1 (Figure 4), which demonstrated that LPS-induced inflammation damaged the structure and barrier functions of porcine intestinal epithelia and the major nutrient transport through a transcellular pathway. Meanwhile, oxidative stress might cause mitochondria injury and energy metabolisms disorder, but because these transporters are energy-dependent, it is possible that this might poses a threat to the process of energy-dependent active transport. However, they were not totally restored by the addition of thymol on the level of transporter mRNA abundance. It does not mean that thymol did not affect the transport activities. Many post-translational modifications (PTMs) could regulate the activity of transporters, such as glycosylation, phosphorylation, trafficking and ubiquitination.[43] We assumed that thymol might mainly regulate the activities of these transporters at the PTMs level, but further research is needed to confirm this assumption. It is understood that the mRNA

abundance might not thoroughly reflect the activity of these transporters. Furthermore, many reports have indicated that nutrient transporters function depends on ions like Na^+ and H^+.[44] We inferred that thymol might not be able to completely rescue the disruption of ions homeostasis caused by LPS stimulation, which explains the insignificant changes in the expression of transporters at the transcription level under the thymol treatment.

A recent study demonstrated that intact epithelial barrier functions are necessary to maintain the "homeostatic tolerance" in response to physiological host–gut microbiome cross-talks and therefore the control of epithelial barrier dysfunction is crucial for preventing gut inflammation.[45] The results also indicated that when the intestinal epithelium is intact, probiotic bacteria can be of benefit to gut health and they may be harmful when the gut barrier is already compromised.[45] So it is very crucial to restore proper gut barrier function in order to prevent gut disorder and diseases in weaned piglets. The TEER reflects variations in the permeability both through transcellular and paracellular pathways, but it is mainly thought to depend on the status of tight junctions.[46] Thus, TEER is considered as a good indicator of the integrity and tightness of intestinal epithelial barrier model.[6] Similarly, the FITC-dextran flux reflects the permeability of cells. The TEER was reduced by LPS stimulation for 1 h. After LPS stimulation, the structure of IECs, as well as tight junctions were disrupted (Figure 7). Thus, the relative TEER value significantly decreased and FD-4 significantly increased (Figure 6A and 6B). However, these effects were prevented by thymol, and the TEER value was normalized by using thymol as a pre-treatment. Also, pre-treatment of the monolayers with thymol significantly decreased the leakage of FITC-dextran and increased ZO-1 and actin staining in the cells (Figure 7). These results indicate that thymol could enhance barrier functions in the IPEC-J2 cells, which were consistent with recent studies with cinnamaldehyde in porcine epithelial cells [47] and with oregano essential oil in pigs.[48] Moreover, thymol increased tight

junction integrity by 5 mg/L and by max. 61.7 ± 5.4% at 100 mg/L in Caco-2 cells, which indicates that thymol might have protective functions in intestinal epithelial cells.[36] Therefore, supplementation with a low dose of thymol might be a potential nutritional approach to improve intestinal barrier functions in weaned piglets.

Various dietary components are known to regulate epithelial permeability by modifying expression and localization of tight junction proteins.[49-50] Although thymol improved the protein level of ZO-1 in the IPEC-J2 cells, the mRNA abundance of ZO-1 was not affected by either LPS or thymol (Figure 5A). Similarly, as a crucial protein of tight junctions, the mRNA abundance of CLND-3 was not significantly upregulated (Figure 5B). The results were consistent with the findings that a carvacrol-thymol blend decreased intestinal oxidative stress and influenced selected microbes without changing the mRNA levels of tight junction proteins in the jejunal mucosa of weaning piglets.[51] This discrepancy between ZO-1 mRNA and ZO-1 protein levels suggested that thymol may regulate ZO-1 expression by the post-transcriptional or post-translational mechanisms in the present study, although tight junction proteins have been reported to be regulated at both the transcriptional and post-transcriptional levels.[52] However, the molecular basis for thymol actions on the barrier functions requires further investigations, especially thymol's molecular target (e.g. odorant receptor or protein kinase).[53] For instance, thymol might be an agonist of transient receptor potential cation channel subfamily M member 8 channel (TRPM8) to inhibit downstream inflammatory responses, and it has been reported for eucalyptol, a plant essential oil.[54] Therefore, we suggested that the sensing and responding to thymol in the gut may also play a role in maintaining intestinal homeostasis.

In conclusion, thymol is derived from cymene and recognized as a bioactive compound of essential oils, which effectively enhanced gut barrier structure and functions under inflammatory

355 status by reducing ROS production and pro-inflammatory cytokine gene expression in the IPEC-
356 J2 cells. Supplementation with a low dose of thymol might be a potential nutritional or therapeutic
357 strategy to improve the intestinal mucosal barrier function and reducing inflammatory responses
358 in weaned piglets. *In vivo* studies with experimentally infected pigs are still needed to validate the
359 beneficial effects of thymol on intestinal barrier functions observed in the present study.

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372 **Notes**

373 The authors declare no competing financial interest.

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536 villus axis in young pigs fed a liquid formula. *Amino acids* **2016,** *48* (6), 1491-1508.

537

538 **Table 1. Primers used in this study.**

Genes	Primer sequences	Product size (bp)	References
TNF-α	5'-ATGGATGGGTGGATGAGAAA-3' 5'-TGGAAACTGTTGGGGAGAAG-3'	151	
IL-8	5'-CACCTGTCTGTCCACGTTGT-3' 5'-AGAGGTCTGCCTGGACCCCA-3'	126	
IL-6	5'-AAGGTGATGCCACCTCAGAC-3' 5'-TCTGCCAGTACCTCCTTGCT-3'	151	
SGLT1	5'-GGCTGGACGAAGTATGGTGT-3' 5'-GAGCTGGATGAGGTTCCAAA-3'	153	Yang et al., 2010 [55]
ASCT2	5'-GCCAGCAAGATTGTGGAGAT-3' 5'-GAGCTGGATGAGGTTCCAAA-3'	206	Yang et al., 2016 [56]
EAAC1	5'-CCAAGGTCCAGGTTTTGGGT-3' 5'-GGGCAGCAACACCTGTAATC-3'	168	
B^0AT1	5'-AAGGCCCAGTACATGCTCAC-3' 5'-CATAAATGCCCCTCCACCGT-3'	102	Yang et al., 2016 [57]
PepT1	5'-CATCGCCATACCCTTCTG-3' 5'-TTCCCATCCATCGTGACATT-3'	143	
ZO-1	5'-GATCCTGACCCGGTGTCTGA-3' 5'-TTGGTGGGTTTGGTGGGTT-3'	200	
CLDN3	5'-CTACGACCGCAAGGACTACG-3' 5'-TAGCATCTGGGTGGACTGGT-3'	123	
CycA	5'-GCGTCTCCTTCGAGCTGTT-3' 5'-CCATTATGGCGTGTGAAGTC-3'	160	Farkas et.al., 2015 [21]
HPRT	5'-GGACTTGAATCATGTTTGTG-3' 5'-CAGATGTTTCCAAACTCAAC-3'	91	Farkas et.al., 2015 [21]

539 Note: TNFα: Tumor necrosis factor α; IL-8: Interleukin 8; IL-6: Interleukin 6; SGLT1:
540 Sodium/glucose cotransporter 1; ASCT2: glutamine transporter; EAAC1: excitatory amino acid
541 transporter 1; B^0AT1: neutral amino acid transporter; PepT1: peptide transporter 1; ZO-1: Zonula
542 occludens-1; LDN3: Claudin 3; CycA: Cyclophilin-A; HPRT: hypoxanthine phosphoribosyl
543 transferase.

Figure captions

Figure 1. Dose-effect of thymol on the viability of IPEC-J2 cells. IPEC-J2 cells were seeded into a 96 well plate and cultured for 10 d. Cells were then treated with thymol at the indicated concentrations for 24 h. Cell viability was measured using WST-1 as described in the Materials and Methods and expressed as a percentage of control. The data were presented as mean ± SD, n = 5.

Figure 2. Effect of thymol on LPS-induced IL-8 secretion, ROS production, and cell viability. IPEC-J2 cells were seeded into 12-well plates (for IL-8) or 96 well plates (for ROS production and viability) and cultured for 10 d. Cells were pre-treated with thymol (50 μM) for 1 h and then stimulated with LPS (10 μg/mL) for 1 h (for ROS production and viability) and 4 h (for IL-8). IL-8 in medium (A), intracellular ROS production (B) and viability (C) were measured as described in the Materials and Methods The data were presented as mean ± SD, n = 4. Different letters represent a significant difference ($P < 0.05$).

Figure 3. Effect of thymol on LPS-induced cytokine gene expression. IPEC-J2 cells were seeded into 12-well plate and cultured for 10 d. Cells were pre-treated with thymol (50 μM) for 1 h and then stimulated with LPS (10 μg/mL) for 4 h. Total RNA was extracted from cells and the mRNA abundance of IL-8 (A), TNF-α (B) and IL-6 (C) was detected by RT-PCR. The data were presented as mean ± SD, n = 4. Different letters represent a significant difference ($P < 0.05$).

Figure 4. Effect of thymol on LPS-induced nutrient transporter gene expression. IPEC-J2 cells were cultured and treated using the same condition as Fig. 3. Total RNA was extracted from cells and the mRNA abundance of SGLT1 (A), ASCT2 (B), EAAC1 (C), PepT1 (D) and B^0AT1 (E)

565 was detected by RT-PCR. Data were presented as mean ± SD, n = 4. Different letters represents a
566 significant difference ($P < 0.05$).

567 **Figure 5**. Effect of thymol on LPS-induced tight junction protein gene expression. IPEC-J2 cells
568 were cultured and treated using the same condition as Fig. 3. Total RNA was extracted from cells
569 and mRNA abundance of ZO-1 (A) and claudin3 (CLDN3) was detected by RT-PCR. Data were
570 presented as mean ± SD, n = 4. Different letters represent a significant difference ($P < 0.05$).

571 **Figure 6**. Effect of thymol on LPS-induced trans-epithelial electrical resistance (TEER) and
572 permeability. IPEC-J2 cells were seeded into millicell membrane cell inserts (24-well) with a
573 density of 1×10^5/well and cultured for 2 weeks. TEER was monitored every other day. LPS (10
574 μg/mL) induced time course change of TEER was measured (A). To test the effect of thymol (50
575 μM) on LPS-induced TEER, cells were pre-treated with thymol (50 μM) for 2 h and then
576 stimulated with LPS for 1 h. TEEER was measured before and after LPS-stimulation (B).
577 Permeability was tested by FD4 (C). Data were presented as a percentage of initial TEER value
578 and given as means ± SD, n = 4. Bars with different letters are significantly different ($P < 0.05$).

579 **Figure 7**. Effect of thymol on the LPS-induced morphological changes of tight junction and actin
580 fiber. IPEC-J2 cells were seeded into coverslips at a density of 1×10^5/well and cultured for 2
581 weeks. Cells were pre-treated with thymol (50 μM) for 2 h and then stimulated with LPS for 1 h.
582 Cells then were the fixed for ZO-1 and actin staining as described in the MATERIALS AND
583 METHODS.

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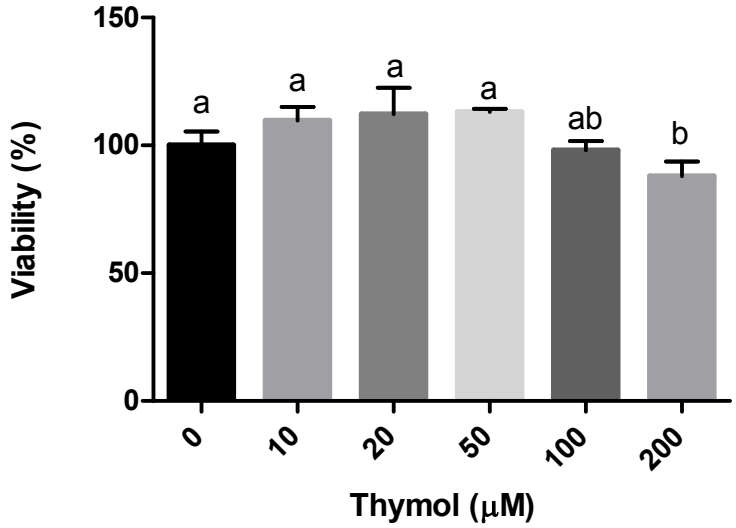
586

587 **Figure 1**



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590 **Figure 2**

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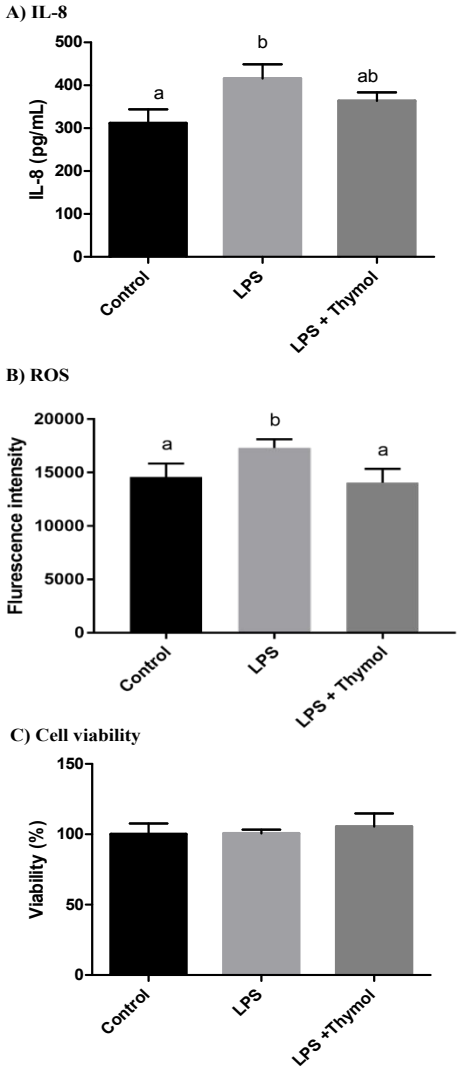

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593 **Figure 3**

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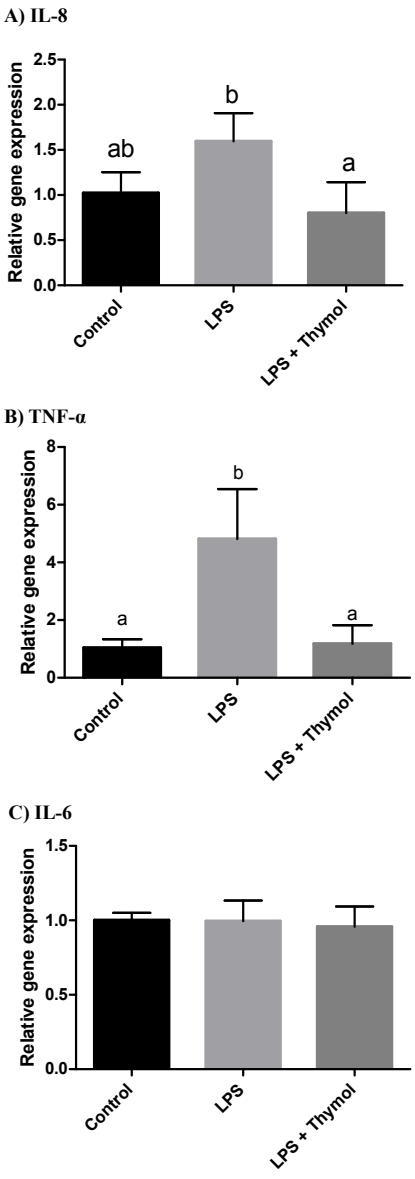

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596 **Figure 4**

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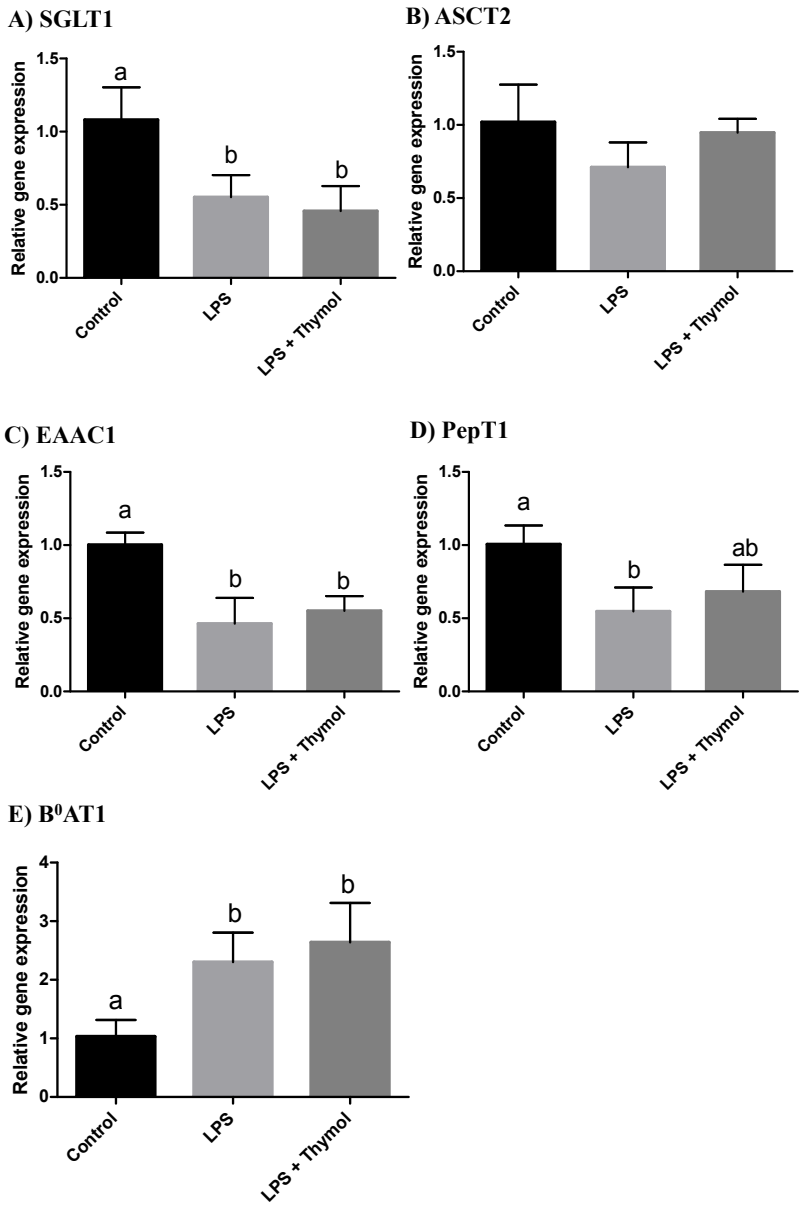

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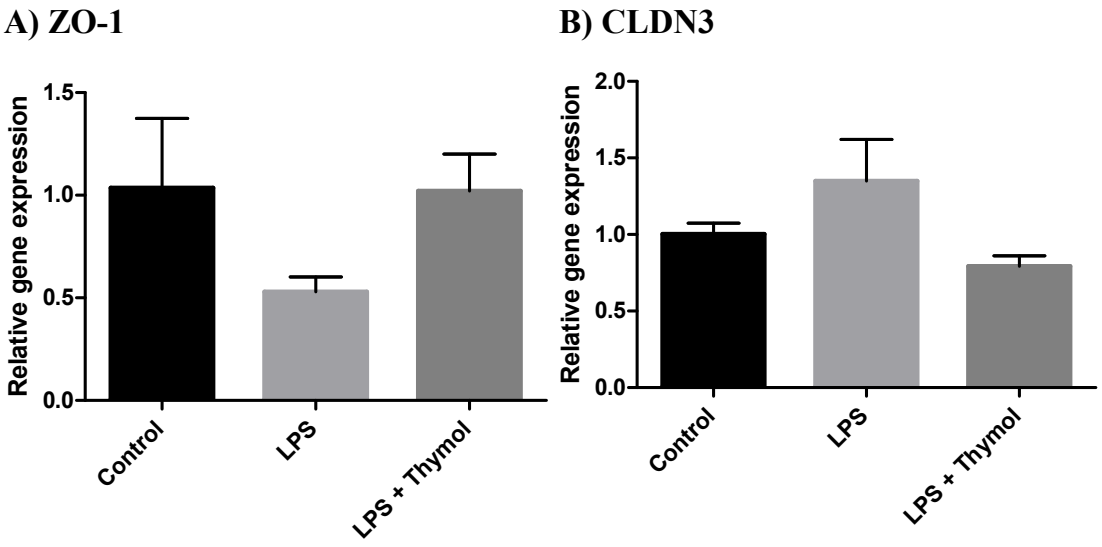
599 **Figure 5**



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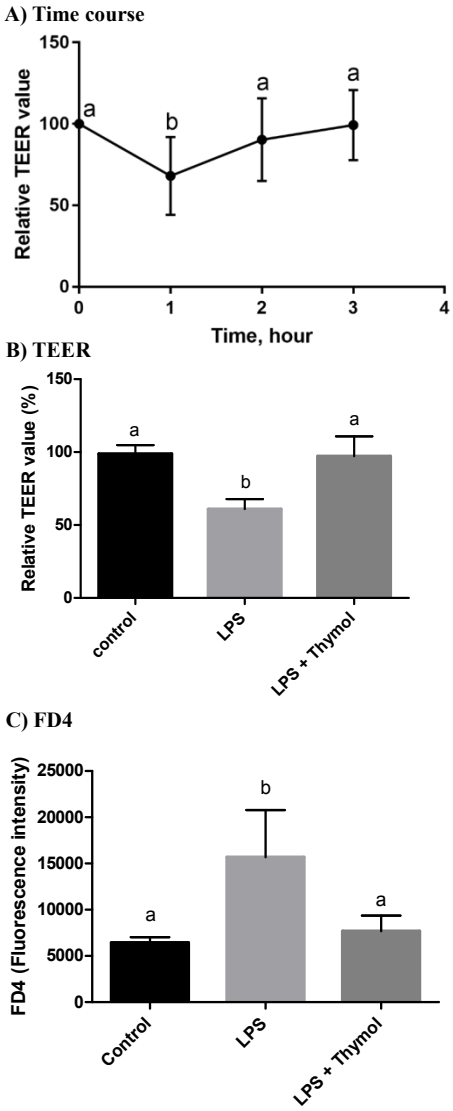
601 **Figure 6**



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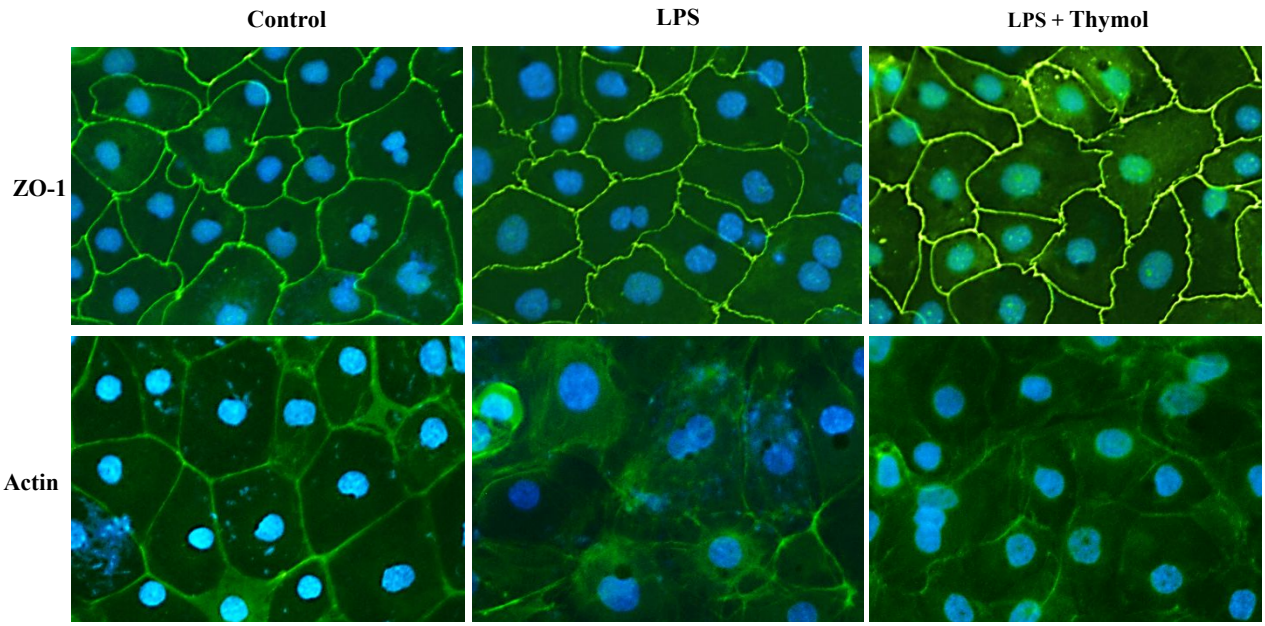
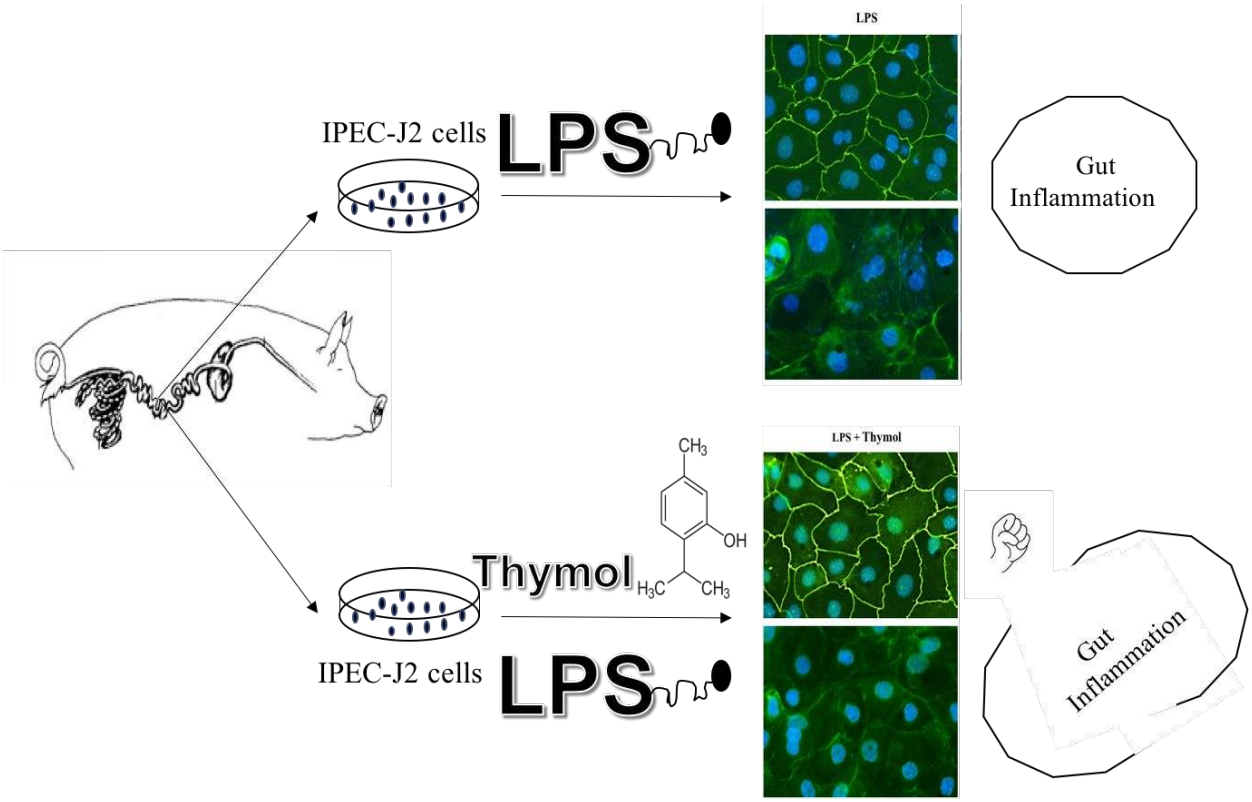
604 **Figure 7**



605 TOC graphic



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