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Evaluating protective effects of botanicals under inflammation and oxidative stress in chicken apical-out enteroids

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ABSTRACT Botanicals (**BOTs**) are well known for their anti-inflammatory and antioxidant activities. They have been widely used as feed additives to reduce inflammation and improve intestinal functions in agricultural animals. However, the effects of BOTs on chicken intestinal epithelial functions are not fully understood. The 3D apical-out chicken enteroids recapitulate the intestinal tissue, and allow convenient access to the luminal surface, thus serving as a suitable model for investigating gut functions. The aim of this study was to identify the roles of BOTs in protecting the intestinal epithelium in chicken enteroids under challenging conditions. Apical-out enteroids were isolated from the small intestines of 18 days-old chicken embryos. Lipopolysaccharide (**LPS**, 10 μg/mL) and menadione (400 μM) challenges were performed in the media with or without BOTs. Paracellular Fluorescein isothiocyanate−dextran 4kD (**FD4**) permeability, inflammatory cytokine gene expression, and reactive oxygen species (**ROS**) generation were analyzed post-BOTs and challenges treatments. Statistical analysis was performed using one-way ANOVA and post hoc multiple comparisons among treatments. The results showed that the LPS challenge for 24 h induced a 50% increase in FD4 permeability compared with nontreated control; thymol, thyme essential oil, and phenol-rich extract significantly ($P < 0.02$) reduced FD4 permeability by 25%, 41%, and 48% respectively, in comparison with LPS treatment. Moreover, the gene expression of inflammatory cytokines was upregulated, tight junction proteins and defensins were downregulated ($P < 0.05$) after 6 h of LPS treatment, while these BOTs treatments significantly restored the LPS-induced gene expression alterations ($P < 0.05$). Menadione oxidative challenge for 1 h significantly increased the ROS level compared with unchallenged control. Enteroids treated with thymol and thyme essential oils showed 30% reduced ROS levels, while the phenol-rich extract reduced them by 60%, in comparison with the challenged group ($P < 0.0001$). These data confirmed the role of BOTs in supporting the barrier function and reducing the disruptive effects of inflammation and oxidation in the chicken intestine.

Key words: chicken enteroids, gut barrier, botanicals, oxidative stress, inflammation

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INTRODUCTION

Intestinal epithelium serves as the largest body interface to the environment and constantly gets exposed to various luminal compounds. Therefore, the integrity of this epithelial barrier is critical for animals to defend against dietary antigens and pathogens. To achieve this, different cell types such as enterocytes, Paneth cells, and goblet cells in the epithelium tightly work together to maintain intestinal homeostasis and normal functions (Salzman et al., 2007; Yang and Yu, 2021). Upon oxidative stress or pathogenic challenge, epithelial cells along with underneath immune cells activate the inflammatory cytokine production pathway, which leads to downregulation in tight junction proteins and increased paracellular permeability (Lu et al., 2008; Gill et al., 2010; Bein et al., 2016). The in vitro monolayer epithelial cells, such as immortalized cell line (Caco2, IPECJ2) (Toschi et al., 2020, 2022) or primary enterocytes culture (Ghiselli et al., 2022), have been used as a 2D epithelial cell model to mimic intestinal barrier and to study the efficacy of feed additives to fight intestinal inflammation or pathogens. However, lacking epithelial components (e.g., mucin production in Caco2) and immune cells (e.g., leukocytes) in these models oversimplified the in vivo intestinal barrier function. In addition, the intrinsic difference between animal species limited

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the proper use of these cell models to investigate intestinal functions in avian species. The recently developed 3D enteroids model from chickens could be an effective alternative. Nash et al., 2021 published a study about the deep characterization of chicken apical-out enteroids, showing that those organoids contain: epithelial cells bound by cell-cell junctions, intraepithelial leukocytes, and an inner core of lamina propria leukocytes (Nash et al., 2021).

Lipopolysaccharide (**LPS**) is a pathogen-associated molecular pattern that can activate inflammatory reaction (Rosadini and Kagan, 2017). Upon LPS binding to the Toll-like receptor 4 (**TLR4**) located on the surface of enterocytes, a classic pro-inflammatory cytokine production and inflammation can be activated through the nuclear factor kappa-light-chain-enhancer of activated B cells (**Nf-kB**) and mitogen-activated protein kinases (**MAPK**) pathways (Lu et al., 2008). Menadione, a polycyclic aromatic ketone, causes intracellular reactive oxygen species (**ROS**) production at various cellular locations via futile redox cycling (Loor et al., 2010). Experimental challenges induced by LPS and menadione have been widely used in the in vitro intestinal models to evaluate cell inflammatory and oxidative status (Bein et al., 2016; Kishore et al., 2019; Ghosh et al., 2020).

Botanicals (**BOTs**) are a well-known class of bioactive compounds with anti-inflammatory and antioxidant properties. Polyphenols, terpenes, and aldehydes are reported to be ROS scavengers. This antioxidant propriety is also connected with their immunomodulatory action. Polyphenols, for example, seem to block the proinflammatory mediators interacting with the Nf-kB and MAPK (Miguel, 2010). Moreover, some aldehydes and polyphenols are also able to block cytokines secretion (Cuevas et al., 2013; Aljaafari et al., 2022). Thymol (**THY**) and carvacrol are 2 terpenes and the main components of thyme essential oil (**TEO**). They have been widely studied for their bactericidal activity (Zengin and Baysal, 2014). These 2 terpenes are able to inhibit the Nf-kB pathway by acting as anti-inflammatory molecules (Liang et al., 2014; Chen et al., 2020; Rossi et al., 2020). Moreover, THY and carvacrol can increase glutathione and superoxide dismutase levels through the Nrf2 pathway to reduce ROS levels inside the cells (Saleh et al., 2021). Another interesting bioactive compounds are capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) and capsaicinoids which are contained in the capsicum oleoresin (**COR**) and they are characterized by antioxidant, and antimicrobial effects (Kim et al., 2003). Ginger essential oil (**GEO**) is commonly obtained from *Zingiber officinale* roots, and it is well known for antioxidant properties (Habibi et al., 2014; Elazab et al., 2022). Jeena et al. (2013) reports high antioxidant activity with increased superoxide dismutase, glutathione, and glutathione reductase enzymes level and a reduction in acute inflammation in mice treated with GEO for 1 mo (Jeena et al., 2013). Lastly, the main components of tea tree oil **(TTO)** are terpenes. They have potent anti-inflammatory and antioxidant activities modulating the metabolism of nitric oxide and ROS (Schewe and Sies, 2009). It has been shown that TTO supplementation could improve the growth performance, cecal microflora composition, immunity, and antioxidant capacity in Partridge Shank chickens (Qu et al., 2019).

The aim of this study was to evaluate the protective effects of BOTs on mitigating oxidative stress and LPS induced inflammation in chicken apical-out enteroids.

MATERIALS AND METHODS

Care and Use of Animals

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Delaware. Cobb500 broiler eggs were incubated at 37.7°C, 55% relative humidity in a semiautomated incubator. On the 18th day of incubation (>80% of incubation), according to the Guide for the Care and Use of Agricultural Animals in Research and Teaching 4th edition, chick embryos were sacrificed by decapitation. As chick embryos older than 14 d can experience pain, decapitation was recommended as a humane method of euthanasia ("AVMA guidelines for the euthanasia of animals"). The Public Health Service Policy on Humane Care and Use of Laboratory Animals applies to activities that involve live vertebrate animals. While embryonic stages of avian species do develop vertebrae before hatching, the University of Delaware IACUC has interpreted "live vertebrate animal" rules to apply to avian species only after hatching. In this context, ethical committee approval was not required.

Intestinal Villus Isolation and Enteroids Culture

The small intestines (jejunum and ileum) were isolated using the landmarks from the end of the duodenum/pancreas loop to the ileal-cecal junction. The tissues were washed and placed in ice-cold Dulbecco's phosphate-buffered saline (**DPBS** - Cat.# D8537 - Sigma-Aldrich St. Louis, MO) to remove blood cells. Mesenteric membranes and connective tissues were peeled off by forceps and removed by DPBS washing. Intestines were cut open longitudinally, chopped into 1 to 2 mm pieces, and washed in ice-cold DPBS. Tissues were digested with 0.5 mg/mL collagenase type I (Cat. # SCR103 - Sigma-Aldrich St. Louis, MO) for 30 min at 37°C. The digested tissue was filtered through cell strainers (Cat.# 734-2762 - VWR, Radnor, PA) to collect intact villus units with sizes between 40 and 100 μm.

The enteroids culture protocol was modified from the method published by Nash et al. (2021). Briefly, the isolated villus units (pooled from more than 60 embryos) were cultured in the complete culture medium constituted by Gibco advanced DMEM/F12 (Cat.# 12634010 - Thermo Fisher Scientific, Waltham, MA), B27 supplement (Cat.# 17504044 - Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine (Cat.# G7513 - Sigma-Aldrich

St. Louis, MO), 50 U/mL Penicillin-Streptomycin (Cat.# P4333 - Sigma-Aldrich St. Louis, MO) and 10 mM HEPES (Cat.# H3375 - Sigma-Aldrich St. Louis, MO). Sphere shape enteroids were formed after 24 h culture. The non-Enteroids single cells and debris were removed after 300 × *g* centrifugation for 5 min. Enteroid pellets were resuspended in complete media and cultured in standard cell culture treated plates at a density of 1,600 enteroids/mL. Media were changed every 2 d. The individual wells serve as experimental unit. Total 4 ∼ 8 replicate wells were included in each treatment condition described below.

BOT Treatments

At d 3 or d 4 of enteroids culture, the following BOTs and their concentrations were added to the complete media. THY 10 ppm equal to 0.07 μM (analytical grade ≥ 98.5% - Sigma-Aldrich St. Louis, MO), TEO 20 ppm (50% thymol − Frey + Lau GmbH, Henstedt−Ulzburg, Germany); phenols-rich grape seed extract 100 ppm (**PRE** - Guilin Layn Natural Ingredients Corp., Shanghai, China); COR 10 ppm (10% capsaicinoids − Frey + Lau GmbH, Henstedt−Ulzburg, Germany); GEO 100 ppm (Frey+Lau GmbH, Henstedt−Ulzburg, Germany) and TTO 100 ppm (Frey+Lau GmbH, Henstedt−Ulzburg, Germany). Stock solutions of all the BOTs were prepared in absolute ethanol to ensure their complete solubility and supplemented in a culture medium with a final concentration of ethanol ≤ 0.15% (v/v), which is considered safe for cell cultures (Nguyen et al., 2020).

Inflammatory Challenge

Chicken enteroids were seeded at a density of 800 organoid/well (500 μL of culture media per well) into 24 well cell culture plates (Cat.# 3524 − Corning Incorporated, Corning, NY) and were maintained at 37.5°C and 5% CO_2 until d 4. On d 4, 10 μg/mL of LPS from *Salmonella minnesota* R595 (Cat.# tlrl-smlps − Invivogen, Toulouse, France) was added in culture media in the presence or absence of BOTs, to induce inflammatory challenge. At 6 h postchallenge, enteroids were harvested for RNA isolation and qPCR analyses; at 24 h postchallenge, enteroids were harvested for Fluorescein isothiocyanate−dextran 4kD (**FD4** - Cat.# 46944 - Sigma-Aldrich St. Louis, MO) paracellular permeability (**PCP**) analyses.

Paracellular Permeability

At the end of the LPS challenge, enteroids were treated with 1 mg/mL FD4 for 2 h. Then the FD4 was removed by 3 steps of DPBS washing, with 300 × *g* centrifugation for 5 min in each step. Enteroid pellets were resuspended in a density of 1,000 enteroids per ml in DPBS and aliquoted in a black clear bottom 96 well plates (Cat.# 3603 − Corning Incorporated, Corning, NY). The fluorescent signal (excitation: 485 nm; emission: 530 nm) from the permeated FD4 inside the enteroids was quantified by SpectraMax M2e microplate reader (Molecular Devices, San Jose, CA), using the supernatant of the last washing step as blanks. Enteroids fluorescent images were observed under an Echo Revolve microscope.

Oxidative Stress Challenge

Chicken enteroids were seeded at a density of 1600 enteroids/mL onto 96 well cell culture plates (100 μL of culture media per well - Cat.# 3585 − Corning Incorporated, Corning, NY) and were maintained at 37.5°C and 5% CO_2 until d 3. On d 3, BOTs were added to culture media and allowed for a 24 h-pretreatment before the challenge. On d 4, oxidative stress challenge was induced by adding 400 μM menadione (Cat.# M5625 - Sigma-Aldrich St. Louis, MO) to the media for 1 h. At the end of the challenge, the BOTs and menadione were removed by 1 step of DPBS washing. The cellular oxidative status (ROS generation) was measured using CellRox-Green Reagent (Cat.# C10444 - Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. Fluorescent signals (excitation: 485 nm; emission: 530 nm) were quantified by SpectraMax M2e microplate reader (Molecular Devices, San Jose, CA). Enteroids fluorescent images were observed under an Echo Revolve microscope.

qPCR

Total RNA was isolated from enteroids using the TRI Reagent/chloroform method (Rio et al., 2010). Briefly, no less than 2,400 intact enteroids were washed from the culture media with DPBS and resuspended in 1 mL of TRI Reagent. Then 200 μL of 1-Bromo-3-chloropropane (Cat.# B9673- Sigma-Aldrich St. Louis, MO) were added and the aqueous phase was recovered after centrifugation at 12,000 × *g* for 15 min at 4°C. RNA was precipitated by isopropanol incubation for 20 min at -20°C, and pelleted by 12,000 × *g* for 15 min at 4°C. The RNA pellet was washed with 70% Ethanol 3 times and eluted in Nuclease-Free Water (Cat.# W4502 - Sigma-Aldrich St. Louis, MO). RNA yield and quality were determined by NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). cDNA samples were reverse-transcribed from 500 ng total RNA by Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (Cat.# K1672 - Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Lastly, qPCR reactions were performed in duplicate using QuantStudio 3 real-time PCR system and PowerUp SYBR Green Master Mix (Cat.# A25741 - Thermo Fisher Scientific, Waltham, MA).

Gene expression was reported as a fold of change using the ΔΔCt method (Livak and Schmittgen, 2001), using the 60S acidic ribosomal protein P0 (***RPLP0***) and Hydroxymethylbilane Synthase (***HMBS***) as an internal

control. To analyze the effects of the inflammatory challenge, specific markers were chosen: Interleukin (***IL***)1β, IL6, IL8, IL10, Interferon-gamma (***INFG***), tumor necrosis factor α (***TNFA***), toll-like receptor 4 (***TLR4***), zonula occludens-1 (***ZO1***) and occludin-1 (***OCCL***). Moreover, also chicken defensins expression was analyzed: avian beta-defensin 3 (***AvBD3***), avian defensin alpha 4 (***DEF4A***), and cathelicidin 2 (***CATH2***).

All the primers (Sigma-Aldrich, St. Louis, MO) were designed using the PrimerBLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and they are listed in Table 1.

Statistical Analysis

For PCP, gene expression, and oxidative stress status, total 4 $\sim$ 8 replicate wells were included in each treatment condition, with the individual culture well as experimental unit. Data were represented as mean $\pm$ standard error (**SEM**). Statistical analysis was performed using Graph-pad Prism 9.4 (https://www.graphpad.com/scientific-software/prism/). Data were analyzed using one-way ANOVA followed by Tukey's multiple comparisons for comparing the means among all groups. Dunnett's multiple comparisons was also performed to compare each BOT-treated group with the LPS group. The level of significance was set at $P < 0.05$ for all the tests.

The FD4 paracellular permeability data were presented and analyzed by percentage after feature scaling, to minimize plate-to-plate variations among repeated measurements. The resulting formula to calculate the normalized percentage of the fluorescence intensity (**FI**) of a specific sample will be $\% = \frac{(Sample\ FI\ read - Minimum\ FI\ read)}{(Maximum\ FI\ read - Minimum\ FI\ read)} * 100$.

RESULTS

Paracellular Permeability Under Inflammatory Challenge

The protective effects of BOTs on paracellular permeability were evaluated by determining FD4 presence inside of the enteroids (Figure 1). After 24 h of LPS challenge, the FD4 permeability was significantly increased by 2 times in comparison to the non-treated control (75.62 $\pm$ 5.02% vs. 25.76 $\pm$ 4.02%, $P < 0.0001$) (Figure 2). The BOTs treatment of THY, TEO, PRE, and COR significantly reduced the LPS-induced FD4 leakage by (24.52 $\pm$ 8.01%, $P = 0.0147$), (40.79 $\pm$ 3.55%, $P < 0.0001$), (47.46 $\pm$ 2.54%, $P < 0.0001$), and (40.60 $\pm$ 5.97%, $P < 0.0001$), respectively. In contrast, TTO and GEO failed to protect the enteroids from the increased FD4 permeability.

Gene Expression After Inflammatory Challenge

To further evaluate the effects of BOTs on enteroids, gene expression of selected cytokines, tight-junction proteins, and defensins was conducted after 6 h of

Table 1. Primer list used for gene expression.

	Gene	Primer sequence (5'→3')	Product length (bp)	Accession No.
Genes of interest	IL1β	F: TGCCTGCAGAAGAAGCCTCG R: CTCCGCAGCAGTTTGGTCAT	137	NM_204524.1
	IL6	F: GCAGGACGAGATGTGCAAGA R: ACCTTGGGCAGGTTGAGGTT	84	NM_204628.1
	IL8	F: AGCTGCTCTGTCGCAAGGTA R: GCTTGGCGTCAGCTTCACATC	124	NM_205498.1
	IL10	F: GTCACCGCTTCTTCACCTGC R: TCCCGTTCTCATCCATCTTCTCG	84	NM_001004414.2
	TNFα	F: CCCTACCCTGTCCCACAACC R: TGGGCGGTCATAGAACAGCA	150	XM_046927265.1
	Interferon-γ	F: ACAACCTTCCTGATGGCGTG R: AGTTCATTCGCGGCTTTGCG	100	NM_205149.1
	Zonula occludens-1	F: TCTGCACAGTGAGGTTGGCT R: GGCTGTCCTGCATCGGTGT	145	XM_004934975
	Occludin-1	F: TGCTTTTGCCCAAGCAGGAA R: TGTGGGAGAGGCACCAGTTG	153	NM_204417
	Toll-like receptor 4	F: CCTGGGTCTAGCAGCCTTCC R: TGGCCCAGATTCAGCTCCTG	129	NM_001030693
Defensins	DEF4A	F: AGGGAAGAGACCAAGAGGTGT R: ATCCACAGTCTTCTGAGCGGA	71	NM_001201399.2
	AvBD3	F: CCTGCTCATCCCCTTCTTCC R: GCATCAACCTCATATGCTCTTCC	171	NM_204650.2
	CATH2	F: CAAGGAGAATGGGGTCATCAG R: CGTGGCCCCATTTATTCATTCA	221	NM_001024830.3
Ref.	RPLP0	F: TCACGGTAAAGAGGGGAGGTG R: CTTGCTCAGTCCCCAGCCTT	143	NM_205179
	HMBS	F: AGCGCTTTCTAAGATTGGGGA R: TGGGGTGAAAGACAACAGCA	185	XM_040690596.2

Designed with PrimerBLAST.

Abbreviations: AvBD3, avian beta-defensin 3; CATH2, cathelicidin 2; DEF4A, avian defensin alpha 4; HMBS, hydroxymethylbilane Synthase; RPLP0, 60S acidic ribosomal protein P0; TNFα, tumor necrosis factor alpha.

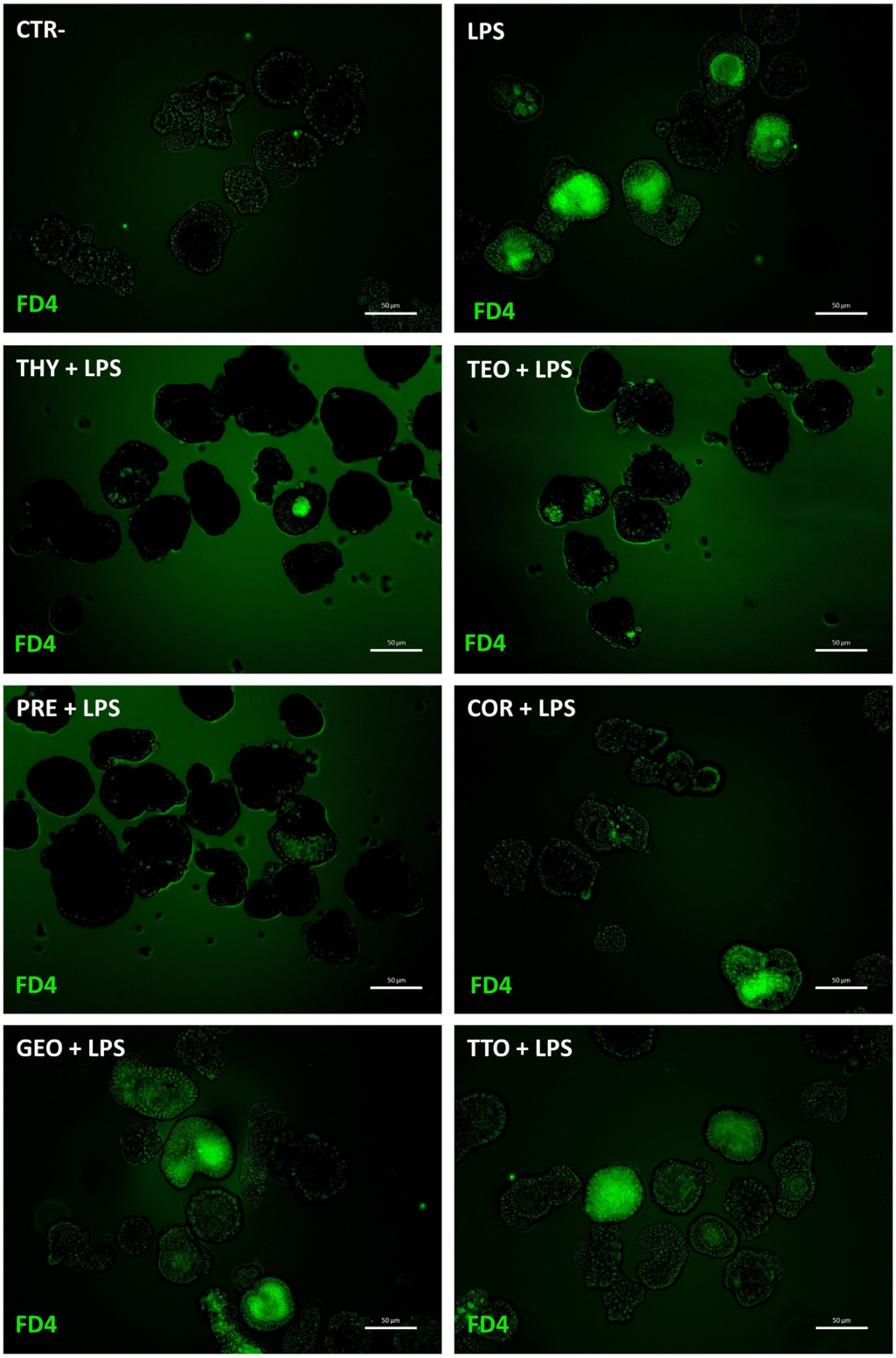

Figure 1. Effect of inflammatory challenge on FD4 permeability inside enteroids. All the botanicals have been tested in presence of LPS 10 μg/mL. Abbreviations: COR, capsicum oleoresin 10 ppm; GEO, ginger essential oil 100 ppm; PRE, phenols rich extract 100 ppm; TEO, thyme essential oil 20 ppm; THY, thymol 10 ppm or 0.07 μM; TTO, tea tree oil 100 ppm.

LPS challenge. The enteroids well responded to the challenge, as indicated by the significantly increased expression ($P < 0.0001$) of proinflammatory cytokines compared to control (Figure 3A). When compared to the LPS challenge group with a Dunnett's comparison, among all BOTs treatments THY, TEO, and PRE exhibited the strongest reductions to the LPS-induced cytokine expression ($P < 0.002$), thus restoring cytokines expression level close to the nontreated control group (Figure 3A). The *TLR4* expression was also significantly reduced in the LPS group compared to nontreated negative control ($P < 0.0001$). All BOTs, except GEO, failed to reduce this TLR4 downregulation.

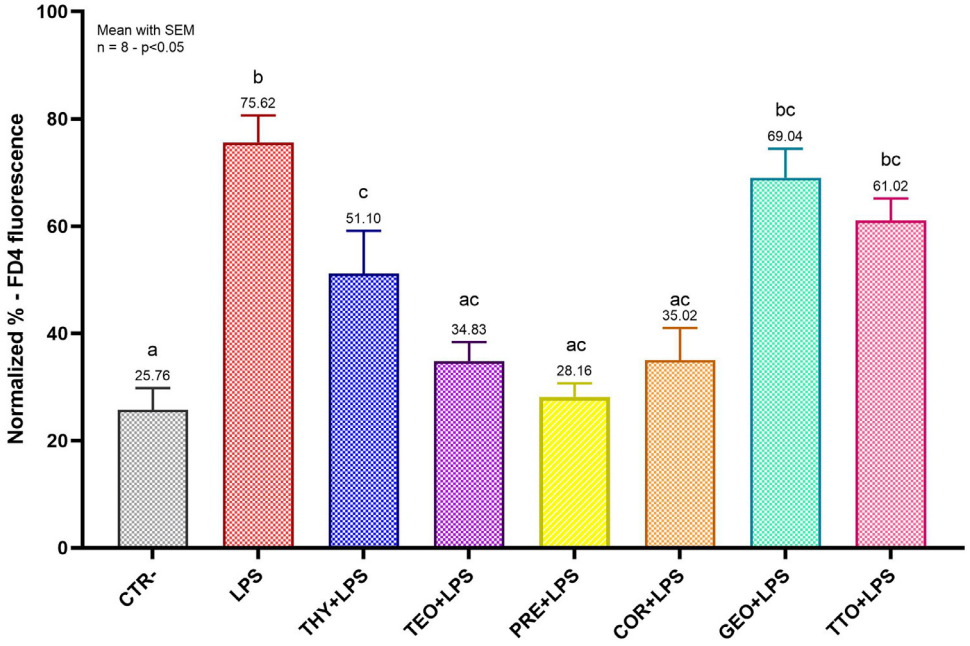

Figure 2. Normalized percentage of FD4 fluorescence recorded inside the organoids with microplate reader. The data were normalized using the feature scaling normalization. All the botanicals have been tested in presence of LPS 10 μg/mL. Abbreviations: COR, capsicum oleoresin 10 ppm; GEO, ginger essential oil 100 ppm; PRE, phenols rich extract 100 ppm; TEO, thyme essential oil 20 ppm; THY, thymol 10 ppm or 0.07 μM; TTO, tea tree oil 100 ppm. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons ($P < 0.05$). Different letters represent a significant difference. Data are represented as mean with SEM (n = 8).

Compared with nontreated control, the gene expression of *ZO1* and *OCCL*, were significantly reduced after LPS challenge ($P < 0.0001$). When compared to the LPS challenge group, THY and TEO treatments almost restored the negative effects of LPS on *ZO1* ($P < 0.04$) and *OCCL* ($P < 0.0003$) expression; COR treatment upregulated *ZO1* ($P < 0.0001$) but not *OCCL* expression; while PRE treatment upregulated only *OCCL* ($P = 0.0001$) expression; GEO and TTO groups showed no difference compared with LPS group (Figure 3B).

The LPS challenge significantly downregulated also the expressions of defensins *DEFB4A, AvBD3*, and *CATH2*, in comparison to nontreated control (Figure 3C). Enteroids treated with THY, PRE and TTO restored defensins gene expression near to negative control levels ($P < 0.02$), while TEO had a protective effect only on *DEFB4A* ($P = 0.0008$). The COR-treated group showed no significant differences with the LPS group, and GEO treatment specifically upregulated *AvBD3* gene expression (Figure 3C).

Intracellular ROS Generation After an Oxidative Stress Challenge

The protective effects of BOTs on enteroids oxidative status were evaluated by measuring intracellular ROS generation (Figure 4). Exposing enteroids to menadione (MEN treatment) for 1 h significantly increased intracellular ROS by 40.83% compared with nontreated control (78.15 ± 3.41% vs. 37.32 ± 1.50%, $P < 0.0001$) (Figure 5). Among BOTs treatments, THY, TEO, PRE, and GEO significantly reduced menadione-induced ROS generation ($P = 0.0001$). PRE was the most effective with a 59.67% reduction (78.15 ± 3.41% vs. 18.48 ± 3.07% - $P < 0.0001$). Lastly, COR and TTO showed no significant effects against menadione-induced ROS production (Figure 5).

DISCUSSION

The recently developed apical-out chicken enteroids model, with a mixed population of intestinal epithelial cell lineages and leukocyte components (Nash et al., 2021), has shown potential in investigating interactions between host and pathogens and luminal accessible compounds. In this study, the responses of chicken enteroids to the dietary bioactive components were evaluated. Moreover, a novel protocol to perform the PCP measurement in intact 3D enteroids with a microplate reader was proposed. In the present study, the 2-h incubation allowed the paracellular leakage of FD4 from media to the inside of enteroids (Figure 1). LPS was added to the culture as a well-known inflammatory stimulus, which led to disruption of barrier-tight junction proteins (Ghosh et al., 2020). As expected, the chicken enteroids in the present study expressed LPS receptor (*TLR4* receptor, Figure 3A), and significantly increased the FD4 leakage after 24 h exposure to LPS (Figure 2). These results in our study indicated advantages of using apical-out enteroids in estimating barrier function. Due to the basal-out nature of Matrigel embedded enteroids, it is difficult to access the luminal surface which hides inside of the 3D enteroid. Therefore, the additional conversion step from 3D enteroids to 2D monolayers is required in the basal-out enteroids model (Braverman and Yilmaz, 2018). As an improvement, directly using

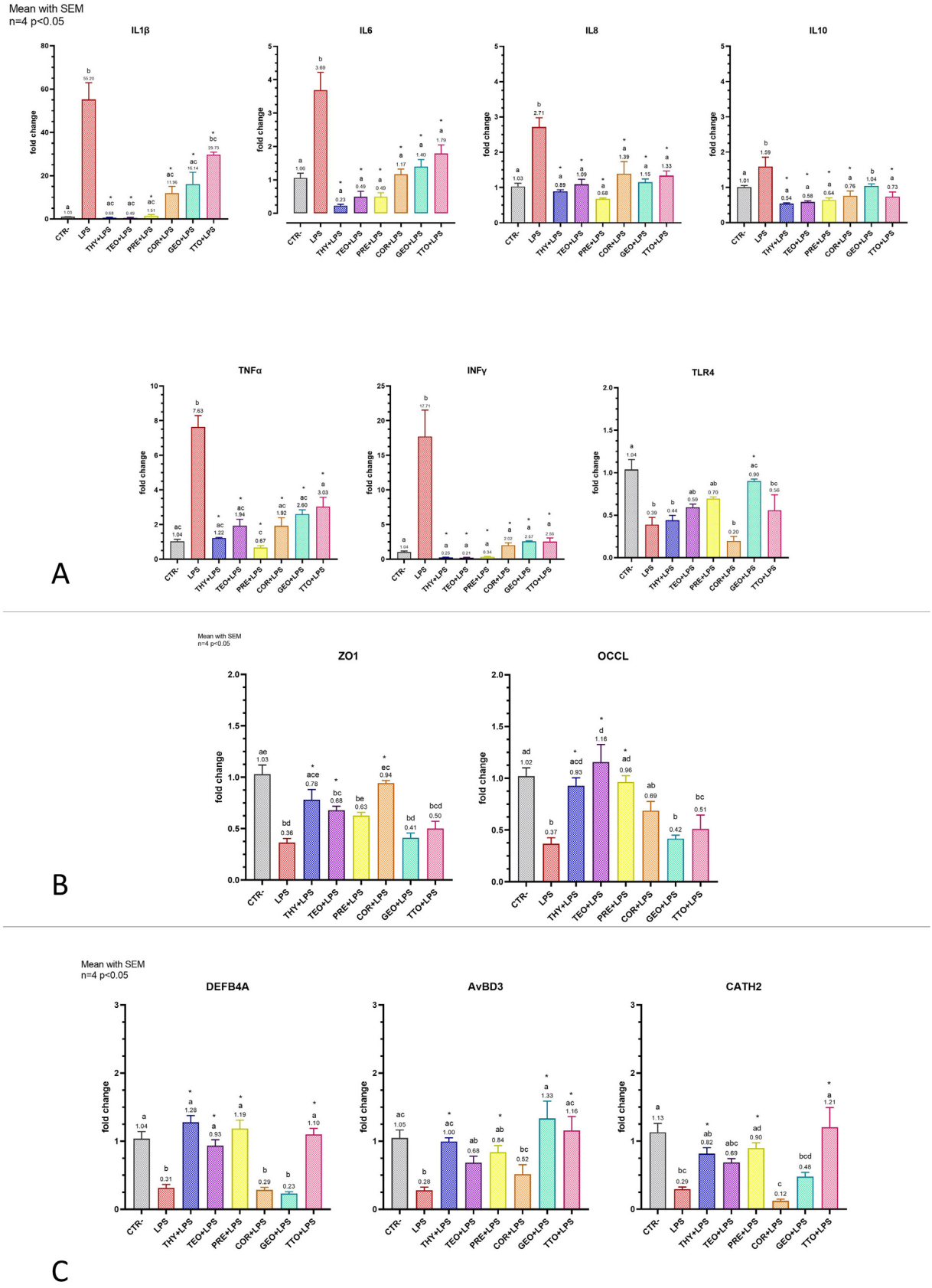

Figure 3. Gene expression of different pro-inflammatory markers (A), tight junctions (B) and defensins (C). All the botanicals have been tested in presence of LPS 10 μg/mL. Abbreviations: AvBD3, avian beta-defensin 3; CATH2, cathelicidin 2; COR, capsicum oleoresin 10 ppm; DEF4A, avian defensin alpha 4; GEO, ginger essential oil 100 ppm; INFγ, Interferon-gamma; OCCL, occludin; PRE, phenols rich extract 100 ppm; TEO, thyme essential oil 20 ppm; THY, thymol 10 ppm or 0.07 μM; TTO, tea tree oil 100 ppm. TNFα, tumor necrosis factor α; TLR4, toll-like receptor 4; ZO1, zonula occludens 1. Data were analyzed using one-way ANOVA with Tukey's multiple comparisons and Dunnett's multiple comparisons ($P < 0.05$). Different letters represent a significant difference comparing the mean of each group with every other group. The asterisk represents significant difference using one-way ANOVA with Dunnett's multiple comparisons comparing each BOT treated group with the LPS group. Data are represented as mean with SEM (n = 4).

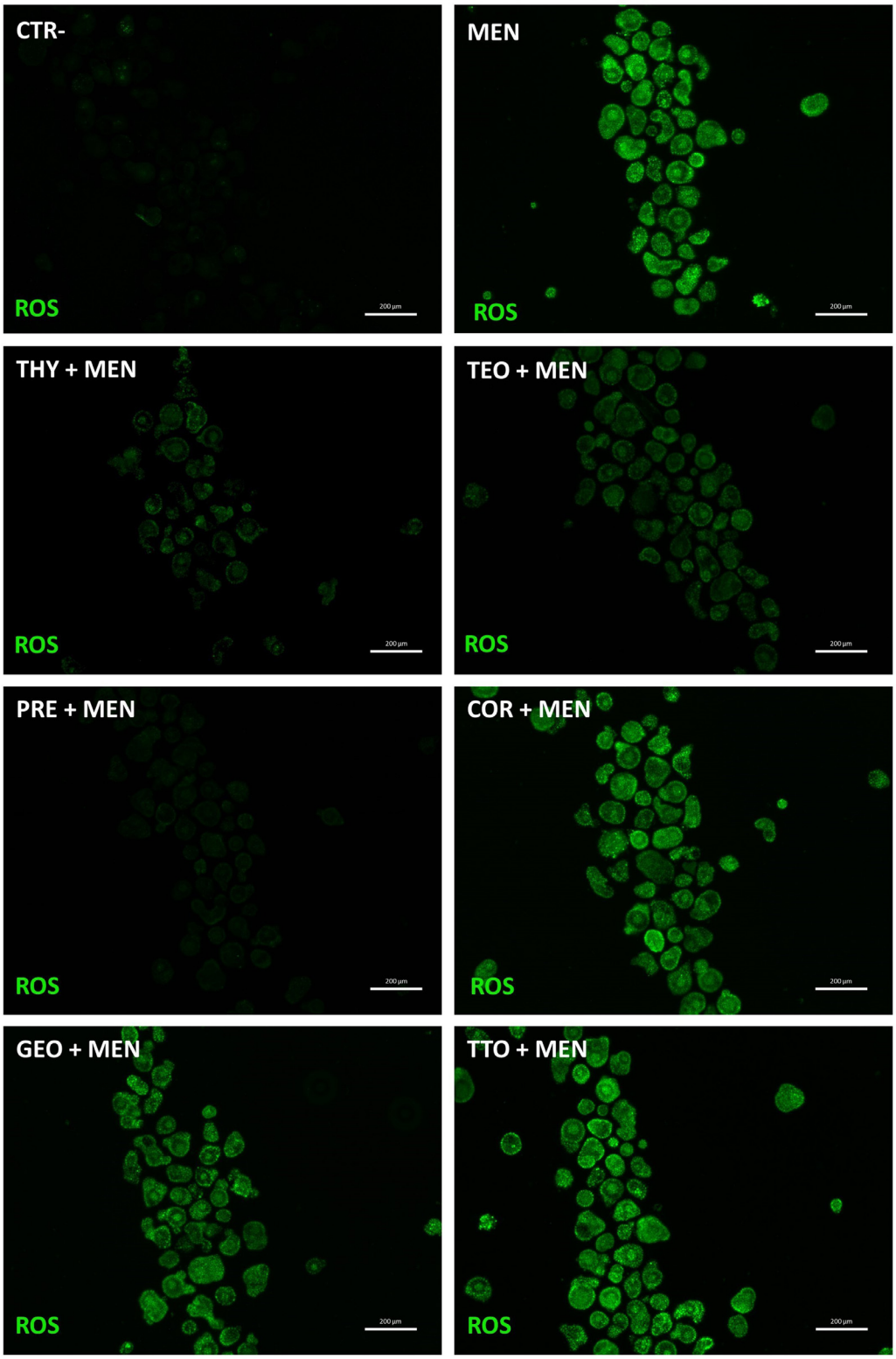

Figure 4. Effect of oxidative challenge on reactive oxygen species (ROS) production permeability on enteroids detected with CellRox-Green Reagent. All the botanicals have been tested in presence of Menadione 400 μM (MEN). Abbreviations: COR, capsicum oleoresin 10 ppm; GEO, ginger essential oil 100 ppm; PRE, phenols rich extract 100 ppm; EO, thyme essential oil 20 ppm; THY, thymol 10 ppm or 0.07 μM; TTTO, tea tree oil 100 ppm.

3D enteroids models to evaluate organoid PCP has been reported by other research groups (Elamin et al., 2012; Bardenbacher et al., 2020), but they lack an easy and fast method to quantify the PCP, creating a limitation for high-throughput screening studies. The PCP measurement in the present study provided the possibility for large-scale screening of the chemicals that improves intestinal barrier functions.

BOTs are widely used as feed additives in animal nutrition because of their antibacterial, antioxidant, and anti-inflammatory properties (Rossi et al., 2020; Tugnoli et al., 2020). For example, THY is widely used

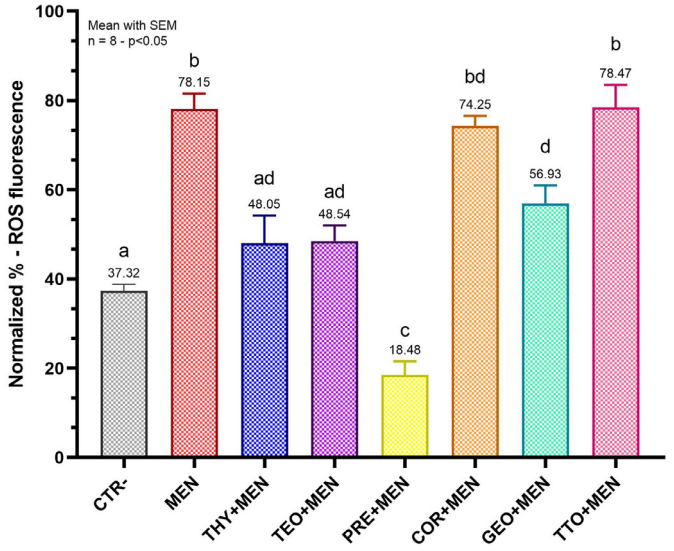

Figure 5. Normalized percentage of reactive oxygen species (ROS) fluorescence detected inside the organoids with CellRox on the microplate reader. All the botanicals have been tested in presence of Menadione 400 μM (MEN). Abbreviations: COR, capsicum oleoresin 10 ppm; GEO, ginger essential oil 100 ppm; PRE = phenols rich extract 100 ppm; TEO, thyme essential oil 20 ppm; THY, thymol 10 ppm or 0.07 μM; TTO, tea tree oil 100 ppm. Data were analyzed using one-way ANOVA Tukey's multiple comparisons ($P < 0.05$). Different letters represent a significant difference. Data are represented as mean with SEM (n = 8).

in poultry productions as antimicrobial, anti-inflammatory and antioxidant to improve chicken gut health (Du et al., 2016; Gholami-Ahangaran et al., 2021). As a botanical used as feed additive, GEO have great benefit in poultry nutrition due to its immunomodulatory, antibacterial, anti-inflammatory, antioxidant and antiparasitic properties (Al-Khalaifah et al., 2022).

Previous works where the human epithelial cell Caco2 were used, have indicated protective functionalities on epithelial integrity and anti-inflammatory activity (Rossi et al., 2020; Toschi et al., 2020, 2022). However, lacking chicken specific epithelial cell model limited the dose optimization, functional evaluation, and mechanistic research of BOTs targeting chicken intestines. This study focused on the activity of thymol and different essential oils and plant extracts tested individually during inflammatory and oxidative stress challenges on the chicken-specific apical-out enteroids. The BOTs to be tested in this study were chosen based on the existing literature, including the molecules and essential oils that are reported to be or to contain among the most promising antioxidant and anti-inflammatory compounds (Rossi et al., 2020). In the present study, LPS inflammatory challenge significantly activated the Nf-kB pathway, and resulted in increasing gene expression of proinflammatory cytokines (Figure 3A). Moreover, the reduction of *TLR4* expression indicated a protective defense mechanism of cells in response to high-dose LPS challenge (Ciesielska et al., 2021). As expected, the addition of THY, polyphenols and capsaicin significantly restored the proinflammatory cytokines and *TLR4* expression level (Figure 3A), which was likely via the strong blockage of the Nf-kB pathway and act as potent anti-inflammatory compounds (Rossi et al., 2020). As a result, these BOTs actively mitigated the inflammation-induced downregulation of selected tight junction protein expression (Figure 3B), thus improving the epithelial tightness, and preventing the epithelial permeability increase caused by the LPS challenge (Figure 2). Those effects have already been reported in vivo for thymol for example (Al-Khrashi et al., 2022). In addition, the polyphenols and terpenes are also reported to be ROS scavengers (Rossi et al., 2020). Given these antioxidant properties, the addition of THY, TEO, and PRE significantly reduced menadione-induced ROS generation as oxidative stress in the present study (Figure 5). Interestingly, GEO and TTO, which contain mainly terpenes and sesquiterpenes respectively, exhibited weaker protective effects on inflammatory marker genes expression, FD4 permeability, and ROS production under inflammatory and oxidative challenges comparing with other BOTs used in the present study. This was in contrast with the previous results that were conducted in the Caco2 cell model (Toschi et al., 2022), in which the 2 oils were able to reduce H_2O_2-induced oxidative stress and improve transepithelial electrical resistance (TEER). These inconsistent results can be explained by different cell compositions between the 2 in vitro cell models. It has been suggested that the biological activity of TTO strongly depends on the cell type, as well as the composition and concentration of the oil (Amirghofran et al., 2012; Anastasiou and Buchbauer, 2017; Aldahlawi et al., 2020; Sandner et al., 2020). A beneficial dose of essential oil in the Caco2 epithelial cells may be toxic to the underneath immune cells in the enteroids. It is worth noting that when targeting on the chicken intestinal functions, the chicken specific enteroids may recapitulate the complicated intestinal tissue better than the human monolayer epithelium.

Chicken intestinal defensins play important roles in vivo in reducing luminal pathogens and contributing to epithelium homeostasis. In this study, the gene expression of defensins, *DEFB4A*, *AvBD3*, and *CHAT2*, were detected in the enteroids. Interestingly, the mRNA expressions of all 3 selected avian defensins were significantly downregulated after 6 h LPS exposure. Contradictory data of defensins gene expression under LPS challenges have been reported previously (Abdelsalam et al., 2012; Zhang et al., 2016; Bar Shira and Friedman, 2018). In ovarian follicular tissue of laying hens, 3 h LPS (10 μg/mL) exposure numerically increased the expression of *AvBD12*, while decreasing the expression of *AvBD10* (Abdelsalam et al., 2012). In chicken embryo (E17) derived intestinal epithelial cells, 6h LPS (10 μg/mL) exposure significantly reduced *AvBD1* expression (Bar Shira and Friedman, 2018). In contrast, chicken embryo (E15) intestinal epithelial cells upregulated expressions of *AvBD* 1, 3, 5, 6, 9, 12, 14 post 6 h LPS (800 μg/mL) treatment (Zhang et al., 2016). Although the underlying mechanism regulating defensin expression is poorly understood, interestingly in the present study, THY, PRE, and TTO were able to maintain the defensin expression near the negative control levels preventing the downregulation induced by the

inflammatory state. *DEFB4A* is known also as beta-defensin-2 in humans. It is a molecule with antimicrobial properties and it can act against both gram-negative and gram-positive bacteria (Cobo and Chadee, 2013). In addition, its known antimicrobial properties have further effects on the healing and protection of the intestinal epithelial barrier as reported by Otte et al. (2008). The ability to protect *DEFB4A* from LPS-induced downregulation could be a key ability of THY, TEO, PRE, and TTO to protect the intestine during pathogen infection.

In conclusion, this study used a novel 3D chicken enteroid model to evaluate the anti-inflammatory and antioxidant properties of different BOTs under challenged conditions. The results confirmed the protective effects of BOTs in the chicken intestine, in terms of paracellular permeability improvement, proinflammatory cytokine expression repression, and intracellular oxidative stress reduction. These data indicated that chicken apical-out enteroids work as a suitable in vitro model to screen feed additive compounds and evaluate intestinal functions. Optimal doses of each BOT and the synergistic effects of those compounds could be further evaluated using the 3D enteroids.

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DISCLOSURES

Yihang Li reports financial support was provided by Vetagro S.p.A. Andrea Piva reports a relationship with Vetagro S.p.A. that includes: board membership. Andrea Piva reports a relationship with University of Bologna that includes: employment. Ester Grilli reports a relationship with Vetagro Inc. that includes: board membership. Ester Grilli reports a relationship with University of Bologna that includes: employment.

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