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Immunomodulatory effects of thymol and cinnamaldehyde in chicken cell lines

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RESEARCH ARTICLE

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Abstract

Thymol and cinnamaldehyde are phytogenic feed additives that have been developed to improve gut health, immunity and growth performance in poultry and swine. This study evaluated the immune modulating effects of a thymol and cinnamaldehyde blend (TCB) in the intestinal system of poultry *in vitro*, using two chicken cell lines, LMH (liver cell line) which has been used to mimic epithelial cell responses, and HD-11 (monocyte/macrophage-like). Cells with high viability (>95%) from established cell lines were cultured in the presence of TCB at concentrations ranging from 1 ng/ml to 100 ng/ml. The viability, transepithelial electrical resistance (TEER) and phagocytic capacity of co-cultured LMH cells, with or without stimulation with lipopolysaccharide (LPS), was subsequently evaluated. The expression of cytokines, chemokines and pattern recognition receptors by HD-11 monocytes/macrophages was measured by RT-PCR and by proteomic analysis. TCB was well tolerated by both cell lines (cell viability >90% after co-culture with TCB at 100 ng/ml for 48 h with or without LPS). Epithelial integrity of LMH cells (as assessed by TEER) was increased by TCB (10 ng/ml) after 4 h incubation, versus untreated controls, and phagocytic capacity of HD-11 cells was increased, in a dose-dependent manner ($P<0.05$). In HD-11 cells, TCB (10 ng/ml) downregulated the relative expression of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8 and the transcription factor cyclooxygenase-2 and upregulated expression of anti-inflammatory IL-10, versus untreated controls ($P<0.05$). In summary, under the tested conditions, TCB enhanced the epithelial barrier integrity of poultry hepatocytes, increased phagocytic activity and production of anti-inflammatory cytokines by monocytes and macrophages. These results indicated how supplementing TCB in poultry diets can increase bird performance, by increasing *in vivo* cell membrane integrity (especially important in the gut) and assisting in immune responses, which can liberate energy for growth.

Keywords: phytogenic, thymol, cinnamaldehyde, anti-inflammatory, gut integrity, immunity

1. Introduction

Since the EU-wide ban on the use of growth-promoting antibiotics in animal feed came in to force in 2006, intestinal diseases, such as necrotic enteritis (NE) caused by the pathogenic bacterium *Clostridium perfringens*, have re-emerged in broilers (Van Immersel *et al.*, 2004). Infected birds exhibit increased morbidity and reduced weight gain which can lead to severe production losses in the poultry industry. There is, therefore, an urgent need for practical and robust solutions to prevent and control the incidence and severity of NE.

Thymol (isopropyl meta-cresol or 2-isopropyl-5-methylphenol) has previously been reported to be one of the most bioactive antimicrobials among all phytochemicals (Falcone *et al.*, 2005). It is the main constituent (comprising up to 50%) of thyme essential oil, a naturally occurring

mixture of compounds with bioactive properties, found in plants such as *Thymus vulgaris* L. (thyme). *Trans*-cinnamaldehyde is another naturally occurring plant-derived compound with antimicrobial activity and has recently been considered as a potential alternative to conventional antibiotics (Doyle and Stephens, 2019). Existing studies have demonstrated that both thymol, cinnamaldehyde and blends of these two compounds (TCB) exhibit antimicrobial activity against *C. perfringens* in poultry *in vivo* (Mitsch *et al.*, 2004). As feed additives, TCBs have been shown to be highly effective in supporting gut health, enhancing feed digestion and growth performance in poultry and swine, in part due to their promotion of a more favourable gut microbiota (Ding *et al.*, 2017; Juneja *et al.* 2016; Ouwehand *et al.,* 2010).

During a NE infection with *C. perfringens*, the organism invades and colonises the gastrointestinal tract (GIT) and produces toxins (e.g. necrotic enteritis B-like toxin, NetB) which depress the bird's immune system and allow the pathogen population to increase further. A *C. perfringens* infection in broilers can impair growth performance and production, as well as lead to increased mortality rates (Huang and Lee, 2018). This is because infected animals divert energy and other nutrients into the immune system, limiting availability for growth. The protective effect exerted by TCB on the GIT is unlikely to solely due to its antimicrobial activity (i.e. direct toxic effect on the pathogen). An increasing number of studies have indicated that phytogenic feed additives (PFAs) have the capacity to modulate the immune system, and that this contributes to their beneficial effects in supporting gut health, fighting infection and ensuring performance, as evidenced by reductions in inflammatory cell infiltration, decreased inflammatory cytokine production and increased plasma immunoglobin concentrations in PFA-supplemented animals (Juhás *et al.*, 2007; Riella *et al.*, 2012). In particular, it has been shown in broilers and mice that both thymol and cinnamaldehyde can improve intestinal integrity and strengthen the mucosal barrier (Strompfova *et al.*, 2014; Wlodarska *et al.*, 2015). In rodents, thymol (administered orally or dermally) inhibited pro-inflammatory cytokines and decreased inflammatory cell recruitment (Juhás *et al.*, 2007; Riella *et al.*, 2012), and cinnamaldehyde enhanced cellular and humoral immunity (Awaad *et al.*, 2014). However, all of these studies were performed *in vivo* and involved an initial challenge and then supplementation in feed, followed by the measurement of all effects (on performance, gut tissues, cells and immune mediators) at the end of the study. This facilitates the correlation of exposure to PFAs and immune modulatory and performance effects in a real-life setting, but does not provide evidence of the direct causative effects of the TCB.

In the present study, the immunomodulatory effects of TCB were examined on two poultry cell lines (LMH, a liver cell line, and HD-11, a macrophage-like cell line) *in vitro* to determine the actual effect of TCB at a cellular level and evaluate the resulting antimicrobial and immunomodulatory changes. LMH cells have been previously used to successfully mimic epithelial and endothelial cell responses, due to morphological and biochemical similarities (Abe *et al.*, 1988). Lipopolysaccharides (LPS) were used as an adjuvant to enhance the antigen-specific immune response and activate the cells prior to treatment with TCB, as non-stimulated or LPS-stimulated cells have distinct physiologies and metabolic pathways. On the one hand, cell activation is critical for the initiation and regulation of the immune response (Adams and Hamilton, 1984; Pross, 2008), whilst on the other hand, excessive cell activation and associated metabolic commitments leads to a net loss of energy which affects animal performance (Pearce and Pearce, 2013). By comparing the effect of TCB on cells in both their non-stimulated and stimulated states, the results would allow better understanding of the value of *in vitro* cell line systems for the evaluation of PFAs as zootechnical feed ingredients.

2. Materials and methods

All experimental procedures were performed in accordance with the established Guidelines of the company sponsor (DuPont Nutrition and Biosciences, Copenhagen, Denmark) as well as the Standard Operation Procedures of the Gut Immunology Lab, R&D Food and Feed Enzymes, DuPont Industrial Biosciences.

Reagents

The TCB was a commercially available blend of 75% thymol and 25% cinnamaldehyde (Enviva® EO) that was manufactured and provided by DuPont Industrial Biosciences (Wilmington, DE, USA). The LPS (from *Escherichia coli* 026:B6) and all cell culture media, equipment and reagents were purchased from Thermo Fisher Scientific (Roskilde, Denmark), unless otherwise stated.

Cell lines and culture conditions

Chicken hepatocyte cell line LMH (ATCC® CRL-2117) was purchased from ATCC (LGC Standards GmbH, Wesel, Germany). Chicken monocyte cell line HD-11 was provided by Professor Michael Kogut (Food and Feed Safety Research, USDA-ARS, College Station, TX, USA). All cells were maintained at 37 °C in a 5% CO_2 atmosphere. LMH cells were maintained in Waymouth MB 752/1 medium supplemented with 20% foetal bovine serum (FBS) and 10% chicken serum. HD-11 cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 5% chicken serum. Both cell cultures were supplemented

with antibiotics (penicillin and streptomycin) to prevent bacterial contamination.

Cytotoxicity assay

In order to determine the dose-levels of TCB that could be applied to the cell lines without significant toxic effects, a cytotoxicity assay was performed over a 48 h time period. Cells of LMH and HD-11 with high viability (>95%, identified by Trypan Blue staining) were harvested from culture and their concentration adjusted to 1×10^6 cells/ml by addition of culture medium. TCB was added at concentrations of 1, 10, 100, 1000, 10,000, 100,000 ng/ml or 1 mg/ml, according to treatment. A second series of cells plus TCB was formulated to which LPS (100 ng/ml) was added. The cultures and co-cultures were incubated for 48 h, after which cells were stained with Trypan Blue and cells were counted using a Countess II FL automated cell counter (Thermo Fisher Scientific).

Transepithelial electrical resistance measurement

The integrity of the epithelial cell monolayer of LMH cells was measured by transepithelial electrical resistance (TEER) using an epithelial Voltohmmeter EVOM2 (Merck, Cambridge, UK). Briefly, on day 1 of the experiment, LMH cells (5.0×10^4 cells/well) were seeded in a complete cultivation medium to quadruplicate wells of semi-permeable cell culture inserts and differentiated for 14 days. On day 4 of cell differentiation, cells were moved to asymmetric serum-conditions using serum-free cultivation medium in the upper chamber of the cell culture insert and complete cultivation medium in the lower chamber. The culture medium was changed every three or four days, until the end of the differentiation period. On day 15, the quality of the cell monolayers was assessed prior to exposure to TCB, in order to ensure that epithelial integrity of the monolayers had been achieved (see Supplementary Materials for more detail). The TEER of the cell monolayers in each replicate well was measured at time zero (i.e. before the addition of TCB or LPS). The TCB (10 ng/ml) was then added, followed by LPS (100 ng/ml) after 2 h in half of the wells. Measurements of TEER were made at 4, 24 and 48 h after addition of TCB. For each replicate well, the resistance of an empty filter without cells (blank) was subtracted from the measured resistance of the treated filter, and the resulting value divided by the surface area of the cell culture insert (0.33 cm^2), according to the following equation:

$$\text{TEER value } (\Omega/cm^{2)} = \frac{\text{Value}_{\text{experiment}} - \text{Value}_{\text{blank}}}{0.33\ cm^2}$$

Experiments were performed twice with four replicate wells per treatment.

Phagocytosis assay

The phagocytic capacity of chicken monocytes/macrophages (HD-11 cells) was analysed using the Vybrant™ Phagocytosis Assay Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions, with slight modifications. Briefly, HD-11 cells were freshly harvested and TCB (10 ng/ml, or 100 ng/ml) was added. Two hours later, LPS (100 ng/ml) was added to half of the wells. Cells with or without TCB and LPS were cultured overnight at 37 °C under 5% CO_2. The next day, fluorescent labelled *E. coli* bio-particles (K-12) were prepared and added to the wells for a further 2 h of incubation. After washing, the fluorescence intensity of the cells in the cell plates was read using a fluorescence plate reader with excitation at 480 nm and emission at 520 nm emission. Net fluorescence intensity was calculated by subtracting background fluorescence (cells only, without fluorescent loading, blank) according to the following equation:

$$\text{Net fluorescent intensity} = \frac{\text{Value}_{\text{experiment}} - \text{Value}_{\text{blank}}}{\text{Value}_{\text{blank}}} \times 100\%$$

Experiments were performed three times with 15 experimental replicates per treatment.

Expression of cytokines, chemokines, toll-like receptors and transcription factors

The expression of cytokines, chemokines and pattern recognition receptors (PRR) was measured by RT-PCR and by proteomic analysis. The methods used for the proteomic analysis and the protein digestion for the determination of PRRs are detailed in Supplementary Material. For the measurement of cytokines, chemokines and cell biomarker expression by RT-PCR, chicken monocytes (HD-11 cells) were initially cultured with TCB (10 ng/ml) for 2 h before adding LPS (100 ng/ml) to half of the cultures. After incubation for a further 6 h, cell pellets were collected for RT-PCR analysis. Extraction of RNA and RT-qPCR were performed at Eurofins AROS (Åarhus, Denmark). Cell RNA was extracted on a QIASymphony SP workstation (Qiagen, Hilden, Germany) using the QIAsymphony RNA Kit and the RNA CT400 automated protocol. The extracted RNA was eluted into 50 µl of H_2O. The quality of the extracted RNA was assessed for each extraction and each assay, before proceeding to PCR, by measurement of its concentration (ng/µl) and the A260/A280 value using a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific), as well as the RNA Integrity Number (RIN) using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The A260/A280 values ranged from 1.965-2.084 and the RIN values ranged from 8.2-9.1. The expression of mRNA was measured by quantitative real-time reverse

transcription polymerase chain reaction (qRT-PCR). Full procedures and conditions of the RT-PCR are described in Supplementary Materials.

Data were normalised to two sets of house-keeping genes according to the following equation:

$$\text{Value} = 2^{-(\text{Ct sample} - \text{Ct house-keeping})} \times 10^6$$

Experiments were performed five times with five experimental replicates per treatment.

Statistical analyses

Paired Student's t-tests (two-tailed) were used to identify significant differences between the means of active (TCB treated, with/without LPS) and control (blank) treatments. A value of $P<0.05$ was used to denote statistical significance and unequal variance was assumed.

3. Results

Cytotoxicity

Cells from both lines exhibited high viability after 48 h exposure to TCB at concentrations up to a maximum of 100 ng/ml, as indicated by consistent cell viability rates of >90% (Table 1). The addition of LPS (100 ng/ml) to the TCB-treated cultures did not produce any cytotoxic effects, as cell viability was maintained at >90% in LPS-treated cultures (Table 1). Higher concentrations of TCB (from 1 ug/ml up to 1 mg/ml) resulted in a gradual decrease in the viability of LMH and HD-11 cells, to ~70% with TCB at 1 mg/ml (data not shown).

Trans epithelial electrical resistance

In non-stimulated chicken LMH cells, TCB enhanced TEER values compared with the untreated control as early as 4 h after the start of the incubation ($P<0.05$, Figure 1). However, in the LPS-stimulated cells, there was no significant effect of TCB treatment on TEER values (Figure 1). This appeared to

Table 1. Cell viability after 48 h cultured with a thymol and cinnamaldehyde blend (TCB).

Cell type	Cell line	Culture condition[1]	TCB[2]		Blank control		*P*-value
			Cell viability, %	SEM	Cell viability, %	SEM	
Chicken hepatocytes	LMH	without LPS	92.7	0.66	93.3	1.20	0.63
		with LPS	94.3	1.52	92.7	2.02	0.65
Chicken monocytes	HD-11	without LPS	92.3	0.88	93.0	1.45	0.52
		with LPS	95.3	1.86	92.0	1.76	0.38

[1] 100 ng/ml; experiments were performed in duplicate with six experimental replicates per treatment.
[2] Cells were cultured with TCB (100 ng/ml) for 48 h.

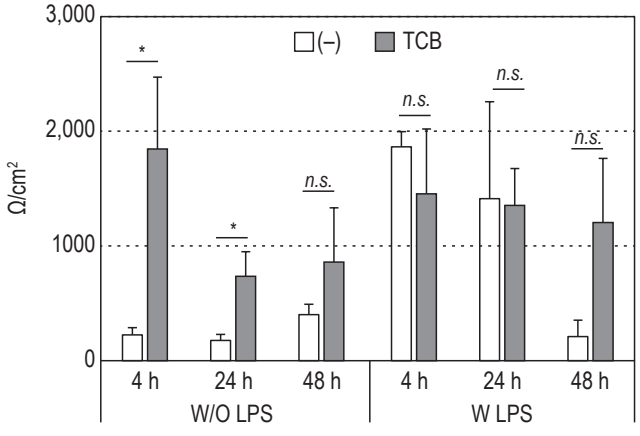

Figure 1. Thymol and cinnamaldehyde blend (TCB) positively modulated epithelial barrier integrity of LMH chicken hepatocytes. Chicken hepatocytes (LMH cells) were cocultured for 2 h with TCB (10 ng/ml) prior to stimulation with/without LPS, as indicated. Cell cultures were incubated for a further 48 h prior to measurement of trans epithelial electrical resistance (TEER) at 4, 24 and 48 h, respectively. Each column represents the mean of 8 replicates from two independent experiments. W/O LPS: LPS was not added to cells; W LPS: LPS was added to cells. Data are shown as means ± SEM. *: $P<0.05$. n.s.: not statistically significant.

be due to an increase (approximately nine-fold) in the mean TEER value of untreated control cells co-cultured with LPS compared with untreated controls without LPS. The effect of TCB-treatment of human epithelial cell line Caco-2 cells on TEER values was studied. In both non-stimulated and LPS-stimulated Caco-2 cells, TCB upregulated TEER values which reached a maximum after 4 h incubation, in resting as well as in LPS challenged cells (P<0.05, non-stimulated; P<0.01, LPS-stimulated; Figure S1).

Phagocytosis

Treatment of HD-11 cells with TCB significantly enhanced the phagocytic activity of cells compared with control (untreated) cells (P<0.05; Figure 2), and this effect was dose-dependent. This effect was additionally observed in LPS-stimulated cells (Figure 2).

Expression of cytokines, chemokines, toll-like receptors and transcription factors

Compared with the untreated controls, treatment of cells with TCB at a concentration of 10 ng/ml upregulated the relative expression of the anti-inflammatory cytokine IL-10 (P<0.05; Figure 3A) and downregulated expression of the pro-inflammatory cytokines IL-1β, IL-8 and the transcription factor cyclooxygenase-2 (COX-2) (P<0.05; Figure 3B). In LPS-stimulated cells, TCB significantly inhibited expression of IL-6 and COX-2 (P<0.05, Figure 3B). Meanwhile, TCB enhanced the expression of the chemokine (C-C motif) ligand (CCL)4 in non-stimulated HD-11 cells, but inhibited CCL4 expression in LPS-stimulated cells (P<0.05; Figure 3C). In addition, TCB numerically downregulated the expression of certain transcription factors, in particular, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), interferon regulatory factor 3, TIR-domain-containing adapter-inducing interferon-β and B-cell lymphoma 2, although the difference between the treatment and control groups did not reach statistical significance (data not shown). TCB has no evident effect on TLR expression (data not shown).

The results of preliminary proteomics analysis revealed that, after LPS stimulation, TCB-treated HD-11 cells exhibited downregulated expression of pro-inflammatory biomarkers, including nitric oxide synthase (P<0.05) and IL-1 receptor associated kinase 4 (P<0.01), and upregulated expression of the mannose-6-phosphate receptor (P<0.01; Figure S2).

4. Discussion

This study sought to determine whether TCB has any direct immunomodulatory effects on poultry cell lines and, in so doing, to ascertain the potential of using *in vitro* cell lines as a tool for studying candidate in-feed PFAs to improve gut health and host-resistance to infection from pathogens. The effects of TCB on both cell lines were studied, since together these form a key component of the innate immune response in chickens to invading microbes (Akira *et al.*, 2001).

There was no evidence of a cytotoxic effect of TCB on LMH liver cells or HD-11 monocytes or macrophages under the test conditions at any of the concentrations (10 ng/ml). Previous *in vitro* cell line studies have shown that thymol (0.05-1.25 μM, equivalent to 10-250 ng/ml of TCB) induced cytotoxicity and DNA damage in human cancer cell lines (Nagoor Meeran *et al.*, 2017). The cytotoxic effect of cinnamaldehyde appeared to vary among cell lines *(Fang*

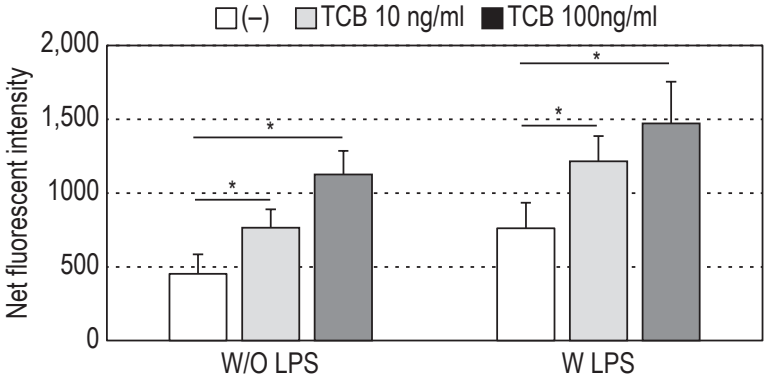

Figure 2. Thymol and cinnamaldehyde blend (TCB) enhances phagocytic activity of HD-11 monoytes/macrophages in a dose-dependent manner. Chicken monocytes (HD-11) were cocultured for 2 h with TCB prior to stimulation with/without LPS, as indicated. The next day fluorescent labelled *E. coli* bioparticles (K-12) were added and cultures incubated for a further 2 h. Each column represents the mean of 15 technical replicates from 3 independent experiments. Net inflorescence intensity is shown (after deduction of background measurement) as an indicator of phagocytic ability. *: P<0.05. n.s.: not statistically significant. W/O LPS: LPS not added to cells; W LPS: LPS added to cells.

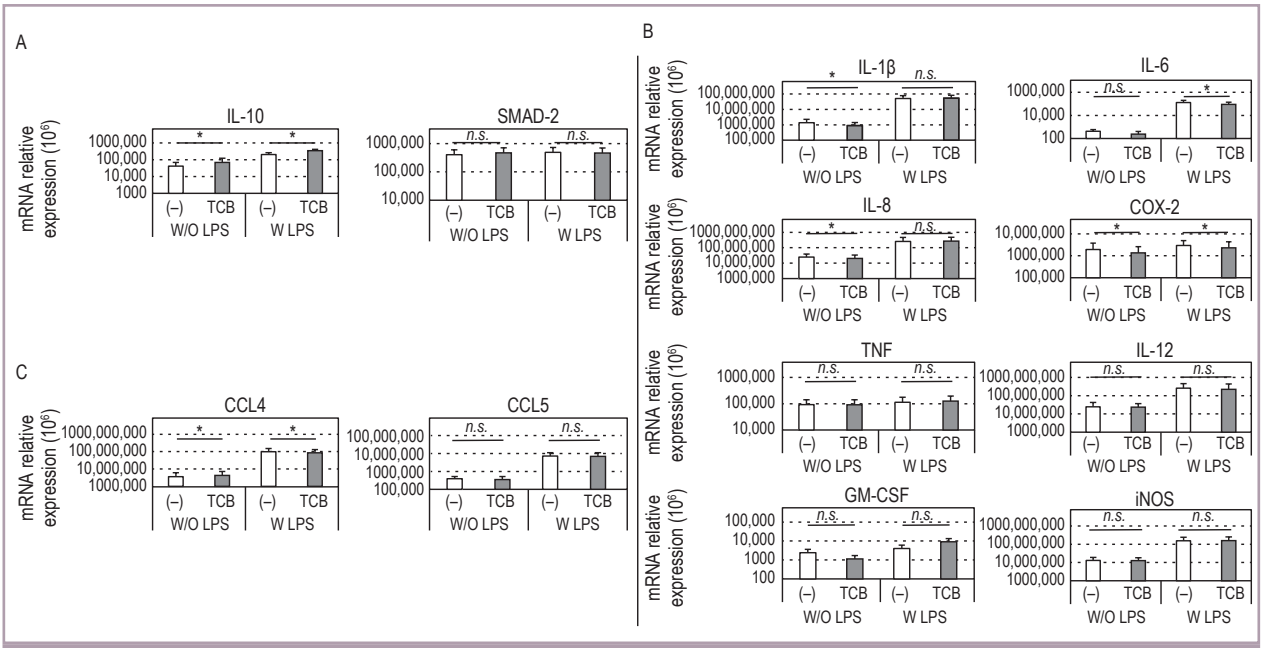

Figure 3. Effects of thymol and cinnamaldehyde blend (TCB) on the relative expression of a variety of cytokines, chemokines and transcription factors by HD-11 monocytes/macrophages, as measured by RT-PCR. (A) anti-inflammatory cytokine interleukin (IL)-10 and transcription factor SMAD-2; (B) pro-inflammatory cytokines IL-1β, IL-6, IL-8, COX-2, TNF, IL-12, GM-CSF and iNOS; (C) CCL4 and CCL5. Chicken monocytes (HD-11) were cocultured for 2 h with TCB (10 ng/ml) prior to stimulation with/without LPS and then further cultured for 6 h. Each column represents the mean of 4 independent experiments each with 4 technical replicates. Data were normalised to 2 sets of house-keeping genes and multiplied by 10^6. Data are shown as means ± SEM. *: *P*<0.05. n.s.: not statistically significant. SMAD-2 = mothers against decapentaplegic homolog 2; GM-CSF = granulocyte-macrophage colony-stimulating factor; iNOS = inducible nitric oxide synthase; TNF= tumours necrosis factor; CCL = chemokine (C-C motif) ligand; COX-2 = cyclooxygenase-2; W/O LPS = LPS not added to cells; W LPS = LPS added to cells.

et al., 2004). These different findings were most likely due to a difference in the sensitivity of the individual cell lines.

The measurement of TEER in gut epithelial cells is a widely accepted quantitative technique for estimating the integrity of tight junction dynamics in cell culture models of endothelial and epithelial monolayers (Srinivasan *et al.*, 2015). However, the lack of an established poultry intestinal epithelial cell line hinders the current application of TEER assays in chickens. It has been reported that the LMH hepatocyte cell line shares morphological and biochemical characteristics with epithelial cells (Abe *et al.*, 1988). The LMH cell line has been used successfully for bacterial adhesion studies as well as cytotoxicity studies (Vaz *et al.*, 2008). The TEER responses of LMH cells to TCB were consistent with those seen for human epithelial cell line Caco-2, which demonstrated that the LMH cell line is a suitable alternative to Caco-2 for the study of epithelial barrier integrity in chickens. The TEER assays revealed that TCB at a concentration of 10 ng/ml enhanced barrier integrity in non-stimulated LMH cells. Stimulation of LMH cells with LPS (without addition of TCB) unexpectedly increased TEER, which may have masked the effect of TCB on TEER in the TCB-treated LPS-stimulated LMH cells. How LPS stimulation increased TEER was unclear, although Guo *et al.* (2018) observed a transient upregulation of the tight junction proteins claudin-1, claudin-15, and ZO-1 in oral epithelial cells following addition of LPS. *In vivo*, the epithelial barrier is exposed to a variety of environmental toxins and enteropathogenic microorganisms (Zhang *et al.*, 2015), and constant exposure to these non-host stimuli can disrupt the proper functioning of epithelial tight junctions. Emerging evidence suggests that increased gut permeability caused by the dysregulation of tight junctions may be the initiating factor in the pathogenesis of a variety of gut inflammatory conditions, as well as in systemic autoimmune disease (Lerner and Matthias, 2015). This being the case, then the capacity of TCB to rapidly restore and reinforce gut epithelial tight junctions may well be advantageous to maintaining host-microbial homeostasis and antimicrobial defence systems.

In NE infections, *C. perfringens* infection causes leaky gut (Eichner *et al.*, 2018) which facilitates transportation of the pathogen into the lamina propria. Monocytes/macrophages, as an important class of phagocytes, act by migrating to infected areas where they ingest and kill micro-organisms (Hirayama *et al.*, 2018). The inhibition of macrophage phagocytosis by *C. perfringens* has been proposed as one of its key defence mechanisms (O'Brien and Melville, 2003).

LPS itself is known to enhance phagocytosis through promoting the differentiation of monocytes to macrophages (Gessani *et al.,* 1993). In the present study, TCB enhanced phagocytic activity of HD-11 monocytes and macrophages in a dose-dependent manner, under both non-stimulated and LPS-stimulated conditions. Further assays are needed to determine the phagocytic activity of HD-11 monocytes and macrophages in combination with TCB against live strains of *C. perfringens.*

Immune cells produce cytokines and express cell-surface markers which regulate and stimulate down-stream immune responses of the host. An imbalance between pro-inflammatory and anti-inflammatory cytokines results in disease progression and tissue damage that limits the resolution of intestinal inflammation. The addition of TCB at a concentration of 10 ng/ml to HD-11 cells resulted in an upregulation of the expression of the anti-inflammatory cytokine IL-10, and the downregulation of pro-inflammatory cytokines and transcription factors including IL-1β, IL-6, IL-8, COX-2, nitric oxide synthase and IL-1 receptor associated kinase 4. In addition, TCB upregulated the mannose-6-phosphate receptor, which mediates anti-bacterial immunity (Zhang *et al.*, 2018) and regulates the activity of $CD8^+$ cytotoxic T cells (CTLs) that protect the host from pathogens (Ara *et al.,* 2018). These *in vitro* observations exemplify how the host may respond to invading pathogens in order to protect itself from excessive inflammation, by establishing a balance between immunity and tolerance. Recent studies (Fasina and Lillehoj, 2019; Lee *et al.*, 2018) demonstrated that both cytokines IL-10 (anti-inflammatory) and IL-6 (pro-inflammatory) were upregulated in a chicken NE model. However, it seems that IL-10 upregulation induced by *C. perfringens* was insufficient to protect the gut mucosa from excessive inflammation and tissue damage. The current results suggested that TCB-supplementation may have the potential to enhance the immune response of the host in terms of the up or down-regulation of cytokines and transcription factors, which may be beneficial to its response to *C. perfringens*.

CCL4 is a chemokine with specificity for CCR5 receptors. It is a chemoattractant for natural killer cells, monocytes and a variety of other immune cells (Kallikourdis *et al.*, 2002). In humans, an elevated expression of CCL4 is often correlated with the inflammatory bowel disease colitis and mediates a mixed immune response (Günaltay *et al.*, 2015). The downregulation of CCL4 by TCB that was observed in the LPS-treated HD-11 cells would, at least theoretically, be expected to attenuate cell recruitment in the lamina propria and subsequently reduce inflammation. This is of even greater significance when considered alongside the apparent marked increase in CCL4 expression of untreated LPS-stimulated HD-11 cells versus non-stimulated cells, which has been reported by An *et al.* (2002). In non-stimulated cells, NF-κB (proinflammatory signalling) pathways were partly inhibited by TCB and yet CCL5 expression was not affected. Thus, the observed upregulation of CCL4 in non-stimulated cells can be considered more likely to be a direct effect of TCB than a general effect of cell activation.

Using poultry cell line-based *in vitro* assays revealed a beneficial immunomodulatory effect of TCB on multiple effectors (Figure 4).

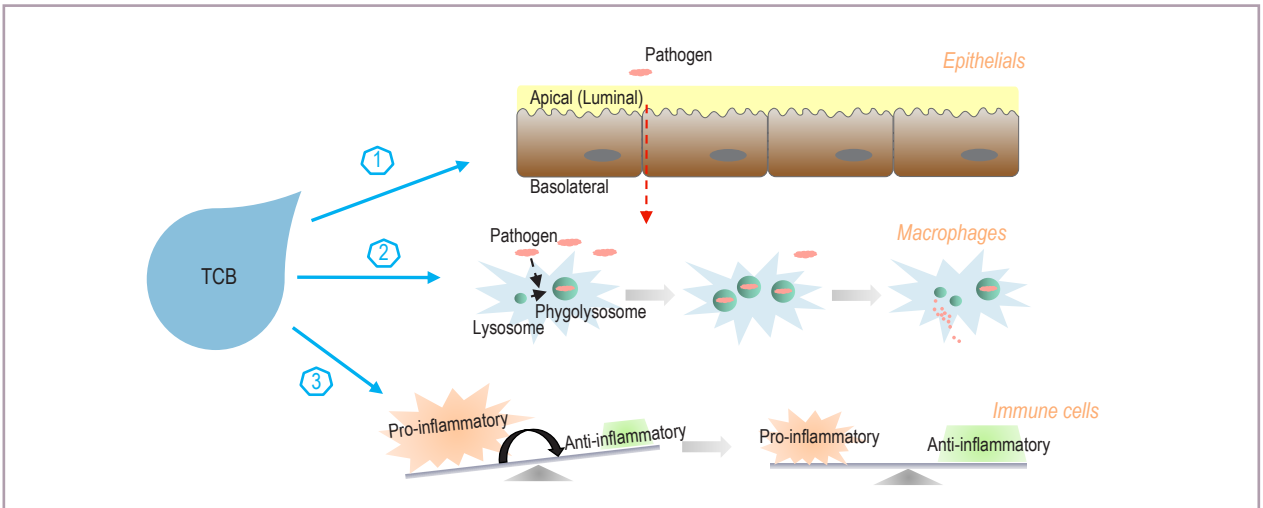

Figure 4. Illustration of the proposed role of thymol and cinnamaldehyde blend (TCB) on immune effectors in the poultry gut. It is proposed that the beneficial effects of TCB are mediated through: (1) upregulation of tight junctions in the epithelial monolayer, thus preventing 'leakage' through the epithelial barrier; (2) marked enhancement of monocyte/macrophage phagocytic activity, thus improving the removal of infiltrating pathogens; and (3) modulation of the expression of pro- and anti-inflammatory cytokines, certain chemokines and transcription factors to achieve a balanced state between tolerance and immunity.

The current results suggested that TCB positively regulated the epithelial barrier integrity (as modelled using the poultry LMH cell line), and can be expected to protect the host from pathogen invasion. TCB enhanced phagocytic activity of monocytes and macrophages, thus promoting pathogen removal. TCB activated these cells for immune surveillance as well as modulating a qualified and precise cytokine, chemokine and allied response, adjusting the balance between immunity and tolerance. All of these effector mechanisms may collectively explain the mode of action of TCB on the immune system and gut health. By comparing cell responses across conditions designed to reflect non-stimulated and LPS-stimulated states, it is possible to make predictions about what might occur in the *in vivo* situation. On the one hand, the immune system is on permanent standby, monitoring the local environment and ready to respond to a potential pathogen threat (Langenkamp *et al.*, 2000), whilst on the other hand, immune cells are programmed to respond to certain activating stimuli, such as LPS or necrotic B-like (NetB) toxins. These cells then capture pathogens from the peripheral tissue, migrate to secondary lymphoid organs and initiate systemic immune responses (Foster *et al.*, 2007). The current data supported the hypothesis that TCB has beneficial effects on both non-stimulated and stimulated immune cells. Further, the observations relating to the modulation of poultry cell immune responses by TCB *in vitro* were well aligned with the reported beneficial effects of TCB on growth performance and gut health from *in vivo* animal trials. These findings indicated that poultry cell line-based assays are useful, cost-effective approaches for screening candidate animal feed phytochemicals for immunomodulatory activity. Determination of how TCB affects the various effectors of the immune response and of the precise mechanisms by which this occurs will lead to a better understanding of the potential utility of PFAs for supporting poultry gut health and nutrition. The present findings offer new insights into feed additive development for the prevention and treatment of infectious and inflammatory diseases that target the intestinal mucosa.

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Declaration of interest

The authors are employees of Du Pont Nutrition and Biosciences, Brabrand, Denmark.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JAAN2020.0001.

Supplementary Materials and methods.

Figure S1. Thymol and cinnamaldehyde blend (TCB) positively modulated epithelial barrier integrity of human Caco-2 cells.

Figure S2. Thymol and cinnamaldehyde blend (TCB) alters relative expression of cell transcription factors by HD-11 monocytes/macrophages, as determined by proteomic analysis.

Table S1. PCR Cycling protocol.

Table S2. Taq Man ID for PCR.

Table S3. PCR probe sequences.

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