In vitro Immunomodulatory effects of thymol and cinnamaldehyde in pig intestinal epithelial cell line (IPEC-J2)

C. Shen, L. G. Christensen, P. B. Rasmussen, and K. M. Kragh

Gut Immunology Lab, Technology & Innovation, DuPont Nutrition & Biosciences, Edwin Rahrs Vej 38, Brabrand, 8220, Denmark

Corresponding author: Chong.Shen@DuPont.com

Abbreviated Title: Phytogenics in pig cell line

Summary

Thymol and cinnamaldehyde are phytogenic feed additives (PFAs) developed to improve gut health and growth performance in poultry and swine. This study aims to evaluate *in vitro* immune modulating effects of thymol and cinnamaldehyde blend (TCB) in the porcine gut epithelial cell line (IPEC-J2). Cytotoxicity, permeability, wound-healing and bacteria adhesion assays were used for evaluation. The expression of cytokines, tight junctions and polymeric immunoglobulin receptor (pIgR) were measured by RT-PCR. IPEC-J2 cells cultured in the presence of TCB at concentrations ranging from 1 ng/ml to 1 μg/ml displayed high viability (> 90%). TCB increased barrier integrity (13.8% less in LPS-induced leak, $P<0.05$) and accelerated the initial speed of wound recovery (day 1, 26% wound recovery in TCB treated *vs* 7% in control, $P<0.05$; day 2, 54 *vs* 39%, $P<0.001$). RT-PCR analysis of cell culture showed that TCB upregulated anti-inflammatory cytokine IL-10 (73.3%, $P<0.05$) in non-stimulated IPEC-J2 cells. While stimulated, pIgR (9.7%, $P<0.05$) and tight junctions claudin-4 (CLDN4, 9.4%, $P<0.05$) were upregulated by TCB. Furthermore, TCB significantly increased *Lactobacillus acidophilus* adherence to gut epithelial cells (285.0%, $P<0.05$). Overall, the

current *in vitro* study shows that TCB induces various immune responses, which may explain its *in vivo* benefits as feed additive.

Keywords: anti-inflammation, essential oils, wound-healing, cytokines, *in vitro* assay

Introduction

Current industrialised pig production brings health challenges from the environment, nutrition, and infection (Lee *et al*., 2016). These stressors can reduce growth performance and alter immune systems at systemic and local levels including the gastrointestinal tract (GIT). Meanwhile, increasing restrictions on the use of antibiotics as feed additives has driven the need to find new solutions, e.g. enhancement of the immune system to protect the host from diseases (Zeng, Zhang, Wang, & Piao, 2015). Numerous studies have shown that phytogenic feed additives (PFAs) like thymol, cinnamaldehyde and eucalyptol are beneficial in swine production (Omonijo *et al*., 2018). This beneficial effect could be attributed to anti-inflammation, anti-oxidative stress, microbiome modulation, and disruption of bacterial quorum sensing (QS).

Thymol and cinnamaldehyde blend (TCB) is a bio-efficacious PFA product which has proven highly efficient in supporting improved gut health, feed digestion and growth performance in poultry and swine (Li *et al*., 2012). This is in part due to the modulation to a more favourable microbiota (Ouwehand *et al*., 2010). The recent poultry cell line-based *in vitro* assays revealed a beneficial immunomodulatory effect of TCB as indicated by positively regulating the epithelial barrier integrity, enhancing phagocytic activity of monocytes/macrophages, and activating immune cells for immune surveillance, as well as tolerance (Shen, Christensen, Bak, Christensen, & Kragh, 2020).

In the present study, the immune modulation effects of TCB is investigated in *in vitro* assays with porcine intestinal immune cell line IPEC-J2. The cells can differentiate in culture and

exhibit enterocytic features, such as microvilli, tight junctions and glycocalyx-bound mucin (Brosnahan & Brown, 2012). LPS was introduced as a cell stimulator so that cell performance in both non-stimulated and stimulated status could be investigated.

Materials and Methods

Reagents

The TCB was a commercially available blend of 75% thymol and 25% cinnamaldehyde (Enviva® EO) that was manufactured and provided by DuPont Nutrition and Biosciences. 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 line, bacterial strains and culture conditions

Pig intestinal epithelial cell line (IPEC-J2, ACC 701) was purchased from DSMZ (Braunschweig, Germany). Cells were maintained in DMEM supplemented with 20% FBS, at 37°C with 5% CO_2 atmosphere. Cell cultures were supplemented with antibiotics (Penicillin and Streptomycin, 100×). Normocin were added every three months (Invivogen, Toulouse France).

The bacterial strains used in this study were *Lactobacillus rhamnosus GG (LGG, DCS3373), Lactobacillus acidophilus (L. acidophilus, DCS856), Clostridium perfringens (C. perfringens., DCS3284),* Enterotoxigenic *Escherichia coli (ETEC) K88 (DCS3370), ETEC O138K81 (DCS3371)* and *Listeria monocytogenes (L. monocytogenes, DCS977)* from the DuPont collection. All bacteria were grown on Brain-Heart-Infusion (BHI) broth at 37ºC under an anaerobic atmosphere (Anaerocult, Merck, Darmstadt, Germany).

Cytotoxicity assay

Cells with viability > 97% were harvested and adjusted to $1*10^6$/ml. TCB (from 1 ng/ml to 1 mg/ml) was added accordingly. Some of them were cocultured with LPS (100 ng/ml). Cell viability was measured after 24 hours by trypan blue staining and counted using a Countess II FL Automated Cell Counter. The data represent one experiment of three replicates in each condition.

Permeability assay

Pig epithelial cells (IPEC-J2) were differentiated on permeable filters (Thincert Pore 0.4 µm, Greiner Bio-one). Briefly, on day 1, cells were seeded at a density of 5.0×10^4 cells/well on cell culture inserts in complete cultivation medium (without phenol red) and differentiated for 21 days. On the 4^{th} day of differentiation, the cells were moved to asymmetric serum-conditions using the serum-free cultivation medium on the apical side and the complete medium on the basal side of the insert. This condition was used until the end of the differentiation by changing the media at three to four days intervals. On day 20, a quality control was performed for each insert prior to the experiment with TEER measurement (Inserts with TEER value above 20,000 Ω/cm^2 are included in the assay). TCB (100 ng/ml) was then first added and cultured overnight and the next day (day 21) LPS (100 ng/ml) was added in some of the conditions for 2 h. FITC-Dextran (4 KD, FD4, 10 µg/ml) was then added on the apical side. After time point 1 h and 4 h culture medium from basolateral side was collected and OD was measured at 485/535 nm. An independent serial dilution of FD4 in medium was measured in parallel and OD/FD4 standard curve was established accordingly. The leak percentage of the barrier was calculated using the following formula:

$$\text{Leak \%} = (\text{Dose}_{\text{basolateral}} / \text{Dose}_{\text{apical}}) \times 100\%$$

The experiments were performed in triplicate giving a total of nine replicates in each condition.

Wound-healing assay

Pig epithelial cells (IPEC-J2) were first pre-cocultured with TCB (100 ng/ml) for 3 days in grid petri dishes. Then the culture medium (day 0) was refreshed and LPS (100 ng/ml) was added in some of the cultures. A 'wound' was made using a cell scraper in a 9×9 square grid, which covers a 1.8 cm ×1.8 cm square area. 'Healing' was recorded by daily microscope observation and recovery area was calculated with the following equation:

$$\text{Recovery\%} = (\text{Number}_{\text{grid with cells}}/81) \times 100\%$$

The experiments were performed in triplicate giving a total of seven replicates for each condition.

RT-PCR

For RT-PCR, pig epithelial cells (IPEC-J2), were first cultured with TCB (100 ng/ml) for 2 h and then LPS (100ng/ml) was added. After 6 h culture, cell pellets were collected for RT-PCR analysis. RNA extraction, quality control and RT-qPCR were performed at Eurofins AROS as described previously (see (Shen et al., 2020) and Supplementary Table S1).

Data were first normalized to two sets of house-keeping genes using the following equation:

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

$\text{Value}_{(-),\ \text{W/O LPS}}$ was then used as background expression (100%). All values were compared to $\text{Value}_{(-),\ \text{W/O LPS}}$ and shown as:

$$\text{\% expression change} = \text{Value} / \text{Value}_{(-),\ \text{W/O LPS}} * 100\%$$

The data represented three independent experiments with nine replicates.

Bacteria adhesion assay

IPEC-J2 cells were seeded in 24-well plates, 1 x 10^5 cells / well. The cells grew for a week until they reached a monolayer confluence. Bacteria suspension was diluted serially and plated onto BHI agar plates (Bacteria $_{loaded}$). The same bacteria suspension in PBS were added to IPEC-J2 cells in each well. After 30 min incubation, the cell monolayer was gently washed 5 times with PBS and lysed with cold 0.1% Triton X-100. The lysates containing total cell-associated bacteria were diluted serially in PBS and plated onto BHI agar plates at 37°C for the enumeration of adherent bacteria (Bacteria $_{adhered}$). In parallel, bacteria suspension was also diluted serially and plated onto BHI agar plates (Bacteria $_{loaded}$). Bacteria binding affinity is calculated using the following equation:

% binding = (CFU Bacteria $_{adhered}$ / CFU Bacteria $_{loaded}$) x 100%

Data are shown after normalization to its blank control and shown as a relative change:

% binding = % binding $_{TCB\ or\ control}$ / % binding $_{control}$ x 100%

The data represented three independent experiments with nine replicates.

Statistical analyses

Student paired t tests were used for all assays. The comparison and the statistics were calculated between each two groups, assuming two-tail and unequal variance data distribution. The values with statistical significance are stated in the figures.

Results and discussion

Epithelial cells form the first line barrier in gut mucosa and initiate the immune response, which is essential in the host response to invading microbes (Akira, Uematsu, & Takeuchi, 2006). We investigated *in vitro* immune modulation effects of TCB in pig intestinal epithelia cell line (IPEC-J2).

149 *Cytotoxicity*

150 IPEC-J2 cells exhibited high viability after 48h exposure to TCB at concentrations up to a
151 maximum of 1 μg/ml, as indicated by consistent cell viability rates of > 90% (Table 1). The
152 addition of LPS (100 ng/ml) to the TCB-treated cultures did not produce any cytotoxic effects;
153 cell viability was maintained at > 90% in LPS-treated cultures (Table 1).

154

155 **Table 1.** Cell viability after 48h cultured with a thymol and cinnamaldehyde blend (TCB)
156 (experiments were performed in duplicate with six experimental replicates per treatment).

Culture condition	TCB[1]		Blank control		*P* - value
	Cell viability, %	SEM	Cell viability, %	SEM	
without LPS	95,3	1,45	93.3	1.20	0.53
with LPS[2]	91.3	0.50	93.7	0,88	0.42

157 [1]Cells were cultured with TCB (100 ng/ml) for 48h.

158 [2]100 ng/ml.

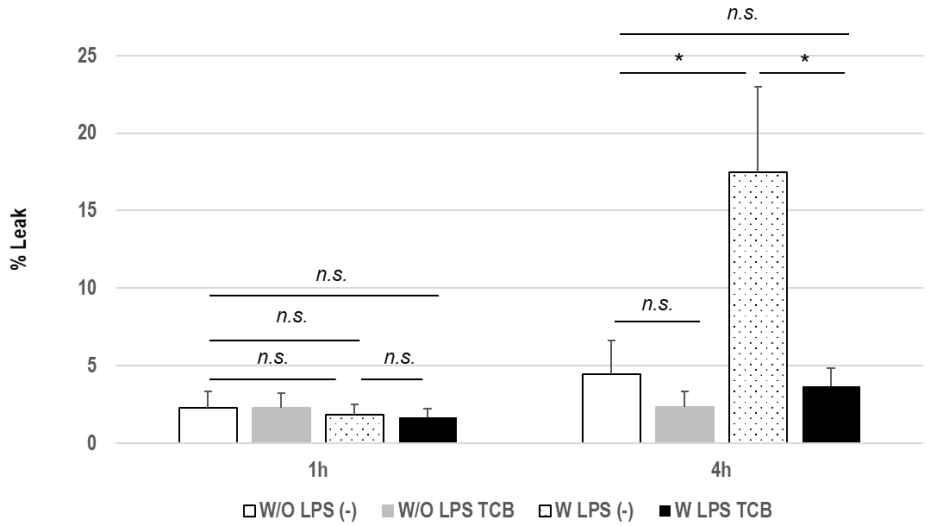
159

160 *Permeability assay*

161 Permeability assay showing paracellular influx, is closely associated to the tight junction (TJ)
162 protein dynamics and gut integrity (Gao *et al*., 2017). LPS was used to cause defects of the tight
163 junction and further led to an increase in intestinal permeability (Guo, Al-Sadi, Said, & Ma,
164 2013). The effects of TCB on intestinal permeability were investigated by measuring the
165 paracellular flux of fluorescent tracers with FD4 across IPEC-J2 cell monolayers at two time
166 points: 1 h and 4 h after coculture. The paracellular flux of FD4 significantly increased ($P<0.05$)
167 in LPS treated cell monolayers at time point 4 h (Figure 1). In comparison, LPS-induced FD4
168 leak was not observed in TCB pre-treated cells ($P<0.05$).

The data thus indicates that TCB abolished the detrimental effect of LPS and restored the barrier integrity. Claudin-4 (CLDN4) is a TJ plays crucial role in modulating paracellular permeability of epithelial cells (Cong et al., 2015). The mitigation of intestinal permeability caused by TCB was concomitant with the increased expression of the CLDN4, as observed in the result of RT-PCR. A recent study (Omonijo *et al.*, 2019) similarly showed that thymol alone could attenuated LPS effect, as evidenced by an increased TEER value as well as a reduced permeability. In addition, the same finding with cinnamaldehyde was also reported (Sun, Lei, Wang, Wu, & Wu, 2017).

Figure 1



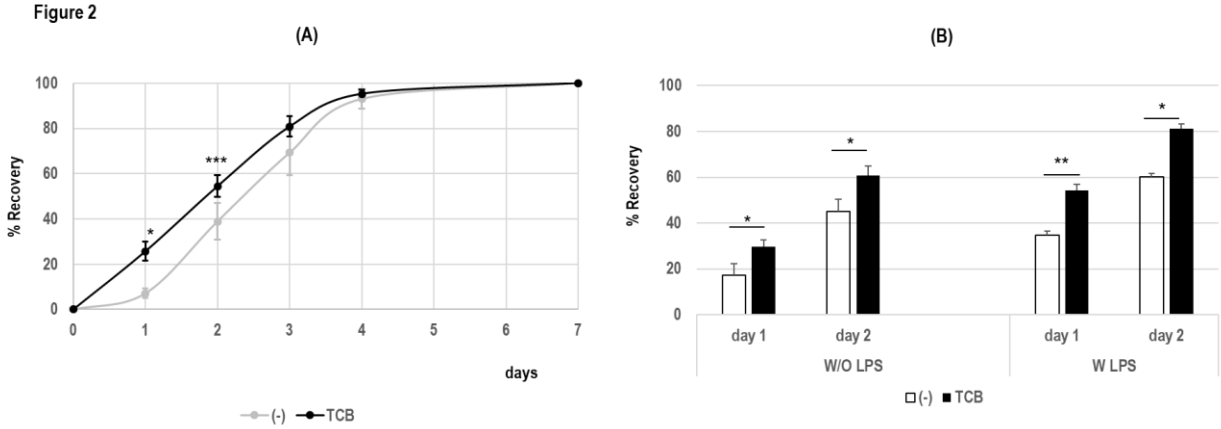
Wound -healing

Upon injury, epithelial wound healing is orchestrated by a series of events that emanate from the epithelium itself as well as by the temporal recruitment of immune cells into the wound bed (Leoni, Neumann, Sumagin, Denning, & Nusrat, 2015). The wound-healing assay mimics cell migration and tissue regeneration during wound healing *in vivo*. In a kinetic study (Figure 2A), cells precultured with TCB (100 ng/ml) had a faster onset of recovery (*vs* control, on day 1 from 7 to 26%, $P<0.05$; on day 2 from 39 to 54%, $P<0.001$) after 'wounding'. In the later

experiments, this promoting effect of TCB has also been observed in LPS challenged cells (day 1: $P<0.01$; day 2, $P<0.05$, Figure 2B).

Our data shows that TCB assisted epithelial cells with rapid restitution, indicating existing cells migrate along the exposed basement membrane to fill in the defects and restore epithelial barrier integrity. Unexpectedly, LPS showed an accelerated *in vitro* wound-healing. Similar results have been shown by other groups in both airway and gut epithelial cells *in vivo* models (Fukata et al., 2005) (Ueki, Koff, Shao, Nadel, & Kim, 2014). Both studies indicated that LPS/TLR4 signaling plays a role in intestinal response to injury and in limiting bacterial translocation, as a response of host against pathogenic microorganisms.

Figure 2



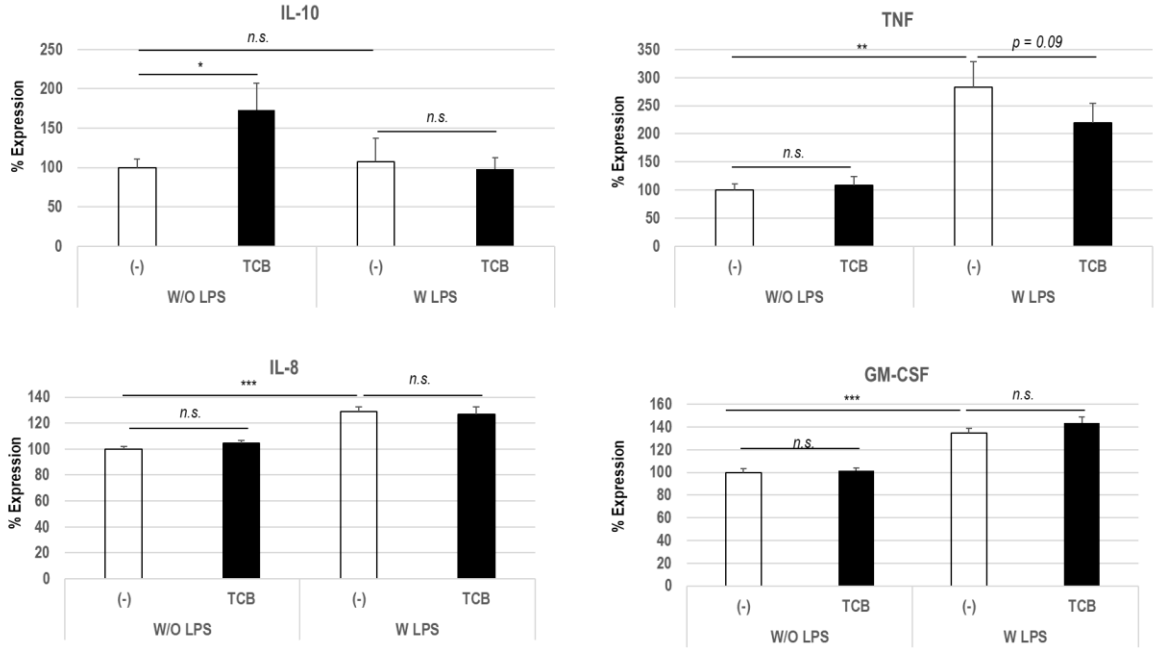
Biomarkers (cytokines, tight junctions and pIgR)

The imbalance between pro-inflammatory and anti-inflammatory cytokines results in disease progression and tissue damage and limits the resolution of intestinal inflammation in pig intestine in PWD (Rhouma, Fairbrother, Beaudry, & Letellier, 2017). Over-production of pro-inflammatory cytokines such as TNF, results in intestinal mucosal injury and dysfunction, and consequently results in poor growth of pigs (Liu, 2015).

Using RT-PCR we further tested the effect of TCB on IPEC-J2 cells, under different circumstances (non-stimulated or LPS-stimulated) at the mRNA level. TCB upregulated anti-

inflammatory cytokines IL-10 (P<0.05, Figure 3A) in non-stimulated cells. TNF, a pro-inflammatory cytokine, was numerically down-regulated by TCB, but only in LPS challenged cells. There is no significant changes of other pro-inflammatory cytokines, such as IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF). For tight junctions (Figure 3B), CLDN4 was upregulated in TCB cultured LPS-stimulated cell (P<0.05), yet non-challenged cells showed the same numerical trend. The expression of OCLN seemed to increase slightly, yet not reaching statistical significance. Finally, pIgR, the carrier of SIgA was upregulated slightly yet significantly (P<0.05) by TCB in LPS-stimulated cells (Figure 3C). Our *in vitro* work showed TCB to upregulate of anti-inflammatory cytokines IL-10 meanwhile pro-inflammatory cytokine TNF was down-regulated. Therefore, TCB has the potential to avoid excessive activation of GI immune system which would be an important way to improve the efficiency of pig production. Another interesting finding is that pIgR was upregulated by TCB. pIgR is an important transporter for dimeric IgA (dIgA), together with secreted component (SC), which forms a secreted form of IgA (SIgA) and transfers dIgA from basal to apical side (Johansen & Kaetzel, 2011). SIgA further interacts with antigens/pathogens, neutralizing their ability to cause disease. Upregulation of pIgR by TCB indicates its potential in pathogens clearance and homeostasis maintenance in microenvironment. Overall, our *in vitro* results suggest that TCB has potential to reformat the cytokines panels *in vivo*.

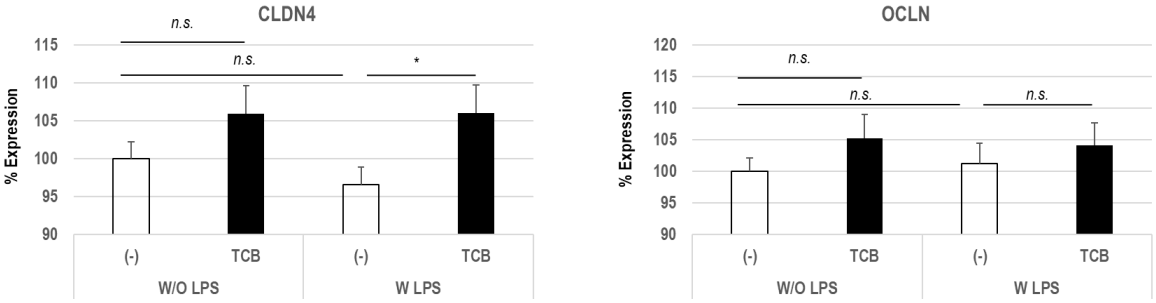
Figure 3A



226

227

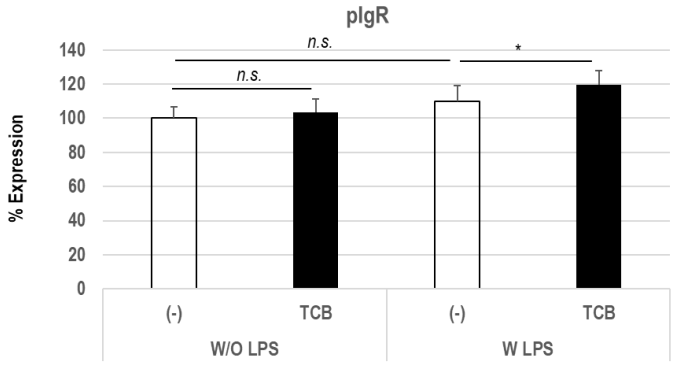
Figure 3B



228

229

Figure 3C



230

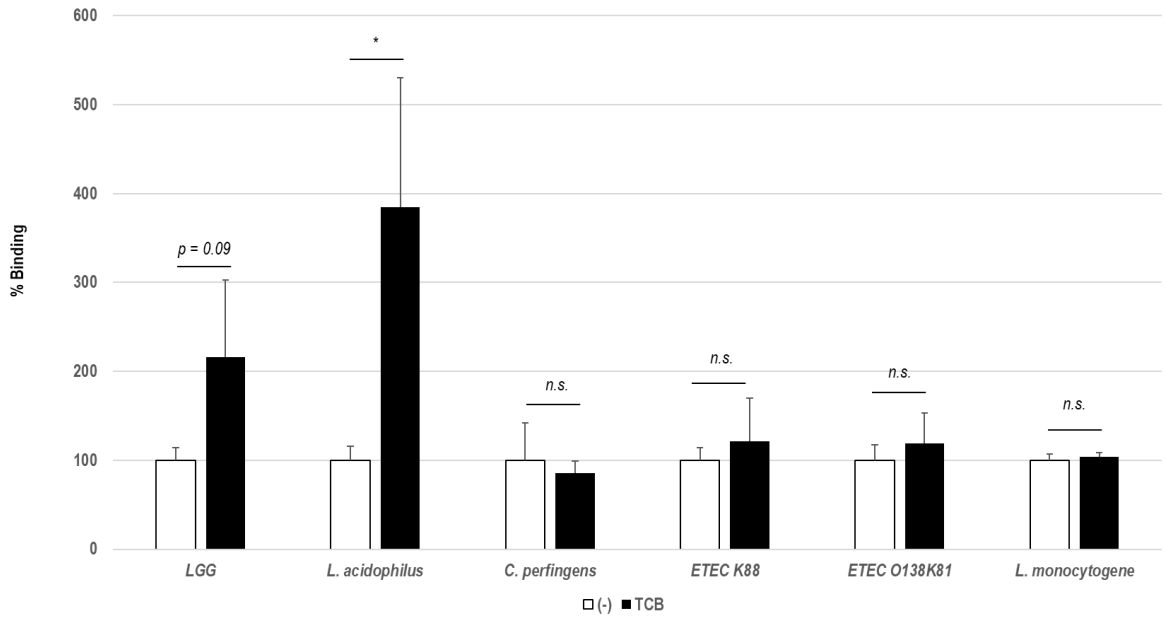
231

Bacteria adhesion assay

The concept of probiotics is a proven solution to improve animal gut health and production performance (Liao & Nyachoti, 2017). *Lactobacillus* is one of the most commonly used bacteria in swine production (Markowiak & Slizewska, 2018). Others (Zhao & Kim, 2015) reported that weanling pigs fed *L. reuteri* and *L. plantarum* (Direct Fed Microbial, DFM) had improved performance. This seemed to be a viable alternative to antibiotics used as growth promoters. However, these positive effects were not observed in all pig experiments (Zimmermann *et al*., 2016). This leads to the discussion of the ability of bacterial strains to adhere to intestinal epithelium, to be the key step in the successful colonization and execution of probiotic effects (Larsen, Nissen, & Willats, 2007).

The effect of TCB on adherence of *Lactobacillus rhamnosus GG, Lactobacillus acidophilus, Clostridium perfringens,* Enterotoxigenic *Escherichia coli K88, ETEC O138K81* and *Listeria monocytogenes* is shown in Figure 4. Preculture of IPEC-J2 cells with TCB significantly enhanced ($P<0.05$) the binding of *Lactobacillus acidophilus,* for which adhesion was increased by more than 2 to 3-fold, as compared to the control without TCB. Adhesion of *LGG* bacteria was numerically increased ($P=0.09$). In comparison, there were no significant changes in attachment of *Clostridium perfringens., ETEC K88, ETEC O138K81* and *L. monocytogenes*. Therefore, TCB enhancing *L. acidophilus* adhesion is considered to be strain specific and might be helpful in improving gut colonization by DFMs.

Figure 4



The former *in vivo* study of TCB, added supplementary to the diet of weaned pigs showed potential as an alternative to traditional antibiotics (Li *et al.*, 2012). This is evidenced by increased weight gain, better nutrient utilization and improved intestinal morphology. Though *in vitro* epithelial cytokine production is not fully comparable to the systemic plasma level *in vivo*, both studies indicate the anti-inflammatory trend of TCB: *in vivo* there is less pro-inflammatory cytokine production (IL-1β and IL-6), while anti-inflammatory cytokine IL-10 is upregulated in *in vitro* cell line. Additionally, a higher ratio of *Lactobacillus* vs *E. coli.* is found *in vivo*, due to the increased count of *Lactobacillus spp.* while *E. coli* remains unchanged. This is well aligned with the observation in the *in vitro* bacterial adhesion assay, indicating IPEC-J2 cells are a powerful, cost-effective tool for probiotic screening.

The previous *in vitro* study based on poultry cell lines similarly showed the immune modulation effect of TCB (Shen *et al.*, 2020, in press). Whether this is related to the same pathway is still unclear. On one hand, IL-10 upregulation is observed in both species and downregulation of inflammatory cytokines, such as IL-1β, IL-6 and IL-8 is more evident in chicken monocytes; on the other hand, the enhancement of barrier integrity by TCB are validated in both species, however the discrepancy of assay used in two studies should not be ignored (poultry: TEER

assay; swine: permeability assay). This is due to the characters of the individual cell lines. For example, lack of the poultry gut epithelial cell line (LMH, chicken hepatocytes used instead) restricted us testing permeability of the cell membrane, while high background TEER value of IPEC-J2 (likely due to the culture with fetal bovine serum (Vergauwen, 2015) might mask the effect of TCB. Permeability assay indeed is more sensitive in leaky cell monolayers and changes in paracellular cell junctions (Benson, Cramer, & Galla, 2013), and from another aspect validates the beneficial effect of TCB in improving barrier integrity in both poultry and swine.

Conclusion

Taken together, our findings of TCB modulation of immune responses *in vitro* in cell lines, as well as the observation from *in vivo* animal trials, indicate a positive role of TCB in modulating the mucosal immune system. We conclude TCB's beneficial functions from multiple aspects: TCB upregulates tight junctions and promotes intestinal wound recovery, thus it greatly improves epithelial integrity and protects the host from pathogen invasion; TCB enhances production of pIgR and facilities *Lactobacillus* adhesion, which might modulate microflora; TCB activates cells for immune surveillance and may modulate a sufficient and more precise response, adjusting the balance between immunity and tolerance. All these aspects may compose TCB's mode of action in establishing immune modulation, holistically.

References

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell, 124*(4), 783-801. doi: 10.1016/j.cell.2006.02.015

Benson, K., Cramer, S., & Galla, H.-J. (2013). Impedance-based cell monitoring: barrier properties and beyond. *Fluids and Barriers of the CNS, 10*(1), 5. doi: 10.1186/2045-8118-10-5

Brosnahan, A. J., & Brown, D. R. (2012). Porcine IPEC-J2 intestinal epithelial cells in microbiological investigations. *Vet Microbiol, 156*(3-4), 229-237. doi: 10.1016/j.vetmic.2011.10.017

Cong, X., Zhang, Y., Li, J., Mei, M., Ding, C., Xiang, R. L., Zhang, L., Wang, Y., & Yu, G. Y. (2015). Claudin-4 is required for modulation of paracellular permeability by muscarinic acetylcholine receptor in epithelial cells. *J Cell Sci, 128*(12), 2271-2286. doi: 10.1242/jcs.165878

Fukata, M., Lechago, J., Abreu, M. T., Lukasek, K., Michelsen, K. S., Naiki, Y., Soliman, A., Arditi, M., & Thomas, L. S. (2005). Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 288*(5), G1055-G1065. doi: 10.1152/ajpgi.00328.2004

Gao, Y., Li, S., Wang, J., Luo, C., Zhao, S., & Zheng, N. (2017). Modulation of Intestinal Epithelial Permeability in Differentiated Caco-2 Cells Exposed to Aflatoxin M1 and Ochratoxin A Individually or Collectively. *Toxins (Basel), 10*(1). doi: 10.3390/toxins10010013

Guo, S., Al-Sadi, R., Said, H. M., & Ma, T. Y. (2013). Lipopolysaccharide causes an increase in intestinal tight junction permeability in vitro and in vivo by inducing enterocyte membrane expression and localization of TLR-4 and CD14. *Am J Pathol, 182*(2), 375-387. doi: 10.1016/j.ajpath.2012.10.014

Johansen, F. E., & Kaetzel, C. S. (2011). Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. *Mucosal Immunol, 4*(6), 598-602. doi: 10.1038/mi.2011.37

Larsen, N., Nissen, P., & Willats, W. G. (2007). The effect of calcium ions on adhesion and competitive exclusion of Lactobacillus ssp. and E. coli O138. *Int J Food Microbiol, 114*(1), 113-119. doi: 10.1016/j.ijfoodmicro.2006.10.033

Lee, I. K., Kye, Y. C., Kim, G., Kim, H. W., Gu, M. J., Umboh, J., Maaruf, K., Kim, S. W., & Yun, C. H. (2016). Stress, Nutrition, and Intestinal Immune Responses in Pigs - A Review. *Asian-Australas J Anim Sci, 29*(8), 1075-1082. doi: 10.5713/ajas.16.0118

Leoni, G., Neumann, P. A., Sumagin, R., Denning, T. L., & Nusrat, A. (2015). Wound repair: Role of immune-epithelial interactions. *Mucosal Immunology, 8*(5), 959-968. doi: 10.1038/mi.2015.63

Li, P., Piao, X., Ru, Y., Han, X., Xue, L., & Zhang, H. (2012). Effects of adding essential oil to the diet of weaned pigs on performance, nutrient utilization, immune response and intestinal health. *Asian-Australasian Journal of Animal Sciences, 25*(11), 1617-1626. doi: 10.5713/ajas.2012.12292

Liao, S. F., & Nyachoti, M. (2017). Using probiotics to improve swine gut health and nutrient utilization. *Anim Nutr, 3*(4), 331-343. doi: 10.1016/j.aninu.2017.06.007

Liu, Y. (2015). Fatty acids, inflammation and intestinal health in pigs. *J Anim Sci Biotechnol, 6*(1), 41. doi: 10.1186/s40104-015-0040-1

Markowiak, P., & Slizewska, K. (2018). The role of probiotics, prebiotics and synbiotics in animal nutrition. *Gut Pathog, 10*, 21. doi: 10.1186/s13099-018-0250-0

Omonijo, F. A., Liu, S., Hui, Q., Zhang, H., Lahaye, L., Bodin, J. C., Gong, J., Niachoti, M., & Yang, C. (2019). Thymol Improves Barrier Function and Attenuates Inflammatory Responses in Porcine Intestinal Epithelial Cells during Lipopolysaccharide (LPS)-Induced Inflammation. *J Agric Food Chem, 67*(2), 615-624. doi: 10.1021/acs.jafc.8b05480

Omonijo, F. A., Ni, L., Gong, J., Wang, Q., Lahaye, L., & Yang, C. (2018). Essential oils as alternatives to antibiotics in swine production. *Anim Nutr, 4*(2), 126-136. doi: 10.1016/j.aninu.2017.09.001

Ouwehand, A. C., Tiihonen, K., Kettunen, H., Peuranen, S., Schulze, H., & Rautonen, N. (2010). In vitro effects of essential oils on potential pathogens and beneficial members of the normal microbiota. *Veterinarni Medicina, 55*(2), 71-78. doi: 10.17221/152/2009-VETMED

Rhouma, M., Fairbrother, J. M., Beaudry, F., & Letellier, A. (2017). Post weaning diarrhea in pigs: risk factors and non-colistin-based control strategies. *Acta Vet Scand, 59*(1), 31. doi: 10.1186/s13028-017-0299-7

Shen, C., Christensen, L. G., Bak, S. Y., Christensen, N., & Kragh, K. (2020). Immunomodulatory effects of thymol and cinnamaldehyde in chicken cell lines. *Journal of Applied Animal Nutrition*, 1-10. doi: 10.3920/jaan2020.0001

Sun, K., Lei, Y., Wang, R., Wu, Z., & Wu, G. (2017). Cinnamicaldehyde regulates the expression of tight junction proteins and amino acid transporters in intestinal porcine epithelial cells. *J Anim Sci Biotechnol, 8*(1), 66. doi: 10.1186/s40104-017-0186-0

Ueki, I. F., Koff, J. L., Shao, M. X. G., Nadel, J. A., & Kim, S. (2014). Pseudomonas Lipopolysaccharide Accelerates Wound Repair via Activation of a Novel Epithelial Cell Signaling Cascade. *The Journal of Immunology, 177*(12), 8693-8700. doi: 10.4049/jimmunol.177.12.8693

Vergauwen, H. (2015). The IPEC-J2 Cell Line. In K. Verhoeckx, P. Cotter, I. Lopez-Exposito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka & H. Wichers (Eds.), *The Impact of Food Bioactives on Health: in vitro and ex vivo models* (pp. 125-134). Cham (CH).

Zeng, Z., Zhang, S., Wang, H., & Piao, X. (2015). Essential oil and aromatic plants as feed additives in non-ruminant nutrition: a review. *J Anim Sci Biotechnol, 6*(1), 7. doi: 10.1186/s40104-015-0004-5

Zhao, P. Y., & Kim, I. H. (2015). Effect of direct-fed microbial on growth performance, nutrient digestibility, fecal noxious gas emission, fecal microbial flora and diarrhea score in weanling pigs. *Animal Feed Science and Technology, 200*(1), 86-92. doi: https://doi.org/10.1016/j.anifeedsci.2014.12.010

Zimmermann, J. A., Fusari, M. L., Rossler, E., Blajman, J. E., Romero-Scharpen, A., Astesana, D. M., Olivero, C.,R., Berisvil, A.,P.,Signorini, M.,L., Zbrun, M.,V., Frizzo, L.,S., & Soto, L. P. (2016). Effects of probiotics in swines growth performance: A meta-analysis of randomised controlled trials. *Animal Feed Science and Technology, 219*, 280-293. doi: https://doi.org/10.1016/j.anifeedsci.2016.06.021

Figure legends

Figure 1. TCB positively modulates *in vitro* epithelial integrity.

Pig epithelial cells (IPEC-J2) were pre-cocultured with TCB (100 ng/ml) overnight, and then some were stimulated by LPS for 2 hours. 10 μg FITC-Dextran powder (4KD, FD4) were then added at the apical side. After time point 1h and 4h culture, medium from basolateral side was collected and OD was measured at 485/535. The experiments were performed 3 times with total 9 replicates in each condition. Data are shown as mean ± SEM. *: $P<0.05$; n.s.: not statistically significant. W/O LPS: LPS was not added to cells; W LPS: LPS was added to cells.

Figure 2. TCB enhances *in vitro* epithelial regeneration.

Pig epithelial cells (IPEC-J2) were pre-cocultured with TCB (100 ng/ml) for 3 days. Then on day 0 culture medium were refreshed and "wound" was made by cell scrapers. The starting empty area was count as 0 %. Cell recovery area was calculated as % daily as shown. Data are shown as mean ± SEM. (A) Kinetics of cell recovery. Each dot represents two experiments with 4 replicates. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$, TCB vs control. (B) Cell recovery under LPS stimulation. In separate experiments, cells were precultured with TCB as described previously. On Day 0 some of the wells were challenged with LPS (100ng/ml) while "wound" was made. Each column represents three experiments with 7 replicates. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. W/O LPS: LPS was not added to cells; W LPS: LPS was added to cells.

Figure 3. Immune modulatory effect of TCB on *in vitro* pig epithelial cells, measured by RT-PCR.

Pig epithelial cells (IPEC-J2) were pre-cocultured 2 hours TCB (100 ng/ml) and then were stimulated by LPS (10 0ng/ml) and further culture for 6 hours. Data are from 3 independent experiments with 9 replicates. Data are normalized to 2 sets of house-keeping gene and shown as a relative % expression after comparison to the Value $_{(-),\ W/O\ LPS}$. 100% expression is considered as a basal expression. Data are shown as mean ± SEM. *: $P<0.05$; **: $P<0.01$; ***:

393 *P*<0.001; n.s.: not statistically significant. W/O LPS: LPS was not added to cells; W LPS: LPS
394 was added to cells. (A) Anti-inflammatory cytokine IL-10 and pro-inflammatory cytokine TNF,
395 IL-8 and GM-CSF; (B) Tight junctions; (C) pIgR.
396 **Figure 4.** TCB facilitates *Lactobacillus acidophilus* adhesion on pig epithelial cells grown *in*
397 *vitro*.
398 IPEC-J2 cells were seeded and grew for a week till reach a monolayer confluence. Bacteria
399 suspension was diluted serially and plated onto BHI agar plates (Bacteria $_{loaded}$). The same
400 bacteria suspension was added to IPEC-J2 cells in each well. After incubation, the cells were
401 washed and lysed. The lysates containing cell-adhered bacteria were diluted serially in PBS and
402 plated onto BHI agar plates at 37°C for the enumeration of adherent bacteria (Bacteria $_{adhered}$).
403 Bacteria binding affinity is calculated with the following equation: Value binding = CFU
404 Bacteria $_{adhered}$ / CFU Bacteria $_{loaded,}$ and then data is shown as relative value % expression after
405 comparison to the Value $_{(-)}$. Data are from 3 independent experiments with 9 replicates. *:
406 *P*<0.05; n.s.: not statistically significant. *LGG: Lactobacillus rhamnosus GG; L. acidophilus:*
407 *Lactobacillus acidophilus; C. perfringens: Clostridium perfringens; ETEC K88:*
408 Enterotoxigenic *Escherichia coli K88; ETEC O138K81:* Enterotoxigenic *Escherichia coli*
409 *O138K81; L. monocytogenes: Listeria monocytogenes.*