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A carvacrol–thymol blend decreased intestinal oxidative stress and influenced selected microbes without changing the messenger RNA levels of tight junction proteins in jejunal mucosa of weaning piglets

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Recent studies indicate that intestinal oxidative stress and microbiota imbalance is involved in weaning-induced intestinal dysfunction in piglets. We have investigated the effect of feeding a carvacrol–thymol blend supplemented diet on intestinal redox status, selected microbial populations and the intestinal barrier in weaning piglets. The piglets (weaned at 21 days of age) were randomly allocated to two groups with six pens per treatment and 10 piglets per pen. At weaning day (21 days of age), six piglets were sacrificed before weaning to serve as the preweaning group. The weaned group was fed with a basal diet, while the weaned-CB group was fed with the basal diet supplemented with 100 mg/kg carvacrol–thymol (1 : 1) blend for 14 days. On day 7 post-weaning, six piglets from each group were sacrificed to determine intestinal redox status, selected microbial populations, messenger RNA (mRNA) transcript levels of proinflammatory cytokines and biomarkers of intestinal barrier function. Weaning resulted in intestinal oxidative stress, indicated by the increased concentration of reactive oxygen species and thiobarbituric acid-reactive substances present in the intestine. Weaning also reduced the population of Lactobacillus genus and increased the populations of Enterococcus genus and Escherichia coli in the jejunum, and increased mRNA levels of tumor necrosis factor α (TNF-α), interleukin 1β and interleukin 6 (IL-6). In addition, decreased mRNA levels of zonula occludens and occludin in the jejunal mucosa and increased plasma diamine oxidase concentrations indicated that weaning induced dysfunction of the intestinal barrier. On day 7 post-weaning, supplementation with the carvacrol–thymol blend restored weaning-induced intestinal oxidative stress. Compared with the weaned group, the weaned-CB group had an increased population of Lactobacillus genus but reduced populations of Enterococcus genus and E. coli in the jejunum and decreased mRNA levels of TNF-α. The results indicated that weaning induced intestinal oxidative stress and dysfunction of the intestinal barrier. Dietary supplementation with 100 mg/kg carvacrol–thymol (1 : 1) decreased the intestinal oxidative stress and influenced selected microbial populations without changing the biomarkers of intestinal barrier in weaning piglets.

Keywords: weaning, carvacrol, thymol, oxidative stress, intestinal barrier

Implications

The current study showed that dietary supplementation with a carvacrol–thymol blend decreased the intestinal oxidative stress and influenced selected microbial populations in weaning piglets. However, the carvacrol–thymol blend had limited ability to decrease weaning-induced intestinal inflammation and failed to improve barrier function of the intestine. It therefore has implications for the application of carvacrol and thymol in the diet of weaning piglets. It also suggested that evaluation of antioxidative, antibacterial and antiinflammatory ability might lead to the development of novel feed additives to promote intestinal health in weaning piglets.

Introduction

During weaning, piglets suffer social, environmental and dietary stress, all of which contribute to a decrease in their performance. Numerous studies have shown that dysfunction of the intestine, which has important immunological,

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193

metabolic and barrier functions, has been demonstrated to play a crucial role in weaning-induced growth check (Wijtten *et al.*, 2011; Campbell *et al.*, 2013). Recent studies have indicated that decreased antioxidant activity and increased production of reactive oxygen species (ROS) is involved in weaning-induced intestinal dysfunction, resulting in intestinal oxidative stress (Wang *et al.*, 2008; Degroote *et al.*, 2012; Zhu *et al.*, 2012). The intestinal oxidative stress is frequently associated with inflammation (Rada *et al.*, 2011; Padgett *et al.*, 2013). Elevated proinflammatory cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6), can induce the decreased abundance of tight junction proteins including zonula occludens (ZO-1) and occludin and thus undermine the integrity of the intestinal barrier (Al-Sadi *et al.*, 2009; Suzuki *et al.*, 2011). In addition to ZO-1 and occludin, plasma diamine oxidase has been proposed as a sensitive circulating biomarker for monitoring the intestinal mucosal barrier (Wolvekamp and de Bruin, 1994).

It has been reported that weaning stress dramatically decreases the amount of *Lactobacillus* and increases the amount of *Escherichia coli* present in the gut (Franklin *et al.*, 2002; Castillo *et al.*, 2007). A recent study reported that *Lactobacillus* was negatively correlated with oxidative stress, while, conversely, *E. coli* showed a strong positive correlation with oxidative stress in the intestines of early weaned piglets (Xu *et al.*, 2014a). In most cases, oxidative stress is characterized by increased generation of ROS which overwhelm the antioxidant capacity, subsequently resulting in damage to cellular macromolecules such as lipids, proteins and DNA. Several kinds of bacteria in the intestines, especially *Enterococcus faecalis* and *E. coli*, have been shown to produce extracellular ROS (Huycke and Moore, 2002), whereas *Lactobacillus* has the ability to inhibit ROS production from fermentation of colon digesta and growth of *E. faecalis* and *E. coli* (Sun *et al.*, 2010).

Both carvacrol and thymol possess *in vitro* ROS-scavenging activity (Mastelic *et al.*, 2008). In addition, it has been demonstrated that either carvacrol or thymol alone, or in combination, has antibacterial properties to inhibit growth of *E. faecalis* and *E. coli* (Michiels *et al.*, 2009; Guarda *et al.*, 2011; Gutiérrez-Fernández *et al.*, 2013). Based on these properties of carvacrol and thymol, we hypothesized that the inclusion of a carvacrol–thymol blend in the diet would scavenge ROS and/or alter the gut microbial populations, such as those of *Lactobacillus*, *E. faecalis* and *E. coli*, thus decreasing oxidative stress and preventing weaning-induced dysfunction of the intestinal barrier, and thereby increasing the performance of weaning piglets.

Material and methods

Animals and experimental design
Gestating sows (Landrace × Yorkshire) were fed daily with average quantity of 2.2 kg of a gestating diet that met the NRC-recommended requirements for nutrients during the period of gestation. After farrowing, sows had free access to a lactation diet that also met NRC-recommended requirements. Each sow nursed 9 to 11 piglets (Duroc × Landrace × Yorkshire) before weaning. All sows had free access to water during the gestation and lactation periods.

A total of 120 piglets from 15 litters with an initial BW of 6.5 ± 0.9 kg were randomly allocated to two groups with six pens per treatment and 10 piglets per pen. The animals in group 1 were fed with a basal diet (weaned group), while the animals in group 2 were fed with a basal diet supplemented with carvacrol–thymol blend (50 mg carvacrol and 50 mg thymol/kg of diet) (weaned-CB group) for 14 days. The carvacrol–thymol blend was provided by Novus International Inc. (St. Louis, MO, USA) as Next Enhance 150® (1 : 1, thymol : carvacrol). According to the manufacturer, Next Enhance 150 contains 50% encapsulated active components (thymol and carvacrol) but no other nutrients. The composition and nutrient levels of the basal diet are shown in Table 1. The feeds used in the current study were prepared weekly and stored in airtight containers. The active components from the feed were analyzed in using gas chromatography MS with an HP 6890 chromatograph (Hewlett Packard, Avondale, PA, USA) connected to an HP 5972 A mass spectrometer (Hewlett Packard) equipped with an HP-5 capillary column (25 m × 0.25 mm) (Hewlett Packard). The experimental diet contained 51.07 mg/kg carvacrol and 43.86 mg/kg thymol, respectively. All of the piglets were given *ad libitum* access to water and feed. At the weaning day (21 days of age), before separation from the sows, six piglets were sacrificed (preweaning group) to collect jejunal digesta and mucosa, plasma, jejunum and jejunal tissue. When the piglets reached 28 days of age, one median-weight weaned piglet from each pen (total of six piglets/group) was sacrificed to collect plasma, jejunum, jejunal digesta and mucosa. The protocol of the experiment was approved by the Huazhong Agricultural University Institutional Animal Care and Use Committee and performed in accordance with national guidelines. The number of the received ethical clearance is 42000600013283.

Determination of diarrhea rate and diarrhea index
The number of pigs with diarrhea was recorded daily throughout the study. The severity of diarrhea was evaluated using the fecal consistency score system (Marquardt *et al.*, 1999). Scores were 0, firm feces, normal; 1, pasty, possible slight diarrhea; 2, semi-liquid, definitely unformed feces; or 3, liquid, very watery and frothy diarrhea. The diarrhea rate was calculated as (the total number of pigs with diarrhea/the total number of all experimental pigs) × 100%. Diarrhea index was calculated as sum of feces score/total number of pigs.

Sample collection
At 0800 h on the day of slaughter, blood was collected in tubes with anticoagulant ethylenediaminetetraacetic acid (EDTA) and plasma was obtained after centrifugation at 3000 × **g** for 20 min at 4°C and stored at −80°C until analysis for redox status. Jejunal digesta were collected under sterile conditions and immediately stored at −80°C

Table 1 *Composition and nutrient levels of the basal diet (as-fed basis)*

Ingredients (%)	Basal diet
Extruded corn	214.00
Broken rice	200.00
Sugar	50.00
Whey powder	100.00
Soybean oil	13.50
Powdered fat	25.00
Extruded soybean	125.00
Dehulled soybean meal	93.80
Fermented bean pulp	84.00
Spray-dried plasma	25.00
Dried porcine soluble	25.00
Limestone	7.26
Calcium formate	5.00
Calcium bicarbonate	14.53
Salt	1.04
Lysine (98.5%)	2.28
Methionine (98.0%)	1.97
Threonine (98.0%)	0.62
Vitamin premix*	6.00
Trace element premix[†]	6.00
Nutrient composition[‡]	
Net energy (kcal/kg)	2500
CP (%)	19.50
Calcium (%)	0.72
Available phosphorus (%)	0.40
Digestible lysine (%)	1.25
Digestible threonine (%)	0.77
Digestible methionine + cystine (%)	0.75

*Provided per kg of diet: vitamin A 9600 IU, vitamin D_3 3400 IU, vitamin E 20 mg, vitamin K_3 0.30 mg, vitamin B_1 1.22 mg, vitamin B_2 9.60 mg, vitamin B_6 2.11 mg, vitamin B_{12} 0.02 mg, vitamin B_3 36 mg, vitamin B_5 54.30 mg, folic acid 0.26 mg, biotin 0.15 mg, choline chloride 120 mg.

[†]Provided per kg of diet: Zn 100.05 mg, Mn 30.53 mg, Fe 100.02 mg, Cu 125.28 mg, I 0.29 mg, Se 0.32 mg. In addition to 1100.05 mg Zn provided by $ZnSO_4$, ZnO was also included in the trace element premix to provide 2250 mg ZnO/kg of diet.

[‡]Nutrient content of diets was calculated using published data for the individual ingredients.

pending the analysis of selected microbial populations. Segments (5 cm in length) of mid-jejunum was obtained, opened longitudinally and rinsed thoroughly with chilled physiological saline before fixed in 4% paraformaldehyde for subsequent histological measurement. In addition, 5 cm mid-jejunum segments were immediately washed with phosphate-buffered saline and collected for further antioxidant-active and thiobarbituric acid-reactive substances (TBARS) analysis. A third, thoroughly rinsed, sample of jejunum tissue was collected in order to obtain jejunal mucosa on a microscope slide, to determine ROS and to analyze the expression of TNF-α, IL-1β, ZO-1 and occludin. The samples were then frozen in liquid nitrogen and stored at −80°C.

Determination of jejunal morphology
The samples were sectioned at 5 µm thickness and stained with hemotoxylin and eosin. These were acquired with 100× magnifications using an Olympus BX51 microscope (Olympus Optical Company, Tokyo, Japan). Intestinal villus height and villus crypt depth were measured using Image-Pro Plus 6.0 image processing and analysis system (Media Cybernetics, Bethesda, MD, USA). For each sample, at least 10 well-oriented were measured and the mean value was calculated.

Determination of antioxidant-active and thiobarbituric acid-reactive substances in jejunum
Following homogenization of the jejunum tissues in saline solution (1 : 10, w : v) and centrifugation at $4000 \times \mathbf{g}$ for 20 min at 4°C, supernatants were obtained and analyzed. The activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) and the contents of TBARS were determined according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) (Wei *et al.*, 2015). SOD activity was determined as its ability to inhibit the reduction of nitro blue tetrazolium. GSH-Px activity was determined based on quantifying the rate of oxidation of GSH to glutathione disulfide (GSSG) by H_2O_2, catalyzed by GSH-Px. TBARS were analyzed based on the reaction with 2-thiobarbituric acid, using an Infinite® M1000 Pro microplate reader (Tecan, Morrisville, NC, USA).

Chemiluminescence measurement of reactive oxygen species
Levels of ROS were measured by a chemiluminescence assay using luminol (5-amino-2, 3 dihydro-1, 4-phthalazinedione; Sigma, St. Louis, MO, USA) as the probe. The measurements were made as described by Du *et al.* (2010), with minor changes to the technique, using a multiple function microplate reader (Mithras LB 940; Berthold Technologies, Bad Wildbad, Germany). Briefly, 50 µl of plasma or 10% jejunal mucosa homogenate (tissue weight (mg) : saline (µl) = 1 : 9) and 20 µl of horseradish peroxidase (HRP) (12.4 U of HRP type VI 310 U/mg; Sigma) were added to 150 µl of Krebs-HEPES buffer (118 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid, 10 mM glucose; pH 7.4). Finally, 50 µl of 1.25 mM luminol was added to the mixture, and chemiluminescence was recorded in the dynamic mode. The area under the curve was calculated using Origin 7.0 software (OriginLab Corp., Northampton, MA, USA).

Quantitative PCR
Total RNA was isolated from the jejunal mucosa of the piglets using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration of total RNA was measured using a NanoDrop 2000/C spectrophotometer (Thermo, Rockford, IL, USA). The complementary DNA (cDNA) was reverse-transcribed from 2 µg of eluted RNA using a TRANScript M-MLV kit (Toyobo, Shiga, Japan) based on the manufacturer's instructions. Following 10-fold dilution, cDNA reverse-transcription was performed to establish the relative quantification of gene amplification. The quantitative PCR for TNF-α, IL-1β, ZO-1 and occludin, as well as β-actin as internal control, was performed using their respective primer pairs. All the primer pairs were designed

195

Table 2 *Primers used for quantitative PCR*

Target group	Sequence of primers (5′ to 3′)	Product size (bp)	Annealing temperature (°C)
Lactobacillus genus	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	341	58
Enterococcus genus	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	144	61
Escherichia coli	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	96	60
TNF-α	CACCACGCTCTTCTGCCTACTG TTGAGACGATGATCTGAGTCCTTGG	115	63
IL-1β	AAAGGGGACTTGAAGAGAG CTGCTTGAGAGGTGCTGATGT	286	58
ZO-1	GGCGCACGGCGAAGGTAATT CTATCAAACTCAGGAGGCGGCACT	405	60
Occludin	GGAGTGATTCGGATTCTGTCTATGCT CGCCTGGGCTGTTGGGTTGA	423	60
β-Actin	CCAGGTCATCACCATCGG CCGTGTTGGCGTAGAGGT	158	60

TNF-α = tumor necrosis factor α; IL-1β = interleukin 1β; ZO-1 = zonula occludens.

using Primer5 software package (Primer-E Ltd, Plymouth, UK). The sequence of primers is presented in Table 2. The reaction was performed in a total volume of 20 µl containing 4 µl of cDNA, 1 µl of forward and reverse primers, 10 µl of iTaq SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA), and 5 µl of nuclease-free water. The PCR analysis was performed at 95°C for 4 min, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at a suitable temperature (Table 2) for 10 s and extension at 72°C for 30 s; a product melting curve was then performed to confirm the specificity of amplification. The results were analyzed by the method of $2^{-\Delta\Delta C_t}$.

Quantification of selected microbial populations
Microbial genomic DNA was extracted from jejunal digesta using a QIAamp DNA stool kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. Briefly, to release bacterial cells from the digesta, 2 g of jejunal digesta was washed three times in saline containing 0.1% Tween 80. During each washing, digesta were shaken vigorously by hand for 5 min and then centrifuged at 4°C for a further 5 min. After removal of the pellet, the bacterial cells were recovered from the pooled washing solutions by centrifugation at $27\,000 \times \mathbf{g}$ for 15 min at 4°C. To lyse the bacterial cells, bacterial samples were homogenized in buffer ASL (stool lysis buffer) and heated at 95°C for 5 min. Following incubation with an InhibitEx (Qiagen) tablet to remove potential PCR inhibitors, the lysates were treated with buffer AL and proteinase K at 70°C for 10 min. DNA was precipitated with ethanol and then purified on a QIAamp spin column and eluted in 50 µl of AE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0).

Genomic DNA from jejunal digesta was amplified by routine PCR using species- and genus-specific primers (Table 2). After PCR amplification with a Taq DNA polymerase kit (Promega, Madison, WI, USA) and electrophoresis on a 1.5% agarose gel, PCR products were purified according to the manufacturer's protocol (Omega, Norcross, GA, USA). The purified PCR products were linked to the pMD18-T vector system (Takara, Shiga, Japan) and then transferred to *E. coli* DH5α (Qiagen) for cloning. After checking the size of the cloned inserts with PCR amplification, the extracted plasmids of the positive clones were sequenced commercially to obtain the positive plasmids.

Serial dilutions of these positive plasmids served to generate standard curves using quantitative real-time PCR (Bio-Rad), permitting estimations of absolute quantification based on respective gene copies. Following 10-fold dilution, microbial genomic DNA was subjected to absolute quantitative PCR assay. The reaction was performed in a total volume of 20 µl containing 4 µl of template DNA, 1 µl of forward and reverse primers, 10 µl of iTaq SYBR Green PCR Master Mix and 5 µl of nuclease-free water. The thermal cycling conditions involved an initial denaturation step at 95°C for 4 min followed by 40 cycles of 95°C for 10 s, annealing temperature (Table 2) for 10 s and 72°C for 30 s; this was then followed by a product melting curve to confirm the specificity of amplification. The mean threshold cycle values from the triplicate of each sample were used for the calculations. The data are presented as gene copy numbers per gram of jejunal digesta.

Determination of activity of diamine oxidase in plasma
Diamine oxidase activity in plasma was measured spectrophotometrically, as described by Hou *et al.* (2012).

Statistical analysis
All results are presented as the mean ± SEM. All statistical analyses were carried out using the SAS statistical package (Version 8.1; SAS Institute Inc., Cary, NC, USA). Comparisons among preweaning, weaned and weaned-CB groups were performed using the one-way ANOVA followed by Tukey's *post-hoc* test. The Student's *t* test was used to test for growth

performance in weaned and weaned-CB groups. The χ^2 test was used to test for diarrhea rate. Differences were considered significant when $P < 0.05$.

Results

Growth performance, diarrhea rate and intestinal morphology of piglets

There was no difference in average daily gain, average daily feed intake, feed conversion ratio, diarrhea rate or diarrhea index between the weaned and weaned-CB groups (Table 3). The weaned and weaned-CB groups showed reduced ($P < 0.05$) villus height and villus : crypt ratio compared with the preweaning group (Table 4). There was also no difference in intestinal morphology between the weaned and the weaned-CB groups (Table 4).

Antioxidant levels in jejunum

As shown in Table 5, there were significantly ($P < 0.05$) elevated ROS and TBARS levels, raised activity of GSH-Px, and decreased activity of SOD in the jejunum of weaned piglets compared with preweaning piglets. Compared with the weaned group, the weaned-CB group had significantly ($P < 0.05$) increased activity of SOD and GSH-Px, but a reduced concentration of TBARS in the jejunum. Compared with preweaning piglets, the weaned-CB piglets had higher activity of GSH-Px, but lower activity of SOD. The ROS concentration in the jejunum of weaned-CB piglets was significantly ($P < 0.05$) lower than that of weaned piglets.

Table 3 *Growth performance and diarrhea rate of weaning piglets fed with basal diet (weaned) or basal diet supplemented with carvacrol–thymol blend (weaned-CB)*

Item	Weaned	Weaned-CB	SEM	P
BW 2 weeks after weaning	8.32	8.51	0.28	0.46
Average daily gain (g/day)	138.1	150.5	8.36	0.52
Average daily feed intake (g/day)	196.9	212.7	4.73	0.17
Feed conversion ratio	1.50	1.43	0.07	0.65
Diarrhea rate (%)	31.21	28.06	1.70	0.35
Diarrhea index	0.51	0.43	0.03	0.16

All results are presented as mean ± SEM ($n = 6$).

Table 4 *Jejunum morphology of 21-day-old (preweaning) and 28-day-old piglets fed basal diet (weaned) or basal diet supplemented with carvacrol–thymol blend (weaned-CB)*

Item	Preweaning	Weaned	Weaned-CB	SEM	P
Villus height (μm)	620.76[a]	353.78[c]	410.63[bc]	31.10	<0.01
Crypt depth (μm)	305.18	368.14	345.55	13.91	0.07
Villi : crypt	2.09[a]	0.96[c]	1.19[bc]	0.13	<0.01

All results are presented as mean ± SEM ($n = 6$).
[a,b,c] Mean values within a row with different superscript letters were significantly different ($P < 0.05$).

There was no difference in the concentration of TBARS in the jejunum of the weaned-CB and the preweaning groups.

Selected microbial populations in jejunal digesta

The weaned group had significantly elevated populations of *Enterococcus* genus and *E. coli* and a reduced population of *Lactobacillus* genus in jejunal digesta, compared with the preweaning group ($P < 0.05$; Figure 1). Compared with the weaned group, the weaned-CB group had significantly ($P < 0.05$) reduced counts of *Enterococcus* genus and *E. coli* and increased counts of *Lactobacillus* genus ($P < 0.05$). The population of *Enterococcus* genus in the weaned-CB group was significantly higher than in the preweaning group. There was no difference in the populations of *E. coli* and *Lactobacillus* genus in the weaned-CB and the preweaning groups.

Messenger RNA levels of tumor necrosis factor α, interleukin 1β and interleukin 6 in jejunal mucosa

The piglets in the weaned group showed a significant increase in messenger RNA (mRNA) levels of TNF-α, IL-1β and IL-6 in jejunal mucosa, compared with those in the preweaning group ($P < 0.05$; Figure 2). Compared with the weaned group, inclusion of carvacrol and thymol in the diet significantly decreased mRNA transcript levels of TNF-α ($P < 0.05$) in jejunal mucosa (Figure 2). The weaned-CB group had a higher mRNA level of IL-6 than the preweaning group. However, mRNA levels of TNF-α and IL-1β in jejunal mucosa did not differ between the preweaning and the weaned-CB groups.

Messenger RNA levels of occludin and zonula occludens in jejunal mucosa

There was a significant ($P < 0.05$) decrease in the mRNA levels of occludin and ZO-1 in the jejunal mucosa of weaned piglets, compared with the preweaning piglets (Figure 3a and b). The mRNA levels of occludin and ZO-1 did not differ between the weaned and the weaned-CB groups. The weaned-CB group had a significantly ($P < 0.05$) reduced mRNA level for ZO-1, but not for occludin, compared with the preweaning group.

Activity of diamine oxidase in plasma

As shown in Figure 3c, the activity of diamine oxidase in plasma significantly increased in the weaned group, compared with the preweaning piglets. The weaned-CB group had a higher activity of diamine oxidase in plasma, compared with the preweaning group ($P < 0.05$). There was no difference in the activity of diamine oxidase in plasma between the preweaning and the weaned-CB groups.

Discussion

Recent studies have indicated that early weaning may induce intestinal oxidative stress, which contributes to intestinal dysfunction (Wang *et al.*, 2008; Zhu *et al.*, 2012). In this study, we have investigated whether supplementing a carvacrol–thymol blend in the diet lowered early

Table 5 *Redox status in jejunum tissue of 21-day-old (preweaning) and 28-day-old piglets fed basal diet (weaned) or basal diet supplemented with carvacrol–thymol blend (weaned-CB)*

Item	Preweaning	Weaned	Weaned-CB	SEM	P
ROS (RLU/mg protein)	1466[b]	5782[a]	2854[ab]	583.64	<0.01
SOD (U/mg protein)	42.45[a]	32.14[c]	37.09[b]	0.67	<0.01
GSH-Px (U/mg protein)	23.37[c]	38.93[b]	47.35[a]	1.32	<0.01
TBARS (nmol/mg protein)	0.35[b]	0.53[a]	0.40[a]	0.03	<0.01

ROS = reactive oxygen species; RLU = relative light unit; SOD = superoxide dismutase; GSH-Px = glutathione peroxidase; TBARS = thiobarbituric acid-reactive substances.
All results are presented as mean ± SEM ($n = 6$).
[a,b,c]Mean values within a row with different superscript letters were significantly different ($P < 0.05$).
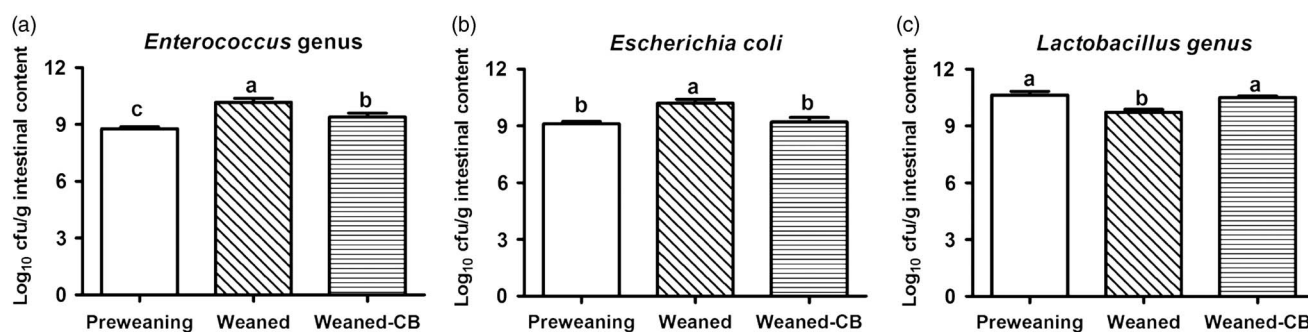
Figure 1 Population of *Enterococcus* genus (a), *Escherichia coli* (b) and *Lactobacillus* genus (c) in jejunum of 21-day-old (preweaning) and 28-day-old (weaned) piglets. Piglets that were 21-day old (preweaning) and 28-day old were fed a basal diet (weaned) or basal diet supplemented with a carvacrol–thymol blend (weaned-CB), were sacrificed and then jejunal digesta were collected to determine major microbiota counts. [a,b,c]Mean values with different superscript letters were significantly different ($P < 0.05$). All results are presented as mean ± SEM ($n = 6$).
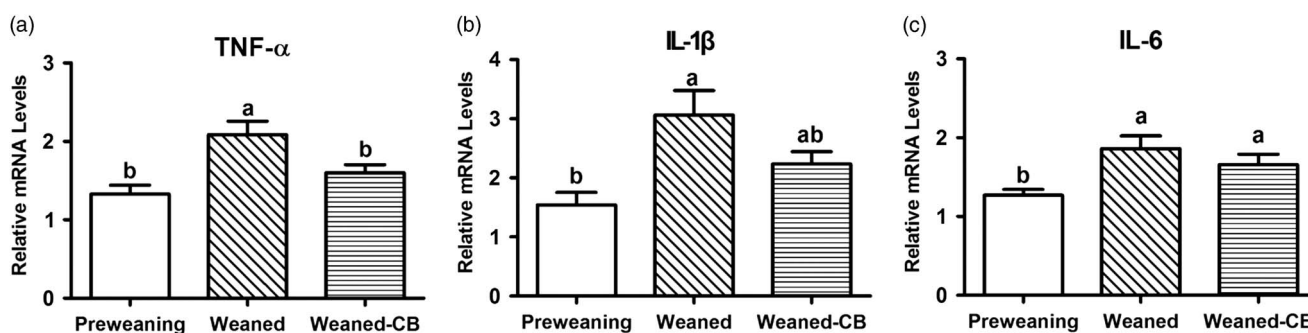

Figure 2 Messenger RNA (mRNA) levels of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interleukin 6 (IL-6) in jejunal mucosa of 21-day-old (preweaning) and 28-day-old (weaned) piglets. Piglets that were 21-day old (preweaning) and 28-day old were fed a basal diet (weaned) or basal diet supplemented with a carvacrol–thymol blend (weaned-CB), were sacrificed and then jejunal digest were collected to determine mRNA levels of TNF-α, IL-1β and IL-6. [a,b]Mean values with different superscript letters were significantly different ($P < 0.05$). All results are presented as mean ± SEM ($n = 6$).

weaning-induced intestinal oxidative stress and subsequent dysfunction of the intestinal barrier in piglets. The results show that early weaning stress induced intestinal oxidative stress, as indicated by increased levels of ROS and TBARS, as well as decreased activity of SOD and GSH-Px in the jejunum. A diet supplemented with 100 mg/kg carvacrol–thymol (1 : 1) blend reduced weaning-induced oxidative stress. Both carvacrol and thymol have free radical scavenging activity (Mastelic *et al.*, 2008; Archana *et al.*, 2009; Safaei-Ghomi *et al.*, 2009). In the current study, increased activity of antioxidants in the jejunum was observed in the weaned-CB group compared with the weaned group. Therefore, the

decreased ROS accumulation in the jejunal mucosa of piglets in the weaned-CB group was at least partly caused by an increased ability to scavenge ROS.

On the other hand, the reduced concentration of ROS in the jejunal mucosa of piglets in the weaned-CB group compared with the weaned group might also have been caused by a reduction in the amount of ROS-producing bacteria (*Enterococcus* genus and *E. coli*) and ROS-scavenging bacteria (*Lactobacillus* genus) in the jejunum. It has been demonstrated that intestinal *Enterococcus* and *E. coli* produce extracellular ROS (Huycke and Moore, 2002), whereas *Lactobacillus* has the ability to inhibit ROS
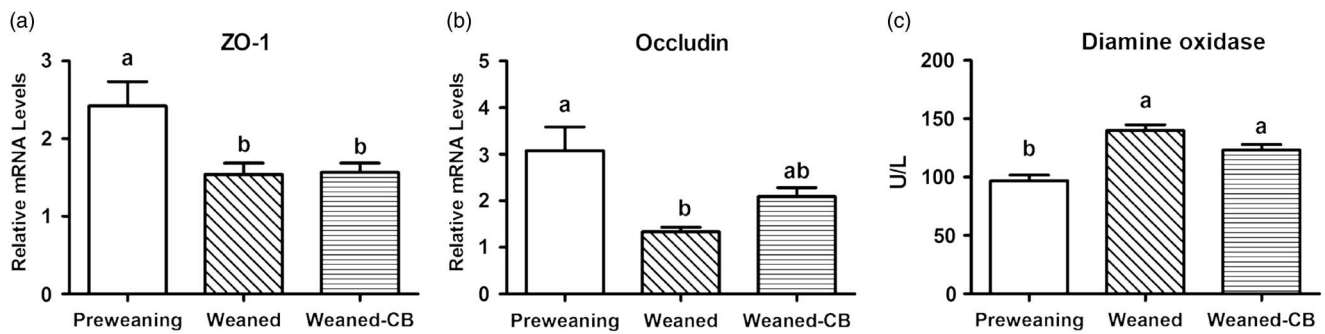
Figure 3 Changes in messenger RNA (mRNA) levels of occludin (a) and zonula occludens (ZO-1) (b) in jejunal mucosa and activity of diamine oxidase (c) in plasma of 21-day-old (preweaning) and 28-day-old (weaned) piglets. Piglets that were 21-day old (preweaning) and 28-day old were fed a basal diet (weaned) or basal diet supplemented with a carvacrol–thymol blend (weaned-CB) and were sacrificed to determine mRNA levels of occludin and ZO-1 in jejunal mucosa and the activity of diamine oxidase in plasma. [a,b]Mean values within a row with different superscript letters were significantly different ($P < 0.05$). All results are presented as mean ± SEM ($n = 6$).

production and the growth of *Enterococcus* and *E. coli* (Sun *et al.*, 2010). In this study, the counts of *Enterococcus* genus and *E. coli* were significantly elevated, and the counts of *Lactobacillus* genus decreased, following weaning. However, these weaning-induced influences on selected microbial populations were weakened in piglets that consumed a diet supplemented with 100 mg/kg carvacrol–thymol (1 : 1) blend. In fact, it has been demonstrated that dietary supplementation with 100 or 150 mg/kg essential oils containing 14.5% thymol and 3.5% cinnamaldehyde decreases populations of *E. coli* and increases the proportion of *Lactobacillus* in the ceca of weaning pigs (Li *et al.*, 2012). It seems like as low as 50 mg/kg carvacrol and thymol or even lower dose affect intestinal microbiota of weaning pigs. *In vitro* studies have showed that both carvacrol and thymol have antimicrobial activity. However, the minimum inhibitory concentrations (MIC) of carvacrol and thymol against *E. coli in vitro* are 250 mg/kg and 375 mg/kg. In addition, MIC of the combination of thymol and carvacrol against strains of *E. faecalis* is 400 mg/ml (Guarda *et al.*, 2011). For *Lactobacilli* genus, *in vitro* studies showed that carvacrol and thymol only had strong antimicrobial activity at 1000 mg/ml (Michiels *et al.*, 2009).

Disturbance of gut microbiota is commonly associated with an increased local inflammatory response and dysfunction of the intestinal barrier in weaning piglets (Peace *et al.*, 2011). Our study also found that weaning significantly increased the mRNA levels of proinflammatory cytokines, including TNF-α, IL-1β and IL-6, which is associated with increased biomarker of intestinal permeability (diamine oxidase in plasma) and decreased biomarkers of intestinal tight junctions (ZO-1 and occludin). However, feeding a carvacrol–thymol blend supplemented diet prevented only the weaning-induced TNF-α expression in the jejunum but did not influence the biomarkers of intestinal permeability and tight junctions. In weaning piglets, intestinal inflammation played an important role in disrupting intestinal tight junctions (Hu *et al.*, 2013). Our results indicate that although a dietary carvacrol–thymol blend decreased the counts of *Enterococcus* genus and

E. coli, and thereby bacterial infection involved in inflammation, it might have limited ability to control intestinal inflammation caused by several other potential mechanisms, including intolerance to particular allergens in feeds and corticotropin releasing factor-induced mast cell activation (Smith *et al.*, 2010).

The effect of supplementation of plant extracts and essential oil blends on performance of weaning pigs has been evaluated in several studies. Most of the studies suggested that supplementation of plant extracts and essential oil blends does not improve performance of weaning pigs, regardless of whether carvacrol or thymol is contained or not (Namkung *et al.*, 2004; Cho *et al.*, 2006; Kommera *et al.*, 2006; Manzanilla *et al.*, 2006; Nofrarias *et al.*, 2006). For example, the dietary supplementation with the plant extract containing 5% (w/w) carvacrol, 3% cinnamaldehyde and 2% capsicum oleoresin at dose of 300 mg/kg does not affect the performance of weaning pigs (Manzanilla *et al.*, 2006; Nofrarias *et al.*, 2006). In addition, feeding a diet supplemented with 1000 mg/kg essential oil blend containing 60% active substance (cymene, terpinene, carvacrol) also failed to improve the performance of weaning pigs (Huang *et al.*, 2010).

In contrast, feeding the diet supplemented with 100 mg/kg essential oil blend containing 18% thymol and cinnamaldehyde increases daily weight gain of weaning pigs compared with those fed with unsupplemented basal diet (Li *et al.*, 2012a and 2012b; Zeng *et al.*, 2015). Those increased performance may be caused by increased nutrient digestibility (Li *et al.*, 2012a; Zeng *et al.*, 2015). Moreover, all the animals in those studies are fed with experimental diet for 4 or 5 weeks, whereas only a 2-week feeding trial was performed in the current study. In fact, feeding the 100 mg/kg essential oil blend containing 18% thymol and cinnamaldehyde for 1 week does not affect the performance of weaning pigs (Li *et al.*, 2012b). In consistence with the effect of performance, the influence of nutrient digestibility is observed after 4 or 5 weeks of feeding the 100 mg/kg essential oil blend supplemented diet (Li *et al.*, 2012a; Zeng *et al.*, 2015).

A previous study showed that feeding a diet supplemented with 2000 or 3000 mg/kg essential oil blend, which contains

60 mg/kg carvacrol and 55 mg/kg thymol, for 3 weeks, increases feed intake and average daily gain of weaning pigs compared with those fed with unsupplemented basal diet (Molnar and Bilkei, 2005). However, supplementation of the essential oil blend at dose of 1000 mg/kg to provide 60 mg carvacrol and 55 mg thymol/kg feed, which is close to the concentration of carvacrol and thymol provided by diet in the current study, does not affect the performance of weaning pigs (Molnar and Bilkei, 2005). These results indicated that the concentration of carvacrol and thymol >100 mg/kg and the feeding period longer than 3 weeks might improve performance of weaning pigs effectively.

It has been demonstrated that feeding a diet supplemented with an antioxidant blend (200 mg vitamin C, 100 mg vitamin E, 450 mg tea polyphenols, 1 g lipoic acid and 5 g microbial antioxidants fermented by *Bacillus*, *Lactobacillus*, photosynthetic bacteria and beer yeast) reduces intestinal oxidative stress and prevents intestinal villus height and villus : crypt ratio in weaning piglets (Xu *et al*., 2014b). Among the ingredients in the antioxidant blend used in that study, both tea polyphenols and lipoic acid exert antiinflammatory effects via inhibition of the nuclear factor-κB (NF-κB) signaling pathway (Goraca *et al*., 2015; Li *et al*., 2015; Marinovic *et al*., 2015). Therefore, the antioxidant blend used in that study might also have reduced weaning-induced intestinal inflammation effectively. Considering the complex mechanism of intestinal barrier dysfunction in weaning piglets, a more complex blended dietary supplement containing antiinflammatory, antioxidative and antibacterial ingredients might enhance the intestinal health of weaning piglets more effectively.

In conclusion, the results of this study showed that weaning stress induced both intestinal oxidative stress and dysfunction of the intestinal barrier, indicated by influenced mRNA levels of tight junction proteins and activity of plasma D-lactate in piglets. Dietary supplementation with a carvacrol–thymol blend reduced weaning-induced intestinal oxidative stress, decreased the populations of *Enterococcus* genus and *E. coli*, and increased the population of *Lactobacillus* genus in the jejunum. However, a carvacrol–thymol blend had limited effects on weaning-induced intestinal inflammation and dysfunction of the intestinal barrier.

Acknowledgments

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201