Beneficial roles of dietary oleum cinnamomi in alleviating intestinal injury

Lei Wang1, Yongqing Hou1, Dan Yi1, Binyaing Ding1, Di Zhao1, Zhongxing Wang1, Huiling Zhu1, Yulan Liu1, Joshua Gong1,2, Houssein Assaad3, Guoyao Wu1,3

1Hubei Collaborative Innovation Center for Animal Nutrition and Feed Safety, Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China, 2Guelph Food Research Centre, Agriculture and Agri-Food Canada, Guelph, Ontario, Canada N1G 5C9, 3Department of Animal Science, Texas A&M University, College Station, TX, USA 77843

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1. ABSTRACT

Cinnamon is a traditional herb used for treatment of many human diseases. The most important chemical compounds of the essential oil are cinnamaldehyde and eugenol. Oleum cinnamomi (OCM, cinnamon oil) is increasingly used as a feed additive to animal diets. Beneficial effects of OCM in protecting tissues from inflammation and injury by endogenous and exogenous agents (such as hydrogen peroxide and lipopolysaccharide (LPS)) may result, in part, from its action on regulating amino acid metabolism in cells to favor the synthesis of glutathione (a major low-molecular-weight antioxidant) from cysteine, glycine and glutamate. In support of this notion, results of recent studies indicate that supplementing OCM (50 mg/kg diet) to a corn- and soybean meal-based diet for piglets weaned at 21 days of age enhances intestinal anti-oxidative capacity and reduces the incidence of diarrhea. Additionally, dietary supplementation with OCM ameliorates LPS-induced mucosal barrier dysfunction and mucosal damage in the small intestine. OCM holds great promise for protecting the gut from injury under conditions of inflammation, infections, and oxidative stress.

2. INTRODUCTION

The intestinal integrity plays an essential role in nutrition, metabolism, and whole-body homeostasis (1). The gastrointestinal tract is part of the first defense of the body against bacteria-derived endogenous and exogenous harmful agents (2, 3). Any damage to the mucosal epithelium can impair intestinal functions, including absorption of nutrients, thereby reducing growth performance and compromising the health of animal (4). Among nutrients, amino acids have received much attention because they are precursors for the synthesis of many molecules with enormous physiological functions (5). For example, glutamate, glutamine, and aspartate are major metabolic fuels for the small-intestinal mucosa (6), whereas cysteine, glycine and glutamate are required for the production of glutathione (a major low-molecular-weight antioxidant) in all cell types (7-11).

Many herbs and spices have antioxidant effects due to their active phenolic components (12). The most important chemical compounds of essential oils are cinnamaldehyde and eugenol. Essential oils are plant extracts containing volatile components. Some of them possess strong antimicrobial activities.
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and immunomodulatory functions (13). Thus, essential oils have been used widely as raw materials in many industries, including food, perfumes, cosmetics, aromatherapy, and phytotherapy (14, 15). In clinical medicine, cinnamon has been used as a traditional herb to treat human disorders, such as spasms, vomiting, infections and digestive or stomach complaints (12, 16-18) because this essential oil exerts anti-oxidative, anti-inflammatory, antispasmodic and anti-ulcerative effects (12). Cinnamon has also been reported to stimulate the immune system, thereby reducing the risk of cardiovascular disease and cancer (16, 19). Water-based extracts of cinnamon bark might bind endotoxin, thereby protecting against endotoxin-induced organ damage (16, 20), and have anti-bacterial effects, such as against Helicobacter pylori (16, 21). Cinnamon essential oil is made from bark and leaves of Cinnamomum verum obtained by steam distillation (22). Substantial evidence suggests that essential oils from both bark and leaves have antioxidant effects and can be used in preventing oxidation-induced decay of food products (23).

The underlying mechanisms for the beneficial effects of cinnamon are being explored using animals challenged with lipopolysaccharide (LPS), also known as endotoxin. LPS is a major component of the outer membrane of Gram-negative bacteria which are widely present in the digestive tracts of humans and animals. Much evidence shows that LPS can cause damage to intestinal mucosa, but effective methods for prevention and treatment are currently lacking (1, 24, 25). In the present article, we review results of recent studies regarding the beneficial effects of oleum cinnaamomi (cinnamaldehyde as the primary active component; Figure 1) on regulating intestinal gene expression (Table 1) and alleviating LPS-induced intestinal injury in animals, particularly early-weaned pigs.

3. THE PORCINE ANIMAL MODEL

Because of ethical concerns over human studies, we have used the pig as an animal model to study effects of nutrients (including amino acids and their derivatives) on intestinal health and function (26-28). Of note, early-weaned pigs exhibit naturally occurring atrophy and mucosal damage in the small intestine during the first week post-weaning (29). In addition, intraperitoneal administration of low doses of lipopolysaccharide (LPS) to weanling pigs causes further perturbations in the intestinal morphology, metabolism, and function (30). Thus, young pigs, particularly early-weaned piglets, are very sensitive to oxidative injury and therapeutic treatments. To minimize impacts of dietary confounding factors (e.g. high-quality animal-source protein and growth factors) and maximize beneficial effects of OCM on the intestine, our experiments normally involve corn- and soybean meal-based diets (1, 24, 25), which provide inadequate amino acids and induce intestinal abnormality and dysfunction in early-weaned pigs.

4. EFFECTS OF OCM ON GROWTH PERFORMANCE AND INTESTINAL ENZYMES IN LPS-CHALLENGED PIGLETS

4.1. General study protocols

Our experiments to determine effects of OCM on piglets were conducted using established protocols (1, 24, 25). At 28 days of age, piglets were assigned randomly into one of three treatment groups: 1) non-challenged control (Control group, piglets fed the basal diet and receiving intraperitoneal administration of sterile saline); 2) LPS-challenged control (LPS group, piglets...
fed the basal diet and receiving intraperitoneal administration of *Escherichia coli* LPS; 3) LPS + 50 mg/kg OCM (LPS+OCM group, piglets fed the basal diet supplemented with 50 mg/kg OCM and receiving intraperitoneal administration of LPS). LPS was dissolved in sterile saline. Oleum cinnamomi (OCM; Sigma-Aldrich, catalog number W225800) was well mixed with the basal diet. The dietary treatments lasted for 4 weeks. On days 21 and 28 of the trial, overnight fasted piglets in the LPS and LPS+OCM groups received intraperitoneal administration of LPS (*Escherichia coli* serotype 055:B5; Sigma Chemical Inc., St. Louis, MO, USA) at the dose of 100 μg/kg BW as a model of gut inflammation (31) and acute oxidative stress (32), whereas piglets in the control group received intraperitoneal administration of the same volume of sterile saline. During days 0-20 of the trial (pre-challenge), all the piglets had free access to feed and drinking water. Incidents of piglets with diarrhea were recorded every day during days 0-20 of the trial (pre-challenge). Piglets with semi-liquid and liquid feces were regarded as diarrhea.

To exclude a possible effect of LPS-induced reduction in food intake, the control and LPS+OCM piglets were pair-fed, during days 21-28 of the trial (post-challenge with LPS), the same amount of feed per kg body weight as LPS piglets. On day 28 of the trial, D-xylose was orally administrated to piglets at the dose of 0.1 g/kg BW (infused with 10% D-xylose at 1 mL/kg BW) 2 h after LPS or saline administration. Absorption of D-xylose into plasma has been used as a marker of *in vivo* intestinal integrity in pigs (33). On days 21 and 28 of the trial, 3 h after LPS or saline administration, blood samples were collected from anterior vena cava into heparinized vacuum tubes (Becton Dickinson Vacutainer System, Franklin Lake, NJ, USA) as described previously (25). Blood samples (7 mL) were centrifuged at 1,000 × g for 10 min at 4°C to separate plasma, and the plasma was then stored at -80°C until analysis (1).

Six hours post-injection of LPS on day 28 of the growth trial, all piglets were sacrificed under anesthesia with an intravenous injection of

### Table 1. Primers for RT-PCR analysis of EGF, claudin-1, occludin, HSP70, ALP and PXR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>Forward: 5'-TgCCATAAgggTgTCaggTATTT -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TgCTTTgCTCTTgCCCTCCTAC -3'</td>
</tr>
<tr>
<td>Claudin-1</td>
<td>Forward: 5'-ggTgCCCTACTTTgTgCTC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCCACACggTTTTgTCCTTTT-3'</td>
</tr>
<tr>
<td>Occludin</td>
<td>Forward: 5'-TATgAgACAgACTACAACgGgGgTgTCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-ATCATAgTCTCCACCACCTCTTgATgTg-3'</td>
</tr>
<tr>
<td>HSP70</td>
<td>Forward: 5'-gACggACgCAgAaggAAgA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-gAAgACAggTgTTTgTg-3'</td>
</tr>
<tr>
<td>ALP</td>
<td>Forward: 5'-CCACTCCCACgTCTTTACCTTT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CTCTCACACCCCACACACCTT-3'</td>
</tr>
<tr>
<td>PXR</td>
<td>Forward: 5'-TgAagAgCAgGgAGAAgA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TgAgGgCAgCACCACA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-CcTCCCTgAGACAgATggTgT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCgAtgCggCCAATATTT-3'</td>
</tr>
<tr>
<td>RPL4</td>
<td>Forward: 5'-gAgAAACgCgTgCgCgAAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-gCCCACACAggAgCAtgT-3'</td>
</tr>
</tbody>
</table>

The oligonucleotide primers were designed from pig gene sequences in the GenBank NM_214020 (for EGF), NM_001244539 (for claudin-1), U79554 (for occludin), NM_001123127 (for HSP70), AY145131 (for ALP), NM_001038605 (for PXR), AF017079 (for GAPDH), and DQ845176 (for RPL4). To avoid amplification of potentially contaminating genomic DNA, the primers were designed to span introns and intron-exon boundaries.
pentobarbital sodium (50 mg/kg BW) for biochemical analyses of the intestinal mucosa (1). Briefly, the piglet abdomen was opened immediately after anesthesia from sternum to pubis, and the whole gastrointestinal tract was immediately removed (1, 25, 34). The small intestine and colon were dissected free of the mesentery and placed on a chilled stainless steel tray. The 10-cm segments were cut at distal duodenum, mid-jejunum, mid-ileum, and mid-colon, respectively (34, 35). The intestinal segments were opened longitudinally and flushed with ice-cold PBS after removal of the contents. Mucosa was collected by scraping using a sterile glass microscope slide, rapidly frozen in liquid nitrogen, and stored at -80°C until analysis. All samples were collected within 15 min after euthanasia.

All assays were performed using the published methods (27, 30, 36-38). Specifically, plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were assessed by an automatic analyzer (7020 Clinical Analyzer, Hitachi High-Technologies Co., Tokyo, Japan). Cortisol and glucagon in plasma were analyzed using commercially available \textsuperscript{125}I RIA kits (Beijing North Institute of Biological Technology, Beijing, China). The detection limits were 2 ng/mL for cortisol and 16.1 pg/mL for glucagon. The coefficients of variation for intra-and inter-assays of cortisol were < 10% and < 15%, respectively. The coefficients of variation for intra-and inter-assays of glucagon were < 10% and < 15%, respectively.

D-xylose in plasma was determined using our established methods (24). Briefly, 50 μL of the collected plasma was added to 5 mL of the phloroglucinol color reagent solution (Sigma Chemical Inc., St. Louis, MO, USA) and heated at 100°C for 4 min. The samples were allowed to cool to room temperature in a water bath. A xylose standard solution was prepared by dissolving D-xylose in saturated benzoic acid (prepared in deionized water) to obtain 0, 0.7, 1.3, and 2.6 mmol/L. The D-xylose standard solution or the sample was added to the colour reagent solution. Absorbance of the resultant mixture at 554 nm was measured using a spectrophotometer (Model 6100, Jenway LTD., Felsted, Dunmow, CM6 3LB, Essex, England, UK). The standard solution of 0 mmol/L D-xylose was considered as the blank.

The intestinal mucosae were used for the analysis of anti-oxidative enzymes and their products. Glutathione peroxidase (GSH-Px) and hydrogen peroxide (\(H_2O_2\)) were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Assays were performed in triplicate (1).

4.2. OCM reduced stress and improved gut function in LPS-challenged piglets

For centuries, cinnamon has been widely used as spice and as an additive in perfumery, flavoring, and pharmaceutical industries (12, 17, 18). Essential oil of cinnamon bark was found to be a unique aromatic monoterpene-rich natural source, with trans-cinnamaldehyde (45.62%) as the major constituents (39). There is evidence that OCM has protective effects on ethanol-induced oxidative and mucosal damage (12). Therefore, we investigated the protective effect of 50 mg/kg OCM on the intestinal mucosal-barrier function after a 6-h LPS challenge in a porcine model. Currently, LPS is widely used to cause intestinal-mucosal damage in many animal and clinical studies, and LPS has been reported to induce stress responses in a porcine model (1, 24, 25). Between days 0 and 21 of the trial (pre-LPS challenge period), average feed intake and daily weight gains of piglets were increased by the OCM supplementation, as compared with the control group. Importantly, dietary OCM supplementation reduced the diarrhea incidence by 58% (Table 2).

Aminotransferase (ALT) and aspartate aminotransferase (AST) are hepatic intracellular enzymes. Their elevations in the plasma reflect the injury of cells, particularly hepatocytes (40-43). Results of our studies showed that LPS increased the activities of ALT and AST in the plasma, indicating that LPS induced liver damage (Table 3). In this regard, it is noteworthy that OCM supplementation significantly suppressed the increases in plasma ALT and AST, indicating that OCM alleviated LPS-induced injury in the liver (Table 3).

Cortisol is a steroid hormone, which is released in response to stress (31). Its primary functions are to increase blood sugar through gluconeogenesis, suppress the immune system, and stimulate the metabolism of fat, protein, and carbohydrate. Substantial evidence suggests that glucagon plays an essential role in blood glucose homeostasis (44, 45). Accordingly, glucagon concentrations in the portal circulation rise in response to many kinds of stress (46). We found that dietary supplementation with OCM prevented the LPS-induced increases in plasma cortisol and glucagon (Table 4). Thus, OCM exerted a protective
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Table 2. Effects of dietary OCM supplementation on growth performance and diarrhea incidence in piglets (pre-challenge)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>OCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight at day 0 of the trial (kg)</td>
<td>7.3±0.26</td>
<td>7.2±0.35</td>
</tr>
<tr>
<td>Average daily feed intake between days 0 and 21 of the trial (g/day)</td>
<td>668±2.9a</td>
<td>707±1.7a</td>
</tr>
<tr>
<td>Average daily weight gain between days 0 and 21 of the trial (g/day)</td>
<td>392±2.3b</td>
<td>413±2.2a</td>
</tr>
<tr>
<td>Prevalence of diarrhea between days 0 and 21 of the trial (%)</td>
<td>20.5±2.1a</td>
<td>8.7±1.3b</td>
</tr>
</tbody>
</table>

The animal use protocol for our research was approved by the Animal Care and Use Committee of Hubei Province. Eighteen crossbred healthy female piglets (Duroc×Landrace×Yorkshire) were weaned at 21 days of age to a corn- and soybean meal-based diet (without antibiotics and zinc oxide), which was formulated to meet National Research Council (2012)-recommended requirements for all nutrients. After a 7-day period of adaptation, piglets (28 days of age, average body weight of 7.29±0.61 kg) were housed individually in stainless steel metabolic cages (1.20×1.10 m²) and maintained in an environmentally controlled room. Each cage was equipped with a feeder and a nipple waterer to allow piglets free access to food and drinking water. Data, expressed as means±SD, n=6, were analyzed by one-way analysis of variance. The normality and constant variance for experimental data were tested by the Levene’s test (36). If data did not have homogenous variance, they underwent logarithm transformation to meet the necessary assumptions for analysis of variance (38). Differences among treatment means were determined by the Duncan’s multiple range tests. The prevalence of diarrhea was analyzed using the X² analysis. Data are means±SD, n=6. See the footnotes of Table 2 for the experimental design. Control (non-challenged control)=piglets fed the basal diet; LPS (LPS challenged control)=piglets fed the basal diet and challenged with Escherichia coli LPS; LPS+OCM (LPS+50 mg/kg OCM)=piglets fed the basal diet supplemented with 50 mg/kg OCM and challenged with LPS. a, bValues within a row with different letters differ (P<0.05).

Table 3. Effects of OCM on concentrations of blood ALT and AST of piglets after LPS challenge (Day 21 of the trial)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>LPS</th>
<th>LPS+OCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/L)</td>
<td>52.8±5.4b</td>
<td>66.8±5.9a</td>
<td>59.1±5.1b</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>50.4±6.4b</td>
<td>66.9±5.0a</td>
<td>47.1±6.4b</td>
</tr>
</tbody>
</table>

Data are means±SD, n=6. See the footnotes of Table 2 for the experimental design. Control (non-challenged control)=piglets fed the basal diet and injected with saline; LPS (LPS challenged control)=piglets fed the basal diet and challenged with Escherichia coli LPS; LPS+OCM (LPS+50 mg/kg OCM)=piglets fed the basal diet supplemented with 50 mg/kg OCM and challenged with LPS. a, bValues within a row with different letters differ (P<0.05). ALT=alanine aminotransferase; AST=aspartate aminotransferase

Table 4. Effects of OCM on concentrations of cortisol and glucagon in plasma

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>LPS</th>
<th>LPS+OCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol, ng/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (Day 21)</td>
<td>41.8±13.67c</td>
<td>280.3±26.68a</td>
<td>179.5±56.82b</td>
</tr>
<tr>
<td>Plasma (Day 28)</td>
<td>62.3±14.53c</td>
<td>266.2±43.86a</td>
<td>184.9±23.35b</td>
</tr>
<tr>
<td>Glucagon, pg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma (Day 21)</td>
<td>116.7±22.08b</td>
<td>269.8±81.17a</td>
<td>180.4±54.56b</td>
</tr>
<tr>
<td>Plasma (Day 28)</td>
<td>127.6±22.83b</td>
<td>213.0±77.94a</td>
<td>135.6±14.18b</td>
</tr>
</tbody>
</table>

Data are means±SD, n=6. See the footnotes of Table 2 for the experimental design. Control (non-challenged control)=piglets fed the basal diet and injected with saline; LPS (LPS challenged control)=piglets fed the basal diet and challenged with Escherichia coli LPS; LPS+OCM (LPS+50 mg/kg OCM)=piglets fed the basal diet supplemented with 50 mg/kg OCM and challenged with LPS. a, bValues within a row with different letters differ (P<0.05).
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Oxidative stress plays an important role in the pathogenesis of LPS-induced intestinal injury and lipid peroxidation via generating reactive oxygen species (ROS) (1). In the current study, LPS challenge reduced glutathione peroxidase (GSH-Px) and increased hydrogen peroxide (H$_2$O$_2$) in the jejunal mucosa (Table 5), indicating that LPS challenge causes intestinal oxidative stress. Under conditions of oxidative stress, lipid peroxidation injures cell membranes with the release of intracellular components, such as lysosomal enzymes, leading to further tissue damage (12). An important finding from the present study is that the adverse effects of LPS-induced intestinal oxidative stress were attenuated by dietary OCM supplementation (Table 5). Consistent with our observations, Ozbayer et al. (12) reported previously that OCM had an antioxidative activity and, thus, decreased lipid peroxidation levels in rats with ulcerative damage.

The 1-h blood D-xylose test is a useful indicator for intestinal absorption capacity and mucosal integrity in young swine (1, 24, 25). In healthy piglets, D-xylose is readily absorbed by the small intestine. However, when malabsorption occurs, D-xylose is not absorbed by the gut, leading to reductions in its concentrations in plasma and urine (33). Notably, we found that dietary supplementation with 50 mg/kg OCM augmented the entry of orally administered D-xylose into the systemic circulation in LPS-challenged piglets (Table 5), which indicates that OCM can enhance the ability of the small intestine to absorb nutrients and barrier function under inflammatory conditions.

### Table 5. Effects of OCM on plasma D-xylose and redox status in the intestinal mucosa

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>LPS</th>
<th>LPS+OCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma D-xylose (µg/mL)</td>
<td>1.5±0.15</td>
<td>1.0±0.14</td>
<td>1.2±0.06</td>
</tr>
<tr>
<td>GSH-Px (U/g protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jejunal mucosa</td>
<td>24.1±2.45</td>
<td>18.0±2.98</td>
<td>21.8±2.31</td>
</tr>
<tr>
<td>Ileal mucosa</td>
<td>50.8±6.68</td>
<td>52.4±4.91</td>
<td>53.9±5.35</td>
</tr>
<tr>
<td>H$_2$O$_2$ (µmol/g protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jejunal mucosa</td>
<td>0.73±0.03</td>
<td>0.87±0.04</td>
<td>0.78±0.06</td>
</tr>
<tr>
<td>Ileal mucosa</td>
<td>0.67±0.08</td>
<td>0.69±0.012</td>
<td>0.70±0.08</td>
</tr>
</tbody>
</table>

Data are means±SD, n=6. See the footnotes of Table 2 for the experimental design. Control (non-challenged control)=piglets fed the basal diet and injected with saline; LPS (LPS challenged control)=piglets fed the basal diet and challenged with Escherichia coli LPS; LPS+OCM (LPS+50 mg/kg OCM)=piglets fed the basal diet supplemented with 50 mg/kg OCM and challenged with LPS. a,bValues within a row with different letters differ (P<0.05). GSH-Px=glutathione peroxidase; H$_2$O$_2$=hydrogen peroxide

### 5. EFFECTS OF OCM ON EXPRESSION OF INTESTINAL GENES IN LPS-CHALLENGED PIGLETS

#### 5.1. General study protocols

Epidermal growth factor (EGF), claudin-1, occludin, heat shock protein 70 (HSP70), alkaline phosphatase (ALP) and pregnane X receptor (PXR) mRNA levels in mucosa were quantified using RT-PCR (1, 30, 34). Approximately 100 mg powdered samples were homogenized and total RNA was isolated using the TRIzol Reagent protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was quantified using the NanoDrop® ND-2000 UV-VIS spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at an OD of 260 nm, and the purity was assessed by determining the OD260/OD280 ratio. All the samples had an OD260/OD280 ratio above 1.8, corresponding to > 95% pure nucleic acids. Meanwhile, the integrity of RNA in each sample was assessed using 1% denatured agarose gel electrophoresis. RNA was used for RT-PCR analysis when it had a 28 S/18 S rRNA ratio ≥ 1.8.

Total RNA was reverse transcribed using the PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer’s instruction. cDNA was synthesized and stored at -20°C until use. The RT-PCR analysis of gene expression was performed using primers for EGF, claudin-1, occludin, HSP70, ALP and PXR, GAPDH, and ribosomal protein L4 (RPL4) (Table 1) and the SYBR® Premix Ex Taq™ (Takara, Dalian, China) on the Applied Biosystems 7500 Real-Time PCR System (Foster City, CA, USA). The total volume of the PCR reaction system was 50 µL. In brief, the reaction mixture contained 0.2 µM of each primer, 25 µL of SYBR® Premix Ex Taq™ (2×) and 4 µL of cDNA in a 50-µL reaction volume. All PCRs were performed in triplicate on a 96-well RT-PCR plate (Applied Biosystems) under the following conditions (two-step amplification): 95°C for 30 sec, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec. A subsequent melting curve (95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec) with continuous fluorescence measurement and final cooling to room
temperature was processed. Amplification products were verified by melting curves and agarose gel electrophoresis. Results were analyzed by the delta delta \( \Delta \Delta CT \) method (36). The standard curves were generated using relative concentration vs. the \( C_T \). The linear correlation coefficient of all genes were \( >0.995 \). Based on the slopes of the standard curves, the amplification efficiencies of the standard ranged from 90\% to 110\% (derived from the formula efficiency = \( 10^{\text{1/slope}-1} \) (37)). Moreover, we used GAPDH and RPL4 as the normalizer in the calculation of relative mRNA levels for target genes. Each biological sample was run in triplicate.

5.2. OCM affected intestinal expression of genes

LPS challenge altered the expression of genes for EGF, claudin-1, occludin, HSP70, ALP and PXR in the intestinal mucosa of LPS-challenged piglets (Table 6). The effects of LPS depended on gene and intestinal segment. The adverse effects of LPS on intestinal gene expression were attenuated by dietary supplementation with OCM. The LPS-induced up-regulation of genes was reversed by OCM supplementation to the values similar to those for the control group in duodenal and jejunal mucosae.

The 70 kDa HSP-70 belongs to a family of ubiquitously expressed heat shock proteins (47). Usually, a high concentration of HSP70 is an indicative of oxidative stress (1, 25, 48). Like other intestinal mucosal proteins (49-53), HSP70 is sensitive to regulation by dietary and physiological factors. We found that the changes in the expression of HSP70 in response to LPS and OCM were the most significant (Table 6). Therefore, dietary supplementation with OCM substantially decreases oxidative stress in the intestinal mucosa of piglets. In contrast to HSP70, mucosal mRNA levels for EGF, ALP, claudin-1, occludin, and PXR were lower (\( P<0.05 \)) in LPS-treated piglets than in the control group. EGF (one of the most important growth factor) is widely distributed on the surface of the intestinal mucosa and is crucial for stimulating enterocyte proliferation and regeneration of the mucosal epithelium (54-56). Previous studies have demonstrated that EGF can promote cell proliferation, repair, and migration during regeneration of the gut after damage (57-59). Our previous research suggested that LPS could decrease EGF concentrations in the plasma of piglets (1). Here, we observed that LPS reduced intestinal mRNA levels for EGF (Table 6). It appears, therefore, that the regulation of EGF expression is one of the major functions whereby OCM alleviates intestinal injury.

Alkaline phosphatase (ALP) is expressed by differentiated enterocytes, but not by undifferentiated epithelial cells or any other cell type. Thus, ALP serves as an enterocyte differentiation marker and is a useful tool for the identification of factors that govern the overall enterocyte differentiation (59). ALP expression is generally repressed under atrophic conditions of starvation and hypothyroidism (50-52). This repression of ALP expression has also been demonstrated in \textit{in vitro} cell models of serum starvation and post-confluence (53). Similarly, we found that ALP gene expression in the intestinal mucosa of piglets was repressed by LPS treatment (Table 6). Implications of this relationship between intestinal injury and ALP expression have not been elucidated. Interestingly, the present study indicates that supplementation with OCM can increase intestinal ALP gene expression in LPS-challenged piglets. Further study is needed to identify the underlying mechanisms.

It is possible that OCM protects intestinal absorption function by regulating expression of key genes in the tight-junction cell signaling. Intestinal epithelial integrity is maintained by cohesive interactions between cells via the formation of tight junctions (24, 60). There is growing evidence that members of the claudin family play a critical role in tight junction formation and determine permeability characteristics in a variety of tissues, including the gut (24, 61, 62). Claudin-1 and occludin integrate diverse processes of gene transcription, tumor suppression, and cell proliferation (63, 64). The results from our previous reports and the present study clearly indicate that the LPS treatment can reduce the abundance of claudin-1 and occludin in the intestinal mucosa of piglets at the levels of both protein (24) and mRNA expression (Table 6). In our studies, dietary supplementation with OCM was able to attenuate the effect of LPS by improving the expression of the claudin-1 and occludin genes (Table 6). These data further support that OCM has beneficial effects on alleviating intestinal injury in LPS-challenged pigs.

Another novel and important finding from our work is the amelioration of PXR gene expression in the intestinal mucosa by dietary treatment of OCM. PXR is a member of the nuclear receptor family of ligand-activated transcription factors, and is highly expressed in the liver and intestine (40).
Dietary oleum cinnamomi in alleviating intestinal injury

As a master gene overseeing detoxification of a large number of xenobiotics, PXR plays a vital role in maintaining intestinal integrity. However, the intestinal expression of genes involved in cellular detoxification is down-regulated in patients with inflammatory bowel diseases (IBD) (65). Previous studies have also demonstrated that LPS reduces the expression of PXR mRNA in the mouse liver (66), placenta (67), fetal liver (68), and intestine (40). Consistent with these reports, our results indicated that LPS significantly inhibited the expression of the PXR gene in the intestinal mucosa of piglets (Table 6). Of note, dietary supplementation with 50 mg/kg OCM prevented the LPS-induced decrease in PXR gene expression in intestinal mucosa of piglets. Similarly, Xu et al. (40) found that LPS-induced decreases in intestinal PXR expression was associated with oxidative stress and that oral infusion with either N-acetylcysteine or ascorbic acid attenuated this adverse effect of LPS in mice. In view of these results, it appears that the antioxidant effects of OCM are associated with the regulation of PXR gene expression. Consequently, dietary supplementation with 50 mg/kg OCM markedly reduced the incidence of diarrhoea in postweaning piglets (Table 2). This significant outcome provides further evidence that the protective effects of OCM are associated with improving intestinal function (including absorption) and health. OCM supplementation offers a novel strategy for ameliorating gut damage under inflammatory conditions.

6. CONCLUSION AND PERSPECTIVE

The small intestine is responsible for the terminal digestion and absorption of nutrients in humans and animals. The gut is also a physical barrier to separate the internal milieu of the organism from the external environment, thereby playing an essential role in excluding food-borne pathogens from entering the body and preventing translocation of luminal microorganisms into the circulation. The intestinal mucosa, which accounts for 20% to 25% of the empty small-intestinal weight (Table 7), expresses a wide array of anti-oxidative, anti-inflammatory, and regulatory proteins to maintain its normal structure and function. Dietary supplementation with 50 mg/kg OCM markedly reduced the incidence of diarrhoea in postweaning pigs. OCM also exerted an anti-oxidative activity, thereby alleviating intestinal injury and improving intestinal absorption in LPS-challenged piglets. The underlying mechanisms

Table 6. Effects of OCM on EGF, claudin-1, occludin, HSP70, ALP and PXR mRNA levels in intestinal mucosa

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>LPS</th>
<th>LPS+OCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.00±0.188&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70±0.139&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.21±0.245&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jejunum</td>
<td>1.00±0.213&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62±0.100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.86±0.173&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum</td>
<td>1.00±0.191</td>
<td>1.32±0.286</td>
<td>1.46±0.560</td>
</tr>
<tr>
<td>Colon</td>
<td>1.00±0.189&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74±0.139&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.21±0.245&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Claudin-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.00±0.142&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64±0.099&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.08±0.197&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jejunum</td>
<td>1.00±0.093&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74±0.094&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.15±0.265&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum</td>
<td>1.00±0.147&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.69±0.160&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.63±0.299&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon</td>
<td>1.00±0.353&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48±0.092&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.02±0.330&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Occludin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.00±0.256</td>
<td>1.05±0.152</td>
<td>1.13±0.202</td>
</tr>
<tr>
<td>Jejunum</td>
<td>1.00±0.294&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41±0.038&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.55±0.052&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum</td>
<td>1.00±0.039&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78±0.133&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.31±0.140&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon</td>
<td>1.00±0.123&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93±0.115&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.76±0.156&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>HSP70</td>
<td></td>
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</tr>
<tr>
<td>Duodenum</td>
<td>1.00±0.464&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.53±0.709&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.35±0.423&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jejunum</td>
<td>1.00±0.320&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.18±1.236&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.14±0.441&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum</td>
<td>1.00±0.265&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.69±0.628&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.56±0.298&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon</td>
<td>1.00±0.245&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.60±0.104&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.35±0.410&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.00±0.238&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.01±0.305&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.59±0.619&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jejunum</td>
<td>1.00±0.184&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73±0.073&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03±0.105&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum</td>
<td>1.00±0.158&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.66±0.143&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.97±0.187&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon</td>
<td>1.00±0.108&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.04±0.321&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.83±0.178&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PXR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>1.00±0.263&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.91±0.334&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.75±0.341&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jejunum</td>
<td>1.00±0.081&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48±0.154&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62±0.056&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ileum</td>
<td>1.00±0.219&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62±0.173&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82±0.237&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon</td>
<td>1.00±0.178&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.79±0.143&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.58±0.576&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are means±SD, n=6. See the footnotes of Table 2 for the experimental design. Control (non-challenged control)= piglets fed the basal diet and injected with saline; LPS (LPS challenged control) = piglets fed the basal diet and challenged with Escherichia coli LPS; LPS+OCM (LPS+50 mg/kg OCM) = piglets fed the basal diet supplemented with 50 mg/kg OCM and challenged with LPS. <sup>a</sup> Values within a row with different letters differ (P<0.05). EGF=epidermal growth factor; HSP70=heat shock protein 70; ALP=alkaline phosphatase; PXR=pregnane X receptor
Table 7. Weights of the empty small intestine and its mucosa in growing pigs

<table>
<thead>
<tr>
<th>Body weight (kg)</th>
<th>Weight of the small intestine¹ (% of body weight)</th>
<th>Weight of the small-intestinal mucosa² (% of body weight)</th>
<th>Ratio of the mucosal weight to the small-intestinal weight (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.36±0.34</td>
<td>0.789±0.060ᵃ</td>
<td>0.236±0.017ᵃ</td>
</tr>
<tr>
<td>50</td>
<td>3.20±0.32</td>
<td>0.693±0.044ᵃᵇ</td>
<td>0.217±0.015ᵃᵇ</td>
</tr>
<tr>
<td>100</td>
<td>3.08±0.42</td>
<td>0.619±0.086ᵇ</td>
<td>0.201±0.020ᵇ</td>
</tr>
</tbody>
</table>

Values are means±SD, n=6. Pigs were fed typical corn- and soybean meal-based diets (29, 69, 70) and were slaughtered at 6 h after the last feeding to obtain the small intestine. The small intestine is defined as that portion of the digestive tract between the pylorus and the ileocecal valve, and consists of the duodenum, jejunum, and ileum. The small-intestinal mucosa consists of the epithelium, the lamina propria, and the muscularis mucosa (71). ¹Including the duodenum, jejunum, and ileum without intestinal content. ²Including the mucosa of the duodenum, jejunum, and ileum.ᵃᵇ: Within a column, means with different superscript letters differ (P<0.05), as analyzed by one-way ANOVA and the Duncan multiple comparison test (72).

are proposed in Figure 2. The beneficial effects of OCM are associated with its following actions: 1) reduced oxidative stress (indicated by increased activities of GSH-Px, decreased concentrations of H₂O₂, decreased gene expression of HSP70, and increased PXR mRNA in the intestinal mucosa); 2) improved mucosal repair via regulating EGF and ALP gene expression (indicated by increases
in EGF and ALP gene expression in the intestinal mucosa); and 3) increased tight junctions (indicated by increased gene expression of claudin-1 and occludin in the intestinal mucosa). OCM holds great promise for protecting the gut from injury under conditions of inflammation, infections, and oxidative stress.

7. ACKNOWLEDGMENTS

This research was jointly supported by National Basic Research Program of China (No. 2012CB126305), National Natural Science Foundation of China (No. 31372319), Hubei Provincial Research and Development Program (No. 2010BB023), Natural Science Foundation of Hubei Province (No. 2013CFA097, 2013CBF325, 2012FFB04805, 2011CDA131), Scientific Research Program of Hubei Provincial Department of Education (D20141701), the Hubei Hundred Talent Program, Agriculture and Food Research Initiative Competitive Grants of (2014-67015-21770) of the USDA National Institute of Food and Agriculture, and Texas AgriLife Research (H-82000). Dr. Houssein Assaad was supported by a postdoctoral training grant (R25T-CA090301) from the National Cancer Institute (U.S.). All these funding agencies had no role in the design, analysis or writing of our published studies. The authors declare that they have no conflict of interest.

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DOI: 10.1007/s00726-012-1255-5

DOI: 10.1007/s00726-008-0155-1


DOI: 10.1186/2193-1801-3-474

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**Abbreviations:** ALT, alanine aminotransferase; ALP, Alkaline phosphatase; AST, aspartate aminotransferase; BW, body weight; HSP 70, Heat shock protein 70; EGF, epidermal growth factor; GSH-Px, glutathione peroxidase; \( \text{H}_2\text{O}_2 \), hydrogen peroxide; LPS, lipopolysaccharide; OCM, oleum cinnamomi; PBS, phosphate buffered saline; PXR, Pregnane X receptor; RT-PCR, Real-time polymerase-chain reaction; SD, standard deviation of the mean

**Key Words:** Oleum cinnamomi, Intestinal mucosa, Piglets, Lipopolysaccharide, Review

**Send correspondence to:** Yongqing Hou, Hubei key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China, Tel: 862783956175, Fax: 862783956175, E-mail: houyq@aliyun.com