Effects of a microencapsulated formula of organic acids and essential oils on nutrient absorption, immunity, gut barrier function, and abundance of enterotoxigenic *Escherichia coli* F4 (ETEC F4) in weaned piglets challenged with ETEC F4

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Abstract

The objective was to study the effects of microencapsulated organic acids (OA) and essential oils (EO) on growth performance, immune system, gut barrier function, nutrient digestion and absorption, and abundance of enterotoxigenic *Escherichia coli* F4 (ETEC F4) in the weaned piglets challenged with ETEC F4. Twenty-four ETEC F4 susceptible weaned piglets were randomly distributed to four treatments including (1) sham-challenged control (SSC; piglets fed a control diet and challenged with phosphate-buffered saline (PBS)); (2) challenged control (CC; piglets fed a control diet and challenged with ETEC F4); (3) antibiotic growth promoters (AGP; CC + 55 mg·kg^{-1} of Aureomycin); and (4) microencapsulated OA and EO [P(OA+EO); (CC + 2 g·kg^{-1} of microencapsulated OA and EO]. The ETEC F4 infection significantly induced diarrhea at 8, 28, 34, and 40 hours post-inoculation (hpi) ($P < 0.05$) in the CC piglets. At 28 days post-inoculation (dpi), piglets fed P(OA+EO) had a lower ($P < 0.05$) diarrhea score compared to those fed CC, but the P(OA+EO) piglets had a lower ($P < 0.05$) diarrhea score compared to those fed the AGP diets at 40 dpi. The ETEC F4 infection tended to increase *in vivo* gut permeability measured by the oral gavaging fluorescein isothiocyanate-dextran 70 kDa (FITC-D70) assay in the CC piglets compared to the SCC piglets ($P = 0.09$). The AGP piglets had higher FITC-D70 flux than P(OA+EO) piglets ($P < 0.05$). The ETEC F4 infection decreased mid-jejunal VH in the CC piglets compared to the SCC piglets ($P < 0.05$). The P(OA+EO) piglets had higher ($P < 0.05$) VH in the mid-jejunum than the CC piglets. The relative mRNA abundance of SGLT1 and B^{0}AT1 was reduced ($P < 0.05$) by ETEC F4 inoculation when compared to the SCC piglets. The AGP piglets had a greater relative mRNA abundance of B^{0}AT1 than the CC piglets ($P < 0.05$). The ETEC F4 inoculation increased the protein abundance of OCLN ($P < 0.05$), and the AGP piglets had the lowest relative protein abundance of OCLN among the challenged groups ($P < 0.05$). The supplementation of microencapsulated OA and EO enhanced

intestinal morphology and showed anti-diarrhea effects at a one-time point in weaned piglets challenged with ETEC F4. Even if more future studies can be required for further validation, this study brings evidence that microencapsulated OA and EO combination can be useful within the tools to be implemented in strategies for alternatives to antibiotics in swine production.

Keywords: *Escherichia coli* F4, essential oils, gut health, microencapsulation, organic acids, weaned piglets

List of Abbreviation

AB/PAS	Alcian blue/The periodic acid–Schiff
ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Antibiotic growth promoters
APN	Aminopeptidase N
ASCT2	Neutral amino acid transporter 2
B^0AT1	Neutral amino acid transporter 1
BW	Body weight
CC	Challenged control
CD	Crypt depth
CLDN1	Claudin 1
CLDN3	Claudin 3
Ct	Threshold cycle
CycA	Cyclophilin-A
ddPCR	Droplet digital polymerase chain reaction
DEPC	Diethylpyrocarbonate
dpi	Days post-inoculation
EAAC1	Excitatory amino-acid carrier 1
EO	Essential oils
ETEC	Enterotoxigenic *Escherichia coli*
FCR	Feed conversion ratio
FITC-D4	Fluorescein isothiocyanate-dextran 4 kDa
FITC-D70	Fluorescein isothiocyanate-dextran 70 kDa

GSH	Glutathione
GSSG	Oxidized glutathione
hpi	Hours post-inoculation
IAP	Intestinal alkaline phosphatase
IL8	Interleukin 8
IL10	Interleukin 10
IL1β	Interleukin 1β
IPEC-J2	Intestinal porcine epithelial cell line-J2
KRB	Krebs ringer buffer
LPS	Lipopolysaccharides
MCFA	Medium chain fatty acids
MGA	Maltase-glucoamylase
MIC	Minimum inhibitory concentration
MUC2	Mucin 2
MUC4	Mucin 4
OA	Organic acids
OCLN	Occludin
P(OA+EO)	Microencapsulated organic acids and essential oils
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PepT1	Peptide transporter 1
PWD	Post-weaning diarrhea
RIPA	Radioimmunoprecipitation assay
SCFA	Short chain fatty acids

SGLT1	Na^+-glucose cotransporter 1
SCC	Sham-challenged control
SI	Sucrase-isomaltase
TAC	Total antioxidant capacity
TBST	Tris-buffered saline with 0.1% Tween 20
TEER	Transepithelial electrical resistance
TLR2	Toll-like receptor 2
TLR5	Toll-like receptor 5
TLR7	Toll-like receptor 7
TSA	Tryptic soy agar
VH	Villus height
V_{max}	Maximal enzyme activity
ZO1	Zonula occludens 1

Introduction

Post-weaning diarrhea (PWD) is one of the most economically important issues in the swine industry (Yang et al., 2014), which is characterized by the frequent release of watery feces resulting in retarded growth performance, damaged gut health, increased morbidity and mortality in young piglets (Pan et al., 2017). Enterotoxigenic *Escherichia coli* F4 (ETEC F4) is one of the common pathogens associated with PWD (Luise et al., 2019). The fimbriae of ETEC can attach to epithelial receptors and release toxins in the intestine of pigs (Jacobsen et al., 2011). Over the last half-century, antibiotic growth promoters (AGP), subtherapeutic doses of antibiotics in the feed or water, have been generally used to control incidences of PWD and to improve the growth rate and feed efficiency of pigs (Cromwell, 2002). However, the overuse of AGP could lead to the spread of antimicrobial-resistant pathogens in both livestock and humans, posing a significant public health threat (Yang et al., 2015). European Union prohibited the use of AGP in 2006, and worldwide authorities are also trying to decrease the use of antibiotics in the livestock industry (Bengtsson and Wierup, 2006; Murphy et al., 2017). However, still therapeutic and subtherapeutic antibiotics for swine are being used in many regions of the world (Lekagul et al., 2019). At the same time, various AGP alternatives have been developed for use in the swine industry (Heo et al., 2013). Pharmacotherapeutic concentrations (high doses) of copper and zinc, which have antimicrobial effects and can improve gut health and growth performance of weaned piglets, were considered as alternatives for AGP (Poulsen and Carlson, 2002; Guan et al., 2017). However, over-supplementation of zinc and copper can induce the generation of antimicrobial-resistant pathogens and cause environmental issues (Ciesinski et al., 2018; Lei and Kim, 2018). Zinc and copper are also being considered to be replaced with other bioactive compounds as is the case with antibiotics. Therefore, cost-effective and eco-

friendly AGP alternatives that induce less antimicrobial resistance are required in the swine industry.

Organic acids (OA), organic compounds with weak acidic properties, have been shown to improve gut health and growth performance of piglets by eliciting antimicrobial effects, increasing enzyme secretions and activities, enhancing intestinal morphology and enhancing gut barrier integrity of piglets (De Lange et al., 2010; Upadhaya et al., 2016). Tricarboxylic acids as citric and dicarboxylic acids as fumaric and malic acids are metabolic intermediates in the Krebs cycle for energy metabolism (Tugnoli et al., 2020). Hence, di or tricarboxylic acids are known to improve gut morphology and gut barrier integrity by providing energy for epithelial cells and by showing antimicrobial effects in pigs (Chen et al., 2018; Li et al., 2019). Sorbic acid (OA) has strong antimicrobial effects and can be an energy source by being subjected to β-oxidation in pigs (Tugnoli et al., 2020). However, most of the OA are absorbed and metabolized in the upper gut (e.g. stomach and duodenum), and dissociated form of OA shows lower positive effects (e.g. antimicrobial effects) than undissociated form of OA, which is especially of interest, in the lower intestine, where many pathogens propagate (Upadhaya et al., 2014). Microencapsulation, which provides a physical barrier for bioactive compounds from their environment until their release, can allow a slow release of OA along the pig gut, which potentially enhance the beneficial effects of OA in the lower intestine, while the effects as acidifiers in the stomach can be attenuated (Bosi et al., 1999).

Essential oils (EO) are bioactive compounds obtained from plants and are known to have antimicrobial, antioxidative, and anti-inflammatory properties, which potentially improve growth rate and gut health of weaned piglets (Puvača et al., 2013; Omonijo et al., 2018; Yang et al., 2020). Among various EO, thymol (main EO component in oregano and

thyme), eugenol (main EO component in clove and basil) and vanillin (main EO of Vanilla) have been frequently used as antimicrobial, antioxidative and anti-inflammatory agents for human and animals (Braga et al., 2006; Si et al., 2006; Tippayatum and Chonhenchob, 2007; Tai et al., 2011). Especially, antimicrobial effects to both Gram-negative and Gram-positive bacteria of those EO are already well documented (Si et al., 2006; Ghosh et al., 2014; Chouhan et al., 2017). Whereas EO have benefits of promoting the growth performance and gut health of pigs, their stability in the feed and along the gut restrains their application to pig diets (Michiels et al., 2008; Omonijo et al., 2018a). However, microencapsulation is thought to increase effectiveness and resolve stability and delivery issues of EO by protecting EO under harsh conditions (e.g. high temperature during pelleting and low pH in the stomach) (Vidhyalakshmi et al., 2009).

The supplementation of OA with EO can show the synergistic effects to improve the growth performance and gut health of animals (Yang et al., 2015; Abdelli et al., 2020). Zhou et al. (2007) reported that OA and EO showed synergistic antimicrobial effects against *Salmonella Typhimurium* potentially because OA and EO have dissimilar modes of action for their antimicrobial effects. The supplementation of OA and EO improved nutrient digestibility and digestive enzyme activities in weaned piglets (Xu et al., 2018). In our previous study, the lipid matrix microparticles containing OA and EO (Jefo Nutrition Inc., QC, Canada) maintained the stability of EO during a feed pelleting process and storage and allowed a slow and progressive release of EO along the gastrointestinal tract of weaned piglets (Choi et al., 2020). Approximately 15% of thymol in the microparticles was released in the stomach and 41% of thymol was delivered to the mid-jejunum section in the study (Choi et al., 2020). However, more studies are still needed to comprehensively understand the mechanisms behind the protection of microencapsulated OA and EO against pathogens in weaned piglets. Therefore, the purpose of the study was to investigate the effects of

microencapsulated OA (fumaric, malic, citric and sorbic acids) and EO (thymol, vanillin and eugenol) on growth performance, immune system, gut barrier function, nutrient absorption, and abundance of ETEC F4 in weaned piglets challenged with ETEC F4.

Materials and Methods

The experimental and animal care protocols (F17-018, AC11280) were reviewed and approved by the Animal Care Committee of the University of Manitoba and piglets were cared for following the Canadian Council on Animal Care guidelines (CCAC, 2009).

Virulence factors of enterotoxigenic *Escherichia coli* F4

The ETEC F4 strain P4 used in this study was isolated from feces of piglets with PWD by the Veterinary Diagnostic Services Laboratory – Government of Manitoba, Canada. In this study, the presence and expression of four virulence genes associated with adhesion including faeG (F4 fimbriae) and enterotoxins including estA (Sta, heat-stable toxin) and estB (STb, heat-stable toxin), elt (LT, heat-labile toxin) in ETEC F4 were verified by polymerase chain reaction (PCR) according to the method previously described by Zhu et al. (2011) with some modifications (Table 1). The genomic DNA from cultured ETEC F4 (1×10^9 CFU) was extracted using PureLink Genomic DNA Kits (Invitrogen, Carlsbad, CA). Total RNA was extracted using an Ambion RiboPure RNA isolation kit (Ambion Inc., Foster City, CA) and the first-strand cDNA was synthesized using oligo (dT) 20 primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each PCR reaction mixture (20 µL) contained 7 µL of 0.1% diethylpyrocarbonate (DEPC)–treated water, 1 µL each of forward and reverse primer (10 $\mu mol \cdot L^{-1}$), 10 µL of DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA), and 1 µL genomic DNA or 1 µL cDNA. PCR thermocycler conditions were as follows: 50°C denature for 2 min, 95°C denature for 5 min, 40 cycles at 95°C for 45 s,

50°C for 45 s, and 72°C for 30 s, and a final extension of 72 °C for 10 min. All PCR products were electrophoresed on a 3% agarose gel in a Tris-borate-EDTA buffer and visualized by staining with SYBR Green (Invitrogen, Carlsbad, CA). All 4 virulence genes (estA, estB, faeG and elt) were expressed in the ETEC F4 used in this study.

Genetic susceptibility screening and piglet selection

The ETEC F4 susceptible piglets were selected as described by Jensen et al., (2006). DNA was extracted from tails obtained on 3d after farrowing using a method described by Truett et al. (2000). The PCR of MUC4 gene was performed using DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA) with 2 mmol·L^{-1} $MgCl_2$, 200 µmol·L^{-1} of each dNTP, 400 µmol·L^{-1} of each primer in a total volume of 25 µL. Thermocycling was performed using 5 min initial denaturation at 95℃, subsequently 95℃ for 30 s, annealing at 65℃ for 30 s and extension at 72℃ for 1 min for 35 cycles. The size of the PCR product obtained from pig genomic DNA was 367 bp and 5 µL of the PCR products were digested with FastDigest XbaI (Thermo Fisher Scientific, Waltham, MA) at 37℃ for 5 min following the supplier's instructions. All digested PCR products were electrophoresed on a 2% agarose gel in a Tris-borate-EDTA buffer and visualized by staining with SYBR Green (Invitrogen, Carlsbad, CA). The resistant allele (R) was indigestible by XbaI, whereas the susceptible allele (S) was digested into 151 bp and 216 bp fragments. The piglets with susceptible alleles and similar body weight (BW) were selected.

Preparation of enterotoxigenic *Escherichia coli* F4

The ETEC F4 was streaked on tryptic soy agar (TSA) from frozen stock and grown anaerobically at 37°C overnight. Afterward, 10 mL of tryptic soy broth (sterile) was inoculated with a single ETEC F4 colony from the streak plate and aerobically grown overnight at 37°C in an orbital shaker (MaxQ SHKE4000; Thermo Fisher Scientific,

Waltham, MA) set at 150 rpm. The culture was inclined at 45°C to promote enough aeration. Thereafter, 300 µL of the overnight culture was used as an inoculant for a fresh 300 mL of tryptic soy broth (sterile), again incubating at 37°C and shaking at 150 rpm by using the orbital shaker. The culture was grown for 2.5 h. Necessary preliminary experiments such as a growth curve and standard curve were generated first before preparing the final ETEC F4 inoculum. After incubation, a small sample was taken for OD measurement at 600 nm (tryptic soy broth as blank) to check the bacterial density using a Pharmacia Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK) according to the standard curve generated earlier. Phosphate buffered saline (PBS, pH 7.4) was used as the diluent to achieve the targeted ETEC F4 concentration (1×10^{7} CFU·mL^{-1}). The culture was transported with ice packs to the site for inoculation.

Animals and experimental design

Twenty-four ETEC F4 susceptible weaned piglets (TN Tempo × TN70; 12 female and 12 castrated male piglets with average BW of 8.52 ± 0.11 kg) at the age of 28 d were obtained from the Glenlea Swine Research Unit at the University of Manitoba and housed individually in a temperature-controlled room within the T.K. Cheung Centre for Animal Science Research at the University of Manitoba. Room temperature was maintained at 29 ± 1 ºC during the first week and then reduced by 1.5 ºC for the rest of the experimental period (8-12 d). Piglets were randomly distributed to 4 treatments to give 6 replicates per each treatment. A corn-soybean meal basal diet was formulated to meet or exceed the NRC (2012) recommendations for 6 to10 kg pigs (Table 2) and fed in a mash form. The four treatments were: 1) sham-challenged control (SCC; piglets fed the basal diet and inoculated with PBS); 2) challenged control (CC; piglets fed the basal diet and challenged with ETEC F4; 3) antibiotic growth promoters (AGP; CC + 55 mg·kg^{-1} of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada)); and 4) microencapsulated OA and EO [P(OA+EO); (CC + 2 g·kg^{-1}

of microencapsulated OA [fumaric (18%), citric, malic and sorbic acids] and EO [thymol (0.5%), vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada)]. Piglets were housed in individual pens and allowed free access to feed and water during the whole experimental period. On d 7 (0 days post-inoculation; dpi) and 11 (4 dpi), individual pig BW and pen feed disappearance were recorded. On d 7, 5 mL of 1×10^7 CFU·mL^{-1} ETEC F4 was administered to challenged piglets with a syringe attached to polyethylene tube held into the upper esophagus (Koo et al., 2017; Koo et al., 2019). Core body temperature was measured by inserting a digital thermometer into the rectum before inoculation and at 3, 24, and 48 hpi (hours post-inoculation). Fecal consistency score (0 = normal; well-formed solid feces, 1 = soft feces; formed soft feces, 2 = mild diarrhea; fluid feces with yellowish color, and 3 = severe diarrhea; watery and projectile feces) was recorded at 0, 3, 8, 16, 24, 28, 34, 40, 48 and 54 hpi as described previously (Marquardt et al., 1999).

In vivo gut permeability

On d 11 (4 dpi), oral gavage of fluorescein isothiocyanate-dextran 70 kDa (10 mg per pig; FITC-D70; molecular weight 70 kDa; (Sigma-Aldrich Co., St. Louis, MO) in 5 mL PBS was conducted. Pigs were allowed to eat and drink for 4 h after which blood samples (serum) were collected from each piglet through jugular vein into heparin-free vacutainer tubes (Becton Dickinson, Rutherford, NJ) wrapped in aluminum foil to block the light and kept at room temperature for 3 h to allow clotting. The blood samples were centrifuged at 750 × g for 15 min to recover serum. The serum (100 µL) was transferred to 96-well plates, and the fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a Bio-Tek PowerWave HT Microplate Scanning Spectrophotometer (BIO-TEK Instruments, Inc., Winooski, VT). The concentrations of

FITC-D70 in the serum samples (ng·mL^{-1}) were calculated based on a standard curve (R^2 = 0.99).

Tissue and digesta sample collection

At the end of the experiment (d 12; 5 dpi) all piglets were anesthetized by an intramuscular injection of ketamine:xylazine (20:2 mg·kg^{-1} BW) and euthanized with a captive bolt gun. The abdomen was immediately opened to expose the whole gastrointestinal tract. A 10 cm segment was taken from the mid-jejunum (400 cm from the stomach-duodenum junction) and put in ice-cold Krebs ringer buffer (KRB; in mmol·L^{-1}: 154 Na^+, 6.3 K^+, 137 Cl^-, 0.3 H_2PO_4, 1.2 Ca^{2+}, 0.7 Mg^{2+}, 24 HCO_3^- - pH 7.4 with 1µmol·L^{-1} of indomethacin) with glucose (10 mmol·L^{-1}) and immediately delivered to the laboratory for the Ussing chamber analysis. Another 10 cm segment of the mid-jejunum was removed and immediately snap-frozen in liquid nitrogen, and then stored at –80°C until further analyses. For measurement of gut morphology measurement, a 2 cm segment of the mid jejunum was collected and fixed in a 10% formaldehyde solution. Digesta from the spiral colon (20 cm from the ileum-cecum junction) was collected and immediately frozen in liquid nitrogen before being stored at –80°C until further analyses.

Ussing chamber

The electrophysiological properties including short-circuit current and transepithelial electrical resistance (TEER) were determined using modified Ussing chambers (VCC-MC8; Physiologic Instruments Inc., San Diego, CA) containing pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes housed in 3% agar bridges and filled with KRB buffer without glucose. Five milliliters of the KRB buffer solution with 10 mmol·L^{-1} D-glucose was added to the serosal chambers, and five milliliters of KRB buffer solution enriched with 10 mmol·L^{-1} D-mannitol instead of D-glucose was added to the mucosal chambers. Both the

mucosal and serosal chambers were continuously gassed with a mixture of 95% O_2 and 5% CO_2. The temperature of the chambers was maintained at 37°C by using a water-jacketed reservoir. The possible potential difference existing between the mucosal and serosal chambers was offset before tissue mounting. After gently stripping off serosal and longitudinal muscle layers using micro-forceps, the tissue was mounted in Ussing chambers employing a tissue slider with an aperture of 1 cm^2. The tissue was left to equilibrate for 10 min followed by the recording of the short-circuit current and TEER for 10 min after mounting. Afterward, 10 $mmol \cdot L^{-1}$ D-glucose was added to the mucosal chamber to measure the sodium-dependent glucose transportation and 10 $mmol \cdot L^{-1}$ mannitol was added to the serosal chamber to maintain osmotic balance across the tissue (Mrabti et al., 2019). The difference of short circuit current generated by Na^+-glucose cotransporter 1 (SGLT1) was determined by subtracting the short circuit current value before stimulation from the peak after stimulation. When D-glucose was added, 0.1 $mg \cdot mL^{-1}$ of FITC-D4 (molecular weight 4 kDa; Sigma-Aldrich Co., St. Louis, MO) was added to the mucosal side, and after 1 h, the sample (1 mL) was obtained from the serosal side to measure intestinal permeability. The KRB samples (100 µL) from the serosal side were transferred to 96-well plates. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a Bio-Tek PowerWave HT Microplate Scanning Spectrophotometer (BIO-TEK Instruments, Inc., Winooski, VT). The concentrations of FITC-D4 in the KRB buffer ($ng \cdot mL^{-1}$) were calculated based on a standard curve ($R^2 = 0.99$). The FITC-D4 flux was measured for 1 h using a slide that has 1 cm^2 of well surface area and was expressed as $\mu g \cdot cm^{-2} \cdot h^{-1} \cdot mL^{-1}$.

Intestinal morphology analysis

The Alcian blue/the periodic acid–Schiff (AB/PAS) staining for measuring villus height (VH), crypts depth (CD) and VH:CD and counting the number of goblet cells was conducted as described by Koo et al. (2020). After fixation in 10% neutral-buffered formalin, samples were embedded in paraffin and a 5 µm section was sliced and subsequently mounted on glass slides. Dewaxed sections were immersed in xylene, 100% ethanol and 95% ethanol for 5 min, 2 cycles in each solution. The samples were immersed in Alcian blue solution (pH 2.5) for 15 min at room temperature and washed with water for 2 min, and placed in the Schiff reagent for 10 min and washed with water for 10 min. Finally, samples were counterstained in hematoxylin for 10 s and washed and dehydrated. For the quantification of AB/PAS staining, each sample was visualized and photographed using an Axio Scope A1 microscope (Carl Zeiss Micro-Imaging GmbH, Göttingen, Germany) coupled with an Infinity 2 digital camera (Lumenera Corporation, Ottawa, ON, Canada). Villus height (VH), crypt depth (CD) and VH:CD were measured, and the number of goblet cells per 100 µm VH and 100 µm CD was counted using Infinity Analyze software (version 6.5.4; Lumenera Corporation, Ottawa, ON, Canada). For each sample, 50 to 150 villi and crypts were measured

Total antioxidant capacity, total GSH and GSH/GSSG Assays

Total antioxidant capacity (TAC) of the mid-jejunal tissue samples was measured in duplicate by using the Colorimetric Microplate Assay Kits for Total Antioxidant Capacity (TA02, Oxford Biomedical Research, Oxford, MI) (Yang, 2011). Briefly, 200 mg of liquid nitrogen pulverized mid-jejunal tissue samples were weighed out with a 1.5 mL Eppendorf tube, homogenized with 1 mL of ice-cold PBS on ice for 30 s, and then centrifuged at 3,600 × g for 12 min at 4 ºC. Aliquot of supernatant was taken for the analysis of their protein content

using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The TAC in the supernatant was measured as the capacity to convert Cu^{2+} to Cu^{+} by all antioxidants according to the manufacturer's protocol. Cu^{+} ion forms a stable complex with bathocuproine that is detected by measuring the absorbance at 450 nm with a 96-well plate reader (Bio-Tek PowerWave HT Microplate Scanning Spectrophotometer, BIO-TEK Instruments, Inc.). The values were compared to a standard curve obtained using uric acid as a reductant and were expressed as mM·mg protein^{-1}.

Total glutathione (GSH) and oxidized glutathione (GSSG) in the mid-jejunal tissues were measured in duplicate by using the Glutathione Colorimetric Detection Kit (Invitrogen, Carlsbad, CA). Briefly, 30 mg of liquid nitrogen pulverized mid-jejunal tissue samples were weighed out with a 1.5 mL Eppendorf tube, homogenized with 750 µL of ice-cold PBS on ice for 30 s, and then centrifuged at 3,600 × g for 10 min at 4 ºC. Aliquot of supernatant was taken for the analyses of protein content using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Afterward, 5-sulfo-salicylic acid dihydrate was added to the obtained supernatant to precipitate protein, and then centrifuged at 3,600 × g for 10 min at 4 ºC. After deproteinization, total GSH and GSSG levels in the resulting supernatant were measured according to the manufacturer's protocol. Reduced GSH was calculated by the equation: Reduced GSH = Total GSH – 2 × GSSG.

Digestive enzyme activity assays

The maximal enzyme activity (V_{max}) of intestinal digestive enzymes including aminopeptidase N (APN), intestinal alkaline phosphatase (IAP), maltase, glucoamylase, and sucrase was determined. Specifically, about 200 mg of liquid nitrogen pulverized and frozen mid-jejunal tissue samples were thawed in an ice-cold homogenizing buffer (50 mmol·L^{-1} D-mannitol and 0.1 mmol·L^{-1} Phenylmethylsulfonyl fluoride at pH 7.4) and homogenized on ice

using a polytron homogenizer. The protein content of the resulting homogenate samples was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). The APN (EC. 3.4.11.2) activity was measured according to the method of Maroux et al. (1973), and IAP (EC 3.1.3.1) activity was measured according to Hübscher and West (1965). The activities of disaccharidases including sucrase (EC 3.2.1.48) and maltase (EC 3.2.1.20) were determined using the procedure of Dahlqvist (1964). The glucoamylase (EC 3.2. 1.20) activity was analyzed according to the method of Lackeyram et al. (2012). The V_{max} was expressed in nmol·mg^{-1}·min^{-1}.

RNA extraction and Real-time PCR Analysis

Total RNA was isolated from 50 mg of liquid nitrogen pulverized mid-jejunal tissue samples using an RNAqueous total RNA isolation kit (Ambion Inc., Foster City, CA). The concentration and OD260:OD280 ratio of extracted RNA samples were measured using a Nanodrop UV-Vis spectrophotometer 2000 (Thermo Fisher Scientific Inc., Ottawa, ON, Canada) and the OD260:OD280 ratios of all RNA samples were between 1.9 and 2.1. The RNA samples were stored at -80°C for further analyses. A total of 1 µg RNA was used to synthesize the first-strand cDNA using an iScript cDNA Synthesis Kit (Biorad, Mississauga, ON, Canada) according to the manufacturer's instructions. All primers were designed with Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are shown in Table 1. The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Real-time PCR was carried out using a SYBR Green Supermix (Biorad, Mississauga, ON, Canada) on a CFX Connect Real-Time PCR Detection System (Biorad, Mississauga, ON, Canada) (Omonijo et al., 2018b). A total of 1 µL cDNA was added to a total volume of 20 µL, containing 10 µL SYBR Green supermix and 300 nmol·L^{-1} of each forward and reverse primers. Thermal condition for all reactions was: denaturation for 3 min at 95 °C, then 40

cycles of 20 s at 95°C, 30 s at 60°C and 30 s at 72°C. Cyclophilin-A (CycA) was used as the internal control to normalize the amount of RNA used in the real-time PCR for all the samples. A melting curve program was conducted to confirm the specificity of each PCR product. The target mRNA abundance was normalized with that of a selected reference gene and relative mRNA abundance was determined by using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001). Threshold cycle (Ct) values were obtained at the cycle number at which the gene is amplified beyond the threshold of 30 fluorescence units. Real-time PCR efficiencies were acquired by amplification of the dilution series of DNase-treated RNA according to formula $10^{(-1/slope)}$ (Pfaffl, 2001). The efficiencies of all primers used in this study were between 96-105%. Negative controls without cDNA were conducted along with each run, and each sample was analyzed in duplicate for each gene.

Western blotting

Relative protein abundance of Zonula occludens 1 (ZO1), OCLN (Occludin), and neutral amino acid transporter 1 (B^0AT1) in the jejunum were detected by western blotting. Briefly, an aliquot of about 50 mg of liquid nitrogen pulverized mid-jejunal tissue samples were homogenized in a radioimmunoprecipitation assay buffer (RIPA lysis buffer; Sigma-Aldrich Co., St. Louis, MO) containing a complete cocktail of proteinase inhibitors, and protein concentration was analyzed by a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's instructions. Protein samples were then denatured in 1× Laemmli buffer with mercaptoethanol at 95℃ for 5 min and loaded into the wells of 4-12% gradient pre-made SDS-PAGE gel (Biorad, Mississauga, ON, Canada) for electrophoresis. After electrophoresis, the proteins were transferred onto the Polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo transfer system (Biorad, Mississauga, ON, Canada). For immunoblotting, the membranes were first

blocked with 5% non-fat dry milk in tris-buffered saline with 0.1% of Tween 20 (TBST) at room temperature for 1 h and then incubated with primary antibodies rabbit anti-ZO1 (1:1,000 dilution; Thermo Fisher Scientific, Waltham, MA), rabbit anti-OCLN (1:500 dilution; Thermo Fisher Scientific, Waltham, MA), rabbit anti-B^0AT1 (1:2,000 dilution, provide by Dr. François Verrey at University of Zurich, Switzerland) (Romeo et al., 2006), and rabbit anti-β actin (1:5000 dilution; Thermo Fisher Scientific, Waltham, MA) at 4℃ overnight. Afterward, the membranes were washed 5 times with TBST and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000 dilution; Thermo Fisher Scientific, Waltham, MA) at room temperature for 1 h, then washed 5 times with TBST. The chemiluminescent signals were achieved by applying Clarity^{Max} Western ECL Substrate (Biorad, Mississauga, ON, Canada) to the membranes and images were captured using a ChemiDoc MP imaging system (Biorad, Mississauga, ON, Canada). The intensity of the bands was quantified using Image Lab 6.0 software (Biorad, Mississauga, ON, Canada). β-actin was used as an internal reference. The relative abundance of these proteins was semi-quantified by calculating the ratio of the band intensity of target and reference proteins. Data were presented as mean ± SEM (n=4).

Measuring ETEC F4 abundance by droplet digital PCR (ddPCR)

DNA from the colon digesta was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The ETEC F4 abundance in the colon digesta was quantified by measuring the gene copy number of F4 specific fimbriae gene (faeG) using the droplet digital PCR (ddPCR) system (Biorad, Mississauga, ON, Canada). Briefly, 25 μL of PCR reaction mixture containing 1 ng (ETEC F4 challenged samples) or 100 ng (control samples without ETEC F4 challenge) of DNA templets, 100 nmol·L^{-1} of each faeG primer and 1× Evagreen Supermix (Biorad, Mississauga, ON, Canada) was prepared and 20 μL of the mixture was transferred into a sample well of

the droplet generator cartridge (DG8 cartridges; Biorad, Mississauga, ON, Canada). Droplet Generation Oil (70 µl) (Biorad, Mississauga, ON, Canada) was added to the oil wells of DG8 cartridges. Droplets were generated using a droplet generator (Biorad, Mississauga, ON, Canada) and were gently transferred onto the 96-well PCR plate (Biorad, Mississauga, ON, Canada). The faeG gene in the droplets was amplified on the C1000 Touch thermal cycler (Biorad, Mississauga, ON, Canada) using the following thermal cycling protocol: 95°C for 5 min, 40 cycles of 95°C for 30 s and 57°C for 1 min, and followed by 4°C for 5 min, 90°C for 5 min and 4°C for 10 min. After thermal cycling, the PCR end products were read by a QX200 droplet reader (Biorad, Mississauga, ON, Canada), and data were analyzed by QuantaSoft (Biorad, Mississauga, ON, Canada). Data were presented as $\log_{10}$(faeG gene copies·µg DNA^{-1}).

Statistical analyses

Statistical analyses were conducted with SAS (version 9.4; SAS Inst. Inc., Cary, NC) with each individual animal as the experimental unit. The normality of data was confirmed using the PROC UNIVARIATE except for the data of diarrhea score. The effects of ETEC F4 inoculation (SCC v. SC) were evaluated by unpaired *t* test. The ETEC F4 challenged group (CC, AGP, and P(OA+EO)) were compared using the PROC MIXED in a completely randomized design. The statistical model included dietary treatments as the main effect with no random effects. The LSMEANS statement with the Tukey-adjusted PDIFF option was employed to calculate and compared differences among treatment mean. Results in tables were shown as least-square means and pooled standard errors of the means, and results in figures shown as mean ± SEM. Diarrhea score was analyzed using the Mann-Whitney U-test to compare SCC v. SC and the Kruskal-Wallis test to compare the ETEC F4 challenged group (CC, AGP, and P(OA+EO)) with Dunn's multiple comparison test (Pant et al., 2011).

Differences were considered significant at $P < 0.05$, and trends ($0.05 \leq P \leq 0.10$) were also presented.

Results

Growth performance, rectal temperature and diarrhea score

As shown in Table 3, there were no differences in the BW, average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) observed among treatment groups during the pre-challenge period ($P > 0.10$). During the post-challenge period (7 to 11 d), ETEC F4 infection tended to decrease ADG of the CC piglets when compared to the SCC piglets ($P = 0.05$). The supplementation of Aureomycin and microencapsulated OA and EO did not affect ADG during the post-challenge period ($P > 0.10$), and there were no differences in the BW and ADFI among treatment groups during the post-challenge period ($P > 0.10$). During the whole period (0 to 11 d), there were no differences in ADG, ADFI and FCR among treatment groups ($P > 0.10$).

As shown in Fig. 1, core body temperature was increased in the CC piglets when compared to the SCC piglets at 3 hpi (40.12 v. 39.5°C, $P < 0.05$). However, the piglets fed AGP and P(OA+EO) had statistically similar core body temperature with the piglet fed the CC diet at 3 hpi. No differences were observed among all treatments at 24 and 48 hpi ($P > 0.10$)

As shown in Fig. 2, inoculation of ETEC F4 induced diarrhea at 8, 28, 34, and 40 hpi ($P < 0.05$) and tended to increase the incidence of diarrhea at the 3 hpi ($P = 0.06$) and 16 hpi ($P = 0.06$) in the CC piglets when compared to the SCC piglets. At 28 dpi, piglets fed P(OA+EO) had a lower ($P < 0.05$) diarrhea score compared to the piglets fed CC. However,

the P(OA+EO) piglets had a lower ($P < 0.05$) diarrhea score compared to the AGP piglets at 40 dpi.

Gut permeability and glucose transport

As shown in Table 4, there were no differences in TEER, SGLT1 dependent short-circuit current and FITC-D4 as measured using the Ussing chamber ($P > 0.10$). Inoculation of ETEC F4 tended to increase *in vivo* gut permeability in the CC piglets compared to the SCC piglets ($P = 0.09$). The AGP piglets had higher FITC-D70 flux than P(OA+EO) piglets ($P < 0.05$).

Intestinal morphology and goblet cells

As shown in Table 5, ETEC F4 inoculation decreased mid-jejunal VH in the CC piglets when compared to the SCC piglets ($P < 0.05$). The piglets supplemented with microencapsulated OA and EO had increased ($P < 0.05$) VH in the mid-jejunum when compared to the CC piglets. However, no differences were found in the CD, VH:CD, the number of goblet cells per 100 µm VH and CD among all treatment groups ($P > 0.10$).

Digestive enzyme maximal activities

As shown in Table 6, no differences were found in the V_{max} of APN, IAP, glucoamylase, and sucrase among all treatment groups ($P > 0.10$**).** The ETEC F4 inoculation tended to decrease V_{max} of maltase ($P = 0.05$) when the CC piglets were compared to the SCC piglets.

Total antioxidant capacity, total GSH and GSH/GSSG

As shown in Table 7, there were no differences in TAC, total GSH, GSSG, reduced GSH and reduced GSH:GSSG observed among treatment groups ($P > 0.10$)**.**

Relative mRNA abundance of genes related to gut barrier function, immune system, nutrient transport, and digestive enzymes

There were no differences in the relative mRNA abundance of ZO1 and OCLN among all treatment groups ($P > 0.10$). The ETEC F4 inoculation decreased the relative mRNA abundance of claudin 1 (CLDN1), CLDN3 and mucin 2 (MUC2) in the NC piglets when compared to the SCC piglets ($P < 0.05$). There were no differences in the relative mRNA abundance of peptide transporter 1 (PepT1), excitatory amino-acid carrier 1 (EAAC1), and neutral amino acid transporter 2 (ASCT2) observed among treatment groups ($P > 0.10$). The relative mRNA abundance of SGLT1 and B^0AT1 was decreased due to ETEC F4 inoculation when compared to the SCC piglets ($P < 0.05$). The AGP piglets had a higher relative mRNA abundance of B^0AT1 when compared to the CC piglets ($P < 0.05$). Among the genes related to the immune system including interleukin 8 (IL8), IL10, IL1β, toll-like receptor 2 (TLR2), TLR5 and TLR7, only the relative mRNA abundance of IL8 tended to increase due to ETEC F4 inoculation ($P = 0.10$) in the CC piglets when compared to the SCC piglets. The ETEC F4 inoculation decreased relative mRNA abundance of maltase-glucoamylase (MGA) and APN ($P < 0.05$) and tended to decrease relative mRNA abundance of SI ($P = 0.05$) in the CC piglets compared to the SCC piglets. However, the relative mRNA abundance of MGA, SI, and APN among CC, AGP and P(OA+EO) piglets were similar ($P > 0.10$).

Relative protein abundance of tight junction proteins and nutrient transporter

As shown in Fig. 3, ETEC F4 inoculation increased the protein abundance of OCLN ($P < 0.05$), and the AGP piglets had the lowest relative protein abundance of OCLN ($P < 0.05$) when compared to the CC and P(OA+EO) piglets ($P < 0.05$). However, the relative protein abundance of ZO1 and B^0AT1 were similar among all treatment groups ($P > 0.10$).

ETEC F4 abundance in the colon digesta

As shown in Fig. 4, ETEC F4 inoculation increased the ETEC F4 gene (faeG) ($P < 0.05$) in the colon digesta in the CC piglets when compared to the SCC piglets. However, there were no differences in the copy number of faeG observed among the piglets challenged with ETEC F4 ($P > 0.10$).

Discussion

This study was to investigate whether the supplementation of microencapsulated OA and EO could alleviate the response to ETEC infection (e.g. diarrhea, inflammation and compromised gut health) in weaned piglets. A model for inducing bacterial infection in weaned piglets was established by inoculating ETEC F4 (Opapeju et al., 2015). The pathogenesis of ETEC F4 in pigs depends on two major factors: ETEC F4 virulence and F4 fimbriae receptors in piglets (Kim et al., 2019). The F4 fimbriae (faeG) attach to the F4 receptors (MUC4) on the intestinal brush borders and induce ETEC F4 colonization in the intestine and then release toxins (estA, estB and elt) (Moonens et al., 2015). The toxins of ETEC F4, including estA, estB, elt and lipopolysaccharides (LPS), can cause the disorders of electrolytes and fluid secretion in the intestine, which results in watery feces (Koo et al., 2019). The presence and expression of virulence factors in ETEC F4 strain P4's were verified in this experiment, and four virulence genes (faeG, estA, estB, and elt) were expressed in the ETEC F4 used in the current study.

The ETEC F4 susceptible piglets were selected by verifying the susceptible alleles of MUC4 according to a previous publication (Jensen et al., 2006). Gibbons et al. (1977) showed that the susceptibility to ETEC F4 was inherited as an autosomal dominant Mendelian trait with the two alleles: S (adhesion, dominant) and R (non-adhesion, recessive). The ETEC F4 induces more clinical symptoms if piglets have susceptible alleles of the

MUC4 gene (Fairbrother et al., 2005). Therefore, it is necessary to choose susceptible piglets for this challenge study to successfully induce diarrhea and minimize variations among piglets (Trevisi et al., 2015; Sterndale et al., 2019). The ultimate purpose of this study was to evaluate dietary OA and EO, microencapsulated in this case, to replace AGP in the swine industry. The AGP (low dosage of antibiotics) are mostly expected to show subtherapeutic effects rather than the therapeutic effects that may alleviate clinical diarrhea (Diarrhea score 2 or 3) or mortality (Adewole et al., 2016). Thus, for implementing the ETEC F4 challenge model, the ETEC F4 dosage that could induce mild diarrhea was inoculated to the piglets in current the study. In our pilot studies (data not published), piglets inoculated with 10 mL of 1 × 10^9 CFU·mL^{-1} and 5 mL of 3 × 10^8 CFU·mL^{-1} of ETEC F4 showed 75% (15 dead piglets out of 20 piglets) and 65% (13 dead piglets out of 20 piglets) of mortality in all treatment groups, respectively. A pilot study showed that the oral gavage of 5 mL of 1 × 10^7 CFU·mL^{-1} ETEC F4 induced mild diarrhea, and thus 5 mL of 1 × 10^7 CFU·mL^{-1} was chosen in this study. In the current study, the symptoms of ETEC F4 infection were successfully achieved, which can be indicated by increased diarrhea score at 3 to 40 hpi, core body temperature at 3 hpi and compromised gut health in the CC piglets compared to the SCC piglets.

The OA and EO microparticles used in this study contained fumaric acid, citric acid, malic acid, sorbic acid as OA, and thymol, vanillin and eugenol as EO, both microencapsulated within a matrix-based of hydrogenated vegetable oil for slow release purpose. In our previous study, the microparticles could maintain their stability during a pelleting process and storage and were slowly released along the pig gut (Choi et al., 2020). Within the active ingredients (OA + EO), OA were the most representative bioactive compounds by comparison to EO with a minimum guarantee of 18% for fumaric acid, the ingredient with the highest proportion in those microparticles. Based on the composition and inclusion levels of microparticles, the dose of OA supplied in the feed was in line with

minimum inhibitory concentration (MIC) levels reported to inhibit the growth of pathogens (He et al., 2011; Gao et al., 2012). The EO supplied at low or even sub-MIC levels proved to be beneficial for sustainable swine production since sub-MIC levels of EO still can disrupt quorum sensing of bacteria, which is responsible for regulating pathogenicity and antibiotic resistance (Szabó et al., 2010). Omonijo et al., (2018b) also reported that thymol even at sub-MIC level could improve barrier integrity and antioxidative capacity and attenuated inflammatory responses in the intestinal porcine epithelial cell line-J2 (IPEC-J2) challenged with lipopolysaccharides (LPS). Similar effects were also recently reported with eugenol by Hui et al., (2020). In addition, the use of a blend of diverse OA and EO can show synergistic effects in antimicrobial effects and may reduce the possibility of the development of resistant microorganisms because microorganisms are hampered to develop resistant systems against numerous targets at the same time (Yap et al., 2014; Yang et al., 2019).

In this study, during the pre-challenge period, the supplementation of Aureomycin and microencapsulated OA and EO did not affect the growth performance. The ETEC F4 infection decreased ADG (46.18%) in the present study, which is consistent with the results of Trevisi et al. (2009) and Rong et al. (2015). The potential reasons for decreased ADG of piglets due to ETEC F4 in the current study could be 1) decreased efficiency of nutrient digestion and absorption (Chen et al., 2018); 2) induced inflammation (Kim et al., 2016); 3) increased diarrhea, which cause loss of water in the body (Cho et al., 2012); and 4) decreased available nutrients for pigs because ETEC F4 may have competed for nutrients with the host (Richards et al., 2005). In the current study, the supplementation of microencapsulated OA and EO did not alter growth performance during the post-challenge period. In contrast, Devi et al. (2015) showed that the supplementation of a blend of EO including cinnamon, fenugreek, and clove improved ADG when compared to the control group, while the supplementation of a coated OA containing formic acid, lactic acid, fumaric acid and citric

acid could not improve growth performance of weaned piglets challenged with ETEC F4. In addition, Kwak et al. (2019) showed that microencapsulated OA and EO attenuated the decrease of ADG and ADFI in the LPS-challenged piglets. However, Ahmed et al. (2013) reported that when a mixture of ETEC KCTC 2571 and *Salmonella Typhimurium* was inoculated to piglets, the blend of EO including oregano (*Origanum vulgare*), anise (*Pimpinella anisum*), orange peel (*Citrus sinensis*), and chicory (*Cichorium intybus*) did not improve the growth performance when compared to the control group. These inconsistent results in piglets with physiological challenges may come from different kinds of OA and EO, challenging inoculums or experimental conditions (e.g. hygiene, experimental period and replicates). However, Xu et al. (2020) reported that the supplementation of the same microencapsulated OA and EO combined with antibiotics improved growth performance of weaned piglets challenged with ETEC F4, which may imply that P(OA+EO) can partially replace antibiotics to improve growth performance in pigs challenged with ETEC F4.

In the current study, ETEC F4 infection increased core body temperature at 3 hpi, and the relative mRNA abundance of IL8 (pro-inflammatory cytokines) was increased when SCC piglets compared to CC piglets. Increased core body temperature and upregulated pro-inflammatory cytokines, which are produced by various cell types such as macrophages, endothelial cells, B cells and mast cells, imply inflammatory reactions in pigs (Akira et al., 1993; Kwak et al., 2019). Potentially, colonization and toxins of ETEC F4 induced inflammatory response in the weaned piglets. Induced inflammatory response potentially was associated with damaged gut barrier integrity, the first line of defense against the hostile environment and prevents noxious antigens and pathogens from permeating into the body (Wijtten et al., 2011). In this study, ETEC F4 infection increased *in vivo* gut permeability measured by using FITC-D70 flux and decreased the relative mRNA expression of genes related to gut barrier integrity such as CLDN1, CLDN3 and MUC2. According to Suzuki et

al. (2011) and Al-Sadi et al. (2014), pro-inflammatory cytokines could modulate the expression of tight junction proteins and gut permeability in pigs, and ETEC F4 toxins would directly modulate the expression of tight junction proteins (Dubreuil, 2017). Furthermore, damaged gut barrier integrity increased the flux of toxins of ETEC F4, which possibly caused a more severe inflammatory response in pigs in the present study. However, the relative protein expression of OCLN was overexpressed due to ETEC F4 infection, which was inconsistent with the data of relative mRNA expression in the present study. According to Wu and Su (2018), ETEC infection increased the expression of tight junction proteins via myosin light chain kinase (MLCK)-myosin II regulatory light chain (MLC20) pathways as a defensive system. However, increased protein expression of tight junction proteins did not affect *in vivo* and *ex vivo* gut permeability. The supplementation of Aureomycin and microencapsulated OA and EO did not show anti-inflammatory responses in the current study. However, the AGP piglets even had higher *in vivo* FITC-D70 flux in the current study compared to the P(OA+EO) piglets. In this experiment, relative protein expression of OCLN was lower in the AGP piglets compared to the CC and P(OA+EO) piglets, which is in line with an increased gut permeability of piglets as a side effect, and this could possibly explain the higher diarrhea score of AGP piglets compared to the P(OA+EO) piglets at 40 dpi.

The FITC-D70 was used to measure gut permeability from the esophagus possibly up to ileum of weaned piglets, and FITC-D4 was used to study intestinal permeability of 1 cm^2 of mid-jejunum in the Ussing chamber assay in the current study. The FITC-D70 cannot permeate the epithelial barrier if the epithelial barrier is closed, however, once gut barrier integrity is damaged due to the inflammation or pathogenic infection, FITC-D70 molecule can permeate the gut barrier and enter blood circulation (Yan et al., 2009; Baxter et al., 2017). Hence, FITC-D70 was used to assess *in vivo* gut permeability because there could be many damaged areas from esophagus to ileum even in SCC piglets. Although the FITC-D4

can diffuse across the intestinal barrier even though the intestine barrier is closed in pigs (Weström et al., 1984), it is expected that a higher amount would permeate across the intestinal barrier if intestinal barrier integrity is damaged. Thus, FITC-D4 may be more appropriate to detect differences in intestinal permeability among the treatments in the small part of samples in the Ussing chamber rather than FITC-D70. Therefore, FITC-D70 was appropriate to measure *in vivo* gut permeability, and FITC-D4 was suitable to measure intestinal permeability in the Ussing chamber assay in this study. The differences in molecular size of FITC-D4 and -D70 and area of analysis could explain the different results of *in vivo* and *ex vivo* gut permeability assay in the current study.

Enterocytes play a crucial role in nutrient digestion and absorption because brush border digestive enzymes and nutrient transporters are expressed in the enterocytes, and therefore, the VH may represent intestinal digestion and absorption capacity (Kong et al., 2018). In this study, ETEC F4 infection decreased the mid-jejunal VH, which is consistent with a study by Yi et al. (2005). This observation could be due to villous atrophy caused by ETEC F4 toxins (Rong et al., 2015). Reduced mid-jejunal VH in CC piglets is potentially associated with the decreased maltase activity and downregulation of genes related to nutrient transporters including SGLT1 (main sugar transport system in the intestine of pigs) and B^0AT1 (a nutrient transporter of leucine, valine, isoleucine, methionine, and proline) (Hwang et al., 1991; Yang et al., 2016) and digestive enzymes (MGA, SI and APN) observed in the current study. In this study, the piglets fed the AGP diet had significantly higher relative mRNA expression of B^0AT1, which may imply that AGP can increase nutrient absorption in piglets. That P(OA+EO) piglets had the highest VH compared to CC and AGP piglets is consistent with the results of Liu et al., (2017) indicating that the microencapsulated OA and EO also improved VH in the broiler chickens. However, increased VH did not translate into

increased growth rate or increased nutrient digestion and absorption parameters in the current research.

The ddPCR assay is a novel and promising absolute quantification method in the animal science field due to its sensitivity, specificity, and speed (Sui et al., 2019). According to the ddPCR analysis in this study, ETEC F4 also existed in the SCC piglets, and ETEC F4 inoculation increased the number of ETEC F4 in the colon digesta, but the supplementation of Aureomycin or microencapsulated OA and EO did not statistically alter the number of ETEC F4 in the colon digesta of piglets. However, the supplementation of microencapsulated OA and EO reduced diarrhea score at 28 hpi compared to the CC piglets, which may imply that microencapsulated OA and EO potentially reduced the pathogenicity of ETEC F4 or modulated gut microbiota in piglets challenged with ETEC F4, while microencapsulated OA and EO could not inhibit the growth of ETEC F4.

In summary, ETEC F4 infection decreased growth performance, induced inflammatory response and diarrhea, damaged gut morphology, impaired digestive enzymes and nutrient transporters, and decreased the gut barrier integrity of weaned piglets. While the supplementation of Aureomycin increased mRNA expression of an amino acid transporter and gut permeability of weaned piglets challenged with ETEC F4. The supplementation of microencapsulated OA and EO enhanced intestinal morphology and showed anti-diarrhea effects at one time point in weaned piglets challenged with ETEC F4. Therefore, whereas the supplementation of microencapsulated OA and EO did not attenuate the induced inflammation, reduced digestive enzyme activities and increased gut permeability in the piglets infected with ETEC F4 in this study, microencapsulated OA and EO combination partially enhanced gut health (e.g. diarrhea score and intestinal morphology) in the piglets infected with ETEC F4. This may imply that microencapsulated OA and EO combination can

be useful within the tools to be implemented in strategies for alternatives to antibiotics in swine production.

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CONFLICT OF INTEREST STATEMENT

The authors declare the following competing interest(s): Ludovic Lahaye and Elizabeth Santin are coauthors in this manuscript. They are employees at Jefo Nutrition Inc. that provided microencapsulated organic acids and essential oils to this work.

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Figure legends

Figure 1. Effects of microencapsulated organic acids and essential oils on core body temperature in weaned piglets. Core body temperature of weaned piglets was measured in the SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate-buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 $mg \cdot kg^{-1}$ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); and P(OA+EO) (microencapsulated organic acids and essential oils): CC + 2 $g \cdot kg^{-1}$ of a selected formula of organic acids [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada) groups during 48 hpi (hours post-inoculation). Each value represents the mean ± SEM. At the same time point, SCC was compared with SC (unpaired t test), and the comparison was presented as * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$. The ETEC F4 challenged groups (CC, AGP and P(OA+EO)) was compared by PROC MIXED followed by the Tukey's multiple comparison test.

Figure 2. Effects of microencapsulated organic acids and essential oils on diarrhea score in weaned piglets. Diarrhea score of weaned piglets was measured in the SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate-buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 $mg \cdot kg^{-1}$ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); and P(OA+EO) (microencapsulated organic acids and essential oils): NC + 2 $g \cdot kg^{-1}$ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada) groups during 54 hpi (hour post-inoculation).

Diarrhea score = 0, normal feces; 1, soft feces; 2, mild diarrhea; and 3, severe diarrhea. Each value represents the mean ± SEM. Bars with different letters at the same time point are significantly different ($P < 0.05$) by PROC MIXED followed by the Tukey's multiple comparison test among SCC, SC and P(OA+EO). At the same time point, SCC was compared with SC using Mann-Whitney U-test, and the comparison was presented as * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$. Bars with different letters at the same time point are significantly different ($P < 0.05$) compared by the Kruskal-Wallis test followed by the Dunn's multiple comparison test among SCC, SC and P(OA+EO).

Figure 3. Effects of microencapsulated organic acids and essential oils on the relative abundance of proteins associated with gut barrier integrity and nutrient transporters in weaned piglets. Mid-jejunal relative protein abundance of zonula occludens 1 (ZO1), occludin (OCLN), and neutral amino acid transporter 1 (B^0AT1) was measured in the SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate-buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 $mg \cdot kg^{-1}$ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); and P(OA+EO) (microencapsulated organic acids and essential oils): CC + 2 $g \cdot kg^{-1}$ of a selected formula of organic acids [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada) groups. Each value represents the mean ± SEM. Bars with different letters are significantly different ($P < 0.05$) by PROC MIXED followed by the Tukey's multiple comparison test among SCC, SC and P(OA+EO). At each time point, SCC was compared with SC (unpaired t test), and the comparison was presented as * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$. The ETEC F4 challenged groups (CC, AGP and P(OA+EO)) was compared by PROC MIXED followed by the Tukey's multiple comparison test.

Figure 4. Effects of microencapsulated organic acids and essential oils on DNA abundance of faeG (F4 fimbriae) in the colon digesta in weaned piglets. DNA abundance of faeG (F4 fimbriae) in the colon digesta (20 cm from the ileum-cecum junction) was measured in the SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate-buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 $mg \cdot kg^{-1}$ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (microencapsulated organic acids and essential oils): CC + 2 $g \cdot kg^{-1}$ of a selected formula of organic acids [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada) groups. Data were presented as $\log_{10}$(faeG gene copies·µg DNA^{-1}). Each value represents the mean ± SEM. At each time point, SCC was compared with SC (unpaired t test), and the comparison was presented as * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$. Bars with different letters at the same time point are significantly different ($P < 0.05$) by PROC MIXED followed by the Tukey's multiple comparison test among SCC, SC and P(OA+EO).

Table 1. Primers used in the study[1]

Genes	Amplicon	Sequence (5' to 3')	References
estA	158	CAACTGAATCACTTGACTCTT TTAATAACATCCAGCACAGG	(Noamani et al., 2003)
estB	113	TGCCTATGCATCTACACAAT CTCCAGCAGTACCATCTCTA	(Noamani et al., 2003)
elt	322	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT	(Reischl et al., 2002)
faeG	215	ACTGGTGATTTCAATGGTTCG GTTACTGGCGTAGCAAATGC	(Zhu et al., 2011)
MUC4	367	GTGCCTTGGGTGAGAGGTTA CACTCTGCCGTTCTCTTTCC	(Jensen et al., 2006)
CycA	160	GCGTCTCCTTCGAGCTGTT CCATTATGGCGTGTGAAGTC	(Farkas et al., 2015)
ZO1	200	GATCCTGACCCGGTGTCTGA TTGGTGGGTTTGGTGGGTT	(Omonijo et al., 2018a)
CLDN1	220	CTGTGGATGTCCTGCGTGT GGTTGCTTGCAAAGTGGTGTT	
CLDN3	123	CTACGACCGCAAGGACTACG TAGCATCTGGGTGGACTGGT	(Omonijo et al., 2018a)
OCLN	93	CTGTGGATGTCCTGCGTGT GGTTGCTTGCAAAGTGGTGTT	(Lee and Kang, 2017)
MUC2	90	CCAGGTCGAGTACATCCTGC GTGCTGACCATGGCCCC	

SGLT1	153	GGCTGGACGAAGTATGGTGT GAGCTGGATGAGGTTCCAAA	(Yang et al., 2010)
PepT1	143	ATCGCCATACCCTTCTG TTCCCATCCATCGTGACATT	(Omonijo et al., 2018a)
B^0AT1	102	AGGCCCAGTACATGCTCAC CATAAATGCCCCTCCACCGT	(Yang et al., 2016)
EAAC1	168	CCAAGGTCCAGGTTTTGGGT GGGCAGCAACACCTGTAATC	(Omonijo et al., 2018a)
ASCT2	206	GCCAGCAAGATTGTGGAGAT GAGCTGGATGAGGTTCCAAA	(Yang et al., 2016)
IL8	126	CACCTGTCTGTCCACGTTGT AGAGGTCTGCCTGGACCCCA	(Omonijo et al., 2018a)
IL10	220	CATCCACTTCCCAACCAGCC CTCCCCATCACTCTCTGCCTTC	(Lee and Kang, 2017)
IL1β	91	TGGCTAACTACGGTGACAACA CCAAGGTCCAGGTTTTGGGT	
TLR2	109	ACATGAAGATGATGTGGGCC TAGGAGTCCTGCTCACTGTA	(Tohno et al., 2005)
TLR5	86	GTTCTTTATCCGGGTGACTT AATAAGTCAGGATCGGGAGA	
TLR7	107	GCTGTTCCCACTGTTTTGCC GAGCTGGATGAGGTTCCAAA	
MGA	118	GCCCCTTCTGCATGAGTTCT CGTCACTTTCTCTGCACCCT	

SI	113	AGAAACTTGCCAGTGGAGCA TCCTGGCCATACCTCTCCAA
APN	114	GGACGATTGGGTCTTGCTGA GGGATGACCGACAGGTTTGT

[1]Note: estA: Sta, heat stable toxin A; estB: STb, heat stable toxin B; elt: LT, heat labile toxin; faeG: F4 fimbriae; MUC4: Mucin 4; CycA: Cyclophilin-A; ZO1: Zonula occludens 1; CLDN1: Claudin 1; CLDN3: Claudin 3; OCLN: Occludin; MUC2: Mucin 2; IL8: Interleukin 8; IL10: Interleukin 10; IL1β: Interleukin 1β; TLR2: Toll like receptor 2; TLR5: Toll like receptor 5; TLR7: Toll like receptor 7; SGLT1: Na^+-glucose cotransporter 1; PepT1: Peptide transporter 1; ASCT2: Neutral amino acid transporter 2; EAAC1: Excitatory amino acid transporter 1; B^0AT1: Neutral amino acid transporter 1; MGA: Maltase-glucoamylase; SI: Sucrase-isomaltose; APN: Aminopeptidase N.

Ingredients, kg, as-fed basis	Basal diet

Table 2. The ingredient composition of the basal diet, kg·ton^{-1}, as-fed basis[1].

Corn	483.84
Soybean meal (480 g crude protein·kg^{-1})	160
Whey permeate	124.2
X-SOY600[2] (600 g crude protein·kg^{-1})	110
Fish meal	65.73
Soybean oil	15
Limestone	14.32
Monocalcium phosphate[3]	5.73
Salt - bulk fine	5
Vitamin-mineral premix[4] (1%)	10
L-lysine 78%	2.83
DL-methionine 99%	1.52
L-threonine	1.32
L-tryptophan	0.51
Total	1,000.00
Calculated net energy and nutrient content	
Metabolizable energy (MJ·kg^{-1})	14.18
Net energy (MJ·kg^{-1})	10.35
Crude protein (%)	22.35
SID[5] Lysine	1.34
SID[5] Methionine	0.5
SID[5] Threonine	0.87
SID[5] Tryptophan	0.27

[1]The diet for antibiotic growth promoters (AGP) was prepared by adding 55 mg·kg^{-1} of Aureomycin 220G (Zoetis Canada Inc., Kirkland, QC, Canada) into the basal diet. The diet for P(OA+EO) was prepared by adding 2 g·kg^{-1} of a selected formula of [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

[2]Soy protein concentration (CJ Selecta, Goiania, State of Goiás, Brazil).

[3]Monocalcium phosphate containing Ca, 21% and P, 17% (The Mosaic Co., Plymouth, MN)

[4]Supplied the following per kilogram of diet: 2,200 IU vitamin A, 220 IU vitamin D_3, 16 IU vitamin E, 0.5 mg vitamin K, 1.5 mg vitamin B1, 4 mg vitamin B2, 12 mg calcium pantothenate, 600 mg choline chloride, 30 mg niacin, 7 mg pyridoxine, 0.02 mg vitamin B12, 0.2 mg biotin, 0.3 mg folic acid, 0.14 mg calcium iodate, 6 mg Cu (copper sulphate), 100 mg Fe (ferrous sulfate), 4 mg manganese oxide, 0.3 mg sodium selenite, and 100 mg zinc oxide.

[5]Standardized ileal digestible amino acids.

Table 3. Effects of microencapsulated organic acids and essential oils on the growth performance of weaned piglets during the pre-challenge period (0-7 d), post-challenge period (7-11 d) and whole period (0-11 d).

Items	SCC[1]	ETEC F4-challenged[2]				
		CC	AGP	P(OA+EO)	SEM	*P* value
Initial BW, kg	8.55	8.46	8.49	8.48	0.25	0.99
Pre-challenge						
BW, kg	9.97	10.25	10.02	10.20	0.35	0.88
ADG, g·d^{-1}	202	257	219	243	29	0.48
ADFI, g·d^{-1}	298	362	317	360	29	0.66
FCR, g·g^{-1}	1.73	1.42	1.55	1.55	0.13	0.75
Post-challenge[3]						
BW, kg	11.75	11.07	10.69	11.7	0.55	0.57
ADG, g·d^{-1}	446*	240	183	354	61	0.24
ADFI, g·d^{-1}	635	538	477	584	43	0.25
Whole period						
ADG, g·d^{-1}	291	251	195	284	36	0.28
ADFI, g·d^{-1}	420	422	364	441	33	0.28
FCR, g·g^{-1}	1.50	1.82	2.08	1.62	0.24	0.19

SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 mg·kg^{-1} of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (microencapsulated organic acid and essential oils): CC + 2 g·kg^{-1} of a selected formula of [(18%) fumaric, citric,

malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada); ADG: average daily gain; ADFI: average daily feed intake; BW: body weight; FCR: feed conversion ratio (Feed to gain ratio).

[1]SCC vs SC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[2]ETEC F4 challenged groups (CC, AGP and P(OA+EO)) was compared by PROC MIXED followed by the Tukey's multiple comparison test.

[3]The FCR during the post-challenge period was unable to be calculated because some pigs showed the minus ADG during the post-challenge period.

Table 4. Effects of microencapsulated organic acids and essential oils on electrophysiological properties including transepithelial electrical resistance (TEER) and Na^+-glucose cotransporter 1 (SGLT1) dependent short-circuit current and flux of fluorescein isothiocyanate–dextran 4 kDa (FITC-D4) of weaned piglets' jejunum mounted in Ussing chambers (*ex vivo*) and flux of fluorescein isothiocyanate–dextran 70 kDa (FITC-D70) in weaned piglets (*in vivo*).

Items	SCC[1]	ETEC F4-challenged				
		CC	AGP	P(OA+EO)	SEM	*P* value
Ex Vivo						
TEER, $\Omega \cdot cm^2$	41.77	50.00	42.19	54.70	14.92	0.88
SGLT1 dependent short-circuit current, $\mu A \cdot cm^{-2}$	80.85	48.84	42.10	54.57	8.08	0.53
FITC-D4 flux, $\mu g \cdot cm^{-2} \cdot h^{-1} \cdot mL^{-1}$	45.11	55.20	46.88	31.82	9.29	0.20
In vivo						
FITC-D70 flux, $\mu g \cdot mL^{-1}$	1,032*	1,357^{ab}	1,682^{a}	1,006^{b}	184.8	0.05

SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 $mg \cdot kg^{-1}$ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (microencapsulated organic acid and essential oils): CC + 2 $g \cdot kg^{-1}$ of a selected formula of [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada);

[1]SCC vs SC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$ by PROC MIXED followed by the Tukey's multiple comparison test among CC, AGP and P(OA+EO).

Table 5. Effects of microencapsulated organic acids and essential oils on morphology including villus height (VH), crypt depth (CD), VH:CD and the number of goblet cells per 100 µm VH and 100 µm CD in the mid-jejunum of weaned piglets.

		ETEC F4-challenged2				
Items	SCC[1]	CC	AGP	P(OA+EO)	SEM	*P* value
VH, µm	478**	364[b]	441[ab]	512[a]	38.75	0.04
CD, µm	278	250	250	270	17.02	0.62
VH:CD	1.96	1.90	1.94	2.11	0.23	0.79
Number of goblet cells per 100 µm VH	2.58	3.64	2.46	2.47	0.48	0.17
Number of goblet cell per 100 µm CD	5.71	6.04	5.62	5.91	6.03	0.75

SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 mg·kg^{-1} of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (microencapsulated organic acid and essential oils): CC + 2 g·kg^{-1} of a selected formula of [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

[1]SCC vs SC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$ by PROC MIXED followed by the Tukey's multiple comparison test among CC, AGP and P(OA+EO).

Table 6. Effects of microencapsulated organic acids and essential oils on the activities

(nmol·L^{-1}·mg protein^{-1}·min^{-1}) of brush border digestive enzymes in the mid-jejunum of weaned piglets.

Items	SCC[1]	ETEC F4-challenged[2]				
		CC	AGP	P(OA+EO)	SEM	*P* value
Aminopeptidase N	0.13	0.10	0.11	0.14	0.10	0.32
Intestinal alkaline phosphatase	0.45	0.40	0.52	0.46	0.04	0.16
Maltase	78.63*	54.04	70.64	76.11	10.78	0.33
Glucoamylase	4.36	3.95	4.02	4.49	0.67	0.78
Sucrase	14.43	11.57	12.90	15.93	4.12	0.69

SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 mg·kg^{-1} of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (microencapsulated organic acid and essential oils): CC + 2 g·kg^{-1} of a selected formula of [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

[1]SCC vs SC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[2]ETEC F4 challenged groups (CC, AGP and P(OA+EO)) was compared by PROC MIXED followed by the Tukey's multiple comparison test.

Table 7. Effects of microencapsulated organic acids and essential oils on the total antioxidant capacity (TAC), total glutathione (GSH), oxidized glutathione (GSSG), and reduced GSH:GSSG in the mid-jejunum of weaned piglets.

Items	SCC[1]	ETEC F4-challenged[2]				
		CC	AGP	P(OA+EO)	SEM	*P* value
TAC, mmol·L^{-1}·mg protein^{-1}	84.88	79.80	82.18	85.51	4.27	0.61
Total GSH, nmol·L^{-1}·mg protein^{-1}	3.26	3.09	2.98	2.95	0.02	0.32
GSSG, nmol·L^{-1}·mg protein^{-1}	0.44	0.42	0.50	0.55	0.19	0.87
Reduced GSH[3], nmol·L^{-1}·mg protein^{-1}	2.37	2.24	1.99	1.85	0.05	0.33
Reduced GSH:GSSG	5.46	5.69	4.06	3.81	0.21	0.44

SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 mg·kg^{-1} of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (microencapsulated organic acid and essential oils): CC + 2 g·kg^{-1} of a selected formula of [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

[1]SCC vs SC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.

[2]ETEC F4 challenged groups (CC, AGP and P(OA+EO)) was compared by PROC MIXED followed by the Tukey's multiple comparison test.

[3]Reduced GSH = Total GSH – 2 × GSSG.

Table 8. Effects of microencapsulated organic acids and essential oils on the relative mRNA abundance of genes associated with gut barrier integrity, nutrient transporters, immune system, and digestive enzymes in the mid jejunum of weaned piglets[1].

Items	SCC[1]	ETEC F4-challenged				
		CC	AGP	P(OA+EO)	*P* value	SEM
Gut barrier integrity						
ZO1	1.02	0.78	0.87	0.72	0.71	0.14
CLDN1	1.04**	0.61	0.50	0.41	0.22	0.08
CLDN3	1.01**	0.75	1.15	0.92	0.24	0.15
OCLN	1.03	0.89	0.86	0.81	0.91	0.15
MUC2	1.01***	0.40	0.49	0.33	0.71	0.18
Nutrient transporters						
SGLT1	1.01***	0.50	0.69	0.52	0.49	0.12
PepT1	1.05	0.64	0.80	0.73	0.82	0.18
B^0AT1	1.02***	0.36^b	0.84^a	0.49^{ab}	0.03	0.09
EAAC1	1.06	0.76	0.81	0.96	0.73	0.18
ASCT2	1.03	1.18	1.11	1.02	0.97	0.46
Immune system						
IL8	1.02*	1.92	1.03	1.02	0.08	0.30
IL10	1.09	1.04	0.73	1.01	0.50	0.20
IL1β	1.02	0.99	0.78	0.72	0.44	0.15
TLR2	1.04	1.35	0.82	1.12	0.32	0.24
TLR5	1.02	0.86	0.52	0.55	0.43	0.20
TLR7	1.05	0.75	0.70	0.65	0.81	0.11

Digestive enzymes						
MGA	1.01***	0.41	0.61	0.53	0.70	0.14
SI	1.04*	0.56	0.65	0.63	0.18	0.13
APN	1.00**	0.49	0.64	0.57	0.39	0.48

SCC (sham-challenged control): pigs fed a control diet and challenged with phosphate buffered saline; CC (challenged control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; AGP (antibiotic growth promoters): CC + 55 mg·kg^{-1} of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (microencapsulated organic acid and essential oils): CC + 2 g·kg^{-1} of a selected formula of [(18%) fumaric, citric, malic and sorbic acids] and essential oils [(0.5%) thymol, vanillin and eugenol] microencapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

[1]Note: ZO1: Zonula occludens 1; CLDN1: Claudin 1; CLDN3: Claudin 3; OCLN: Occludin; MUC2: Mucin 2; SGLT1: Na^+-glucose cotransporter 1; PepT1: Peptide transporter 1; ASCT2: Neutral amino acid transporter 2; EAAC1: Excitatory amino acid transporter 1; B^0AT1: Neutral amino acid transporter 1; IL8: Interleukin 8; IL10: Interleukin 10; IL1β: Interleukin 1β; TLR2: Toll like receptor 2; TLR5: Toll like receptor 5; TLR7: Toll like receptor 7; MGA: Maltase-glucoamylase; SI: Sucrase-isomaltose; APN: Aminopeptidase N.

[1] SCC vs SC (unpaired *t* test): * $0.05 < P < 0.10$, ** $P < 0.05$, *** $P < 0.01$

[a,b] Values within a row with different superscripts differ significantly at $P < 0.05$ by PROC MIXED followed by the Tukey's multiple comparison test among CC, AGP and P(OA+EO)

Figure 1

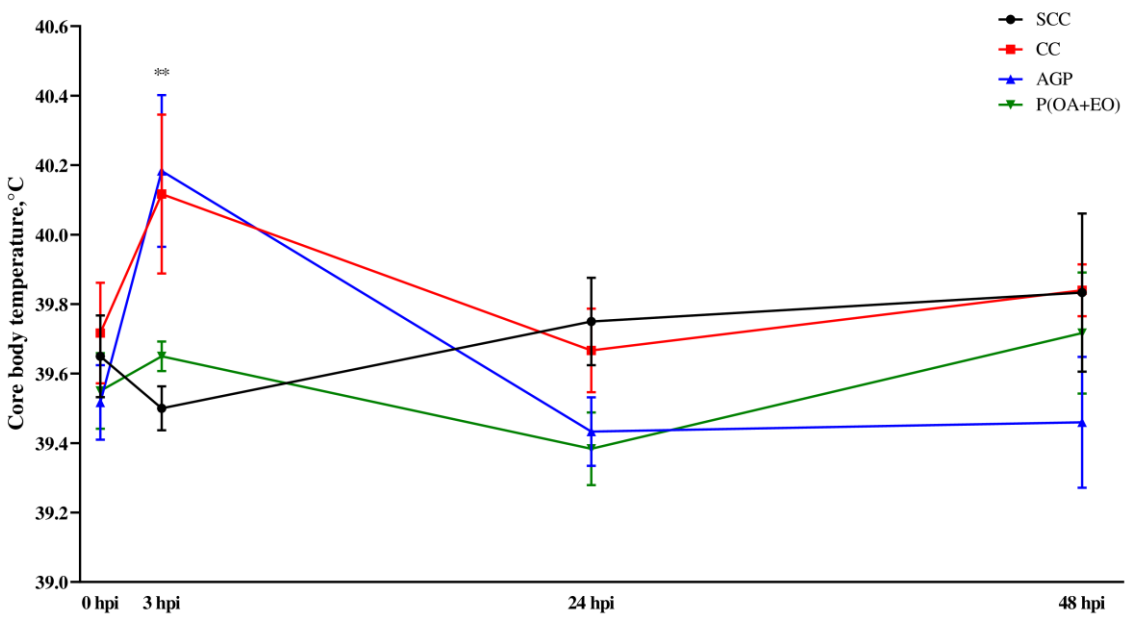

Figure 2

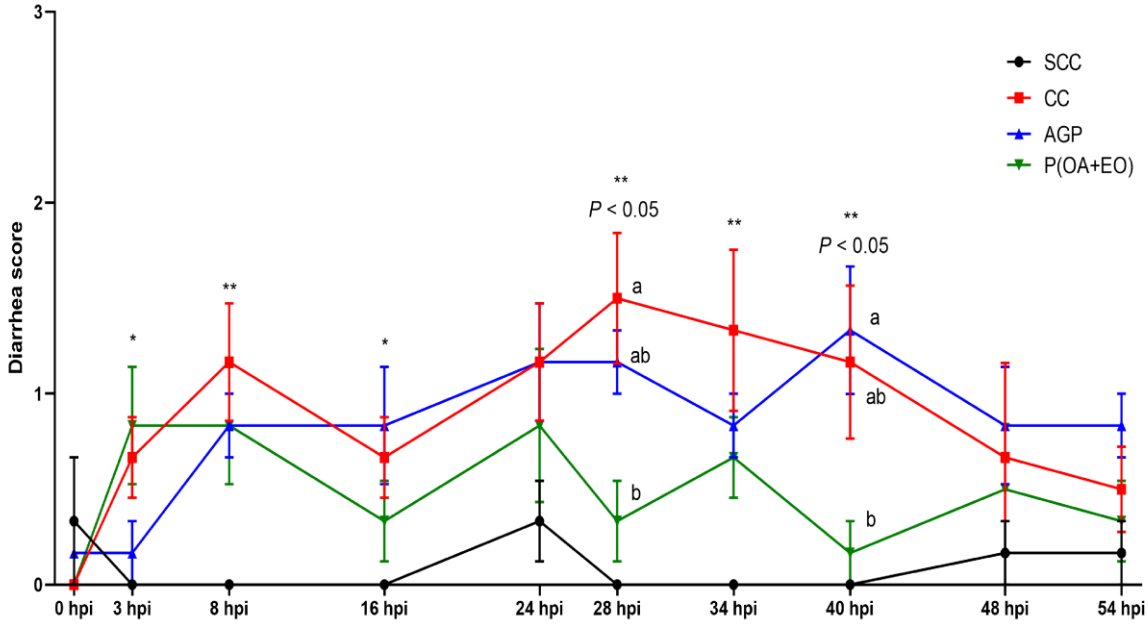

Figure 3

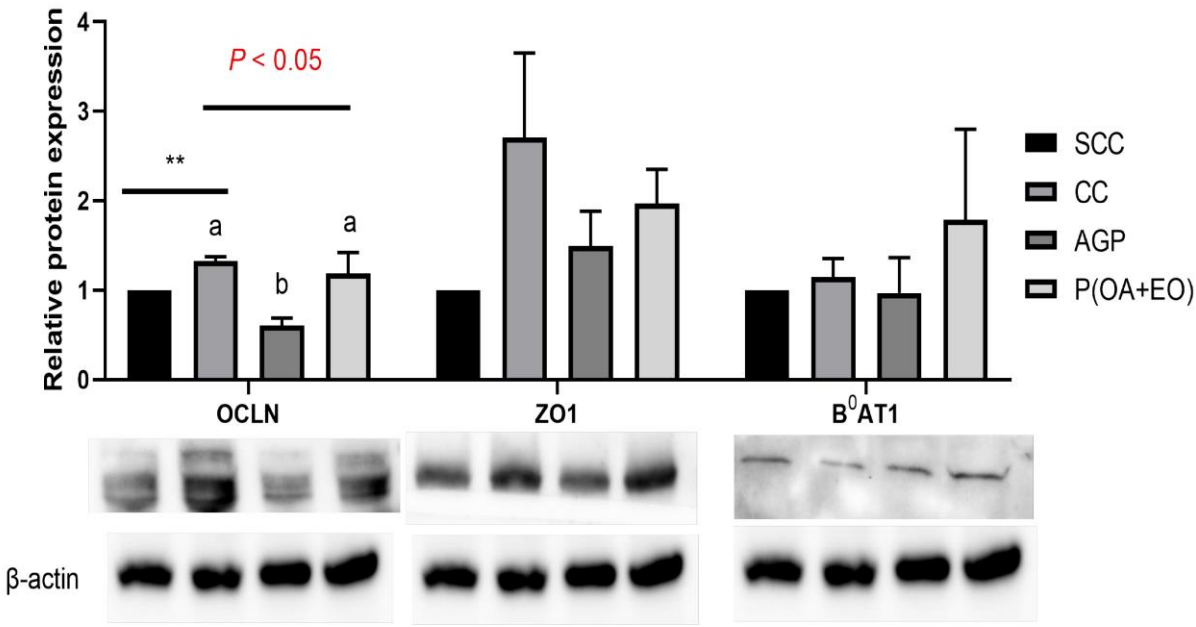

Figure 4