

**LIPID MATRIX MICROENCAPSULATION FOR
EFFECTIVE DELIVERY OF ESSENTIAL OILS
AND ORGANIC ACIDS TO IMPROVE GUT
HEALTH IN WEANED PIGLETS**

By

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ABSTRACT

Essential oils (EO) are considered as one of the most promising antibiotic alternatives in the swine industry due to their gut health-promoting effects. However, EO are very volatile, evaporate quickly during feed processing and storage, and are rapidly absorbed in the upper gastrointestinal tract in pigs. Micro-encapsulation (e.g., lipid matrix micro-encapsulation) has been popularly used to deliver bioactive compounds (e.g., EO and vitamins) to the animal's gut. However, there is a lack of information on the stability of EO during feed processing and storage, and the intestinal release of EO from the lipid matrix microparticles in weaned piglets. More studies are still needed to comprehensively understand the mechanisms behind the protection of micro-encapsulated EO against pathogens in weaned piglets. Therefore, the purposes of the thesis were to 1) evaluate the stability of thymol microencapsulated in combination with organic acids (OA) in commercially available lipid matrix microparticles during feed pelleting process and storage; 2) determine the intestinal release of thymol from the lipid matrix microparticles with *in vitro* and *in vivo* approaches; and 3) investigate the effects microencapsulated OA and EO on growth performance, immune system, gut barrier function, nutrient absorption, and microbiota in weaned piglets challenged with enterotoxigenic *Escherichia coli* (ETEC) F4. The lipid matrix microparticles were able to maintain the stability of thymol during a feed pelleting process and storage (12 weeks) and allow a slow and progressive intestinal release of thymol in the weaned piglets. Moreover, the supplementation of micro-encapsulated OA and EO alleviated diarrhea and inflammation response, and improved gut barrier integrity, intestinal morphology, enzyme activities, and nutrient transport in the weaned piglets experimentally infected with ETEC F4. In conclusion, micro-encapsulated OA and EO can improve gut health in weaned piglets with physiological challenges and can be used as an alternative to antibiotics for swine production.

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LIST OF ABBREVIATIONS

AB/PAS	Alcian blue/The periodic acid–Schiff
ADFI	Average daily feed intake
ADG	Average daily gain
AGP	Antibiotic growth promoters
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
APN	Aminopeptidase N
ASCT2	Neutral amino acid transporter 2
B ⁰ AT1	Neutral amino acid transporter 1
BW	Body weight
Ca	Calcium
CaSR	Calcium sensing receptors
CD	Crypt depth
CD 4 ⁺	Cluster of differentiation 4 ⁺
CLDN1	Claudin 1
CLDN3	Claudin 3
Ct	Threshold cycle
Cu	Copper
CycA	Cyclophilin-A
ddPCR	Droplet digital PCR
DEPC	Diethylpyrocarbonate
DF	Dietary fiber
DGGE	Denaturing gradient gel electrophoresis
DHA	Docosahexaenoic acid
dpi	Day post-inoculum
EAAC1	Excitatory amino-acid carrier 1

EO	Essential oils
EPA	Eicosapentaenoic acid
ETEC	Enterotoxigenic <i>Escherichia coli</i>
FCR	Feed conversion ratio
FISH	Fluorescent in situ hybridization
FITC-D4	Fluorescein isothiocyanate-dextran 4 kDa
FITC-D70	Fluorescein isothiocyanate-dextran 70 kDa
FUT1	Fucosyltransferase 1
GC-FID	Gas chromatography – flame ionization detector
GIP	Glucose dependent insulintropic peptide
GLP1	Glucagon like peptide 1
GLP2	Glucagon like peptide 2
GSH	Glutathione
GSSG	Oxidized glutathione
hpi	Hour post-inoculum
IAP	Intestinal alkaline phosphatase
IL10	Interleukin 10
IL1 β	Interleukin 1 β
IL6	Interleukin 6
IL8	Interleukin 8
IPEC-J2	Porcine intestinal epithelial cells
KRB	Krebs ringer buffers
LCFA	Long chain fatty acids
LPS	Lipopolysaccharides
LTB4	Leukotriene B4
MCFA	Medium chain fatty acids
MGA	Maltase-glucoamylase

MHC	Major histocompatibility complex
MLCK	Myosin light chain kinase
MUC2	Mucin 2
MUC4	Mucin 4
MUPP1	Multi-PDZ domain protein 1
NMR	Nuclear magnetic resonance
NSP	Non-starch polysaccharides
OA	Organic acids
OCLN	Occludin
P	Phosphorous
P53	Tumor protein 53
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PepT1	Peptide transporter 1
PGC1 α	Peroxisome proliferator-activated receptor gamma coactivator 1 α
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene difluoride
PWD	Post-weaning diarrhea
Q-PCR	Quantitative PCR
RIPA	Radioimmunoprecipitation assay
ROS	Oxygen reactive species
SBM	Soybean meal
SCFA	Short chain fatty acids
SGF	Simulated gastric fluid
SGLT1	Na ⁺ -glucose cotransporter 1
SI	Sucrase-isomaltase
SIF	Simulated intestinal fluid

SSCP	Single strand conformation polymorphism
T1R2	Type 1 taste receptors 2
T1R3	Type 1 taste receptors 3
TAC	Total antioxidant capacity
TBST	Tris-buffered saline with 0.1% Tween 20
TEER	Transepithelial electrical resistance
TGGE	Temperature gradient gel electrophoresis
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR7	Toll-like receptor 7
TNF- α	Tumor necrosis factor- α
T-RFLP	Terminal-restriction fragment length polymorphism
TSA	Tryptic soy agar
VH	Villus height
V_{max}	Maximal enzyme activity
ZnO	Zinc oxide
ZO1	Zonula occludens 1
ZO2	Zonula occludens 2
ZO3	Zonula occludens 3

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1.0 CHAPTER 1 GENERAL INTRODUCTION

Weaning is one of the most demanding and complex phases during which piglets confront diverse stressors such as a sudden separation from their dam, sharing space with new litters, and a diet change from liquid milk to a solid feed (Vente-Spreewenbergh et al., 2003). During the weaning phase, piglets frequently have diarrheic syndromes and other intestinal disturbances because piglets have an anatomically and functionally immature “gut ecosystem” (Domeneghini et al., 2006). The correct and timely functional development of the “gut ecosystem” is essential for piglets to remain protected from the dramatic changes that occur in the weaning phase (Domeneghini et al., 2006). The term “gut ecosystem” is based on the idea that various components of the gut such as gut morphology, digestive enzymes, nutrient transporters and sensors, immune system, and gut barrier integrity are interconnected and interact with each other (Fig. 1.1).

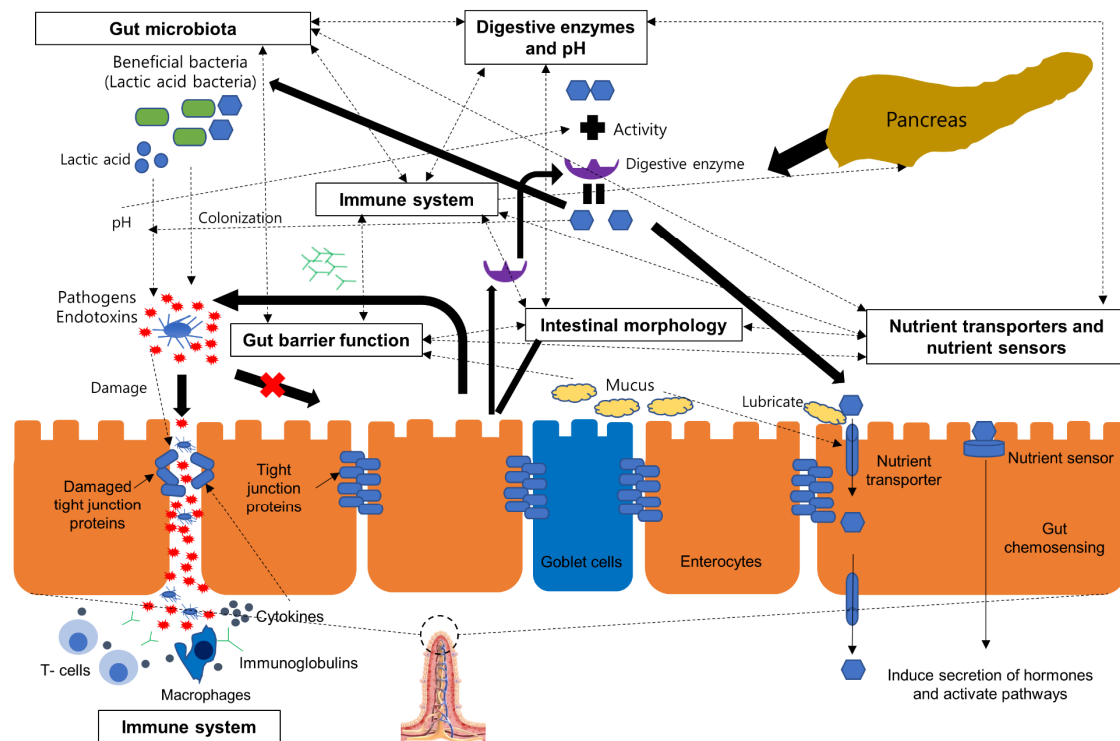


Figure 1.1 Schematic illustration of the gut ecosystem of pigs. The components of the gut ecosystem including intestinal morphology, digestive enzymes, gut barrier function, nutrient transporters and sensors, and gut microbiota are complexly interconnected and interact with each other. Because digestive enzymes, immunoglobulins, and mucus are secreted from villus and crypts, gut morphology affects digestive enzymes, the immune system and gut barrier function (Kong et al., 2018). In addition, gut morphology affects nutrient transporting because mucus secreted from goblet cells has functions of lubricating nutrients to be transported (Kim and Ho, 2010). Digestive enzymes can affect gut microbiota and the immune system by modulating gut pH and releasing more beneficial nutrients from substrates. Gut barrier function can be modulated by gut microbiota because pathogens and toxins damage tight junction proteins and by the immune system since cytokines modulate the expression of the tight junction proteins (Al-Sadi et al., 2009). Gut microbiota influences the pH of the gut because beneficial bacteria produce lactic acid and short-chain fatty acids, and it affects the immune

system because pathogens and toxins can damage the immune system (Flint et al., 2012). Because the expression of nutrient transporters and sensors are affected by the available nutrients, nutrient transporter and sensors can be affected by digestive enzymes and gut microbiota which compete for nutrients with the host (Zhang et al., 2013). Nutrients sensors can affect the development of the gut ecosystem and secretion of digestive enzymes by releasing diverse hormones (Janssen and Depoortere, 2013). The components of the gut ecosystem are closely interconnected.

Antibiotic growth promoters (AGP) have been supplemented to piglet's diet because of their effectivity in augmenting growth rate, controlling diarrhea and reducing mortality due to diseases (Cromwell, 2002). However, the concerns of drug residues in meat products and producing drug-resistant bacteria which can be delivered to both livestock and humans have led to the ban or restriction of AGP use in the swine industry (Thacker, 2013). European Union has banned the use of AGP in animal production and many authorities and countries are expected to follow (Bengtsson and Wierup, 2006). Canada have also restricted the use of AGP in livestock production since December 2018 (Omonijo, 2018). However, according to World bank (2017), global antibiotic consumption in livestock was approximated to range from 63,000 to over 240,000 metric tons yearly, and these quantities may have increased due to increased population and developed economy (Murphy et al., 2017; Vieco-Saiz et al., 2019). While the ban of antibiotics was essential to prevent the transmission of antibiotic-resistant bacteria from the livestock, the prohibition on AGP in animal feed induced a reduction in the efficiency of animal production because of higher frequency of infection in the animals (Cheng et al., 2014). Therefore, there is an urgent need to find appropriate alternatives for antibiotics. Alternatives for AGP should have antimicrobial and growth-promoting effects without causing bacterial resistance and side effects to livestock and humans (Yang et al., 2015). Diverse AGP alternatives such as EO (Dong et al., 2019), organic acids (OA) (Rasschaert et al., 2016; Upadhaya et al., 2016), medium chain fatty acids (MCFA) (Kuang et al., 2015), probiotics (Zhou et al., 2015), prebiotics (Liu et al., 2018), bacteriophages (Kim et al., 2017a; Lee et al., 2017) and their synergistic effects have been studied and some of these are practically applied in the swine industry.

Essential oils (EO), which are synthesized via secondary metabolic pathways of plants and play an important role in defending the plant against pathogens, have gained a lot of

attention in several fields due to their diverse and relevant biological activities (Li et al., 2019). Among the benefits of EO, antimicrobial effects have made EO to be used in the medical and food industry (Swamy et al., 2016). For instance, thymol and carvacrol effectively controlled the oral pathogens and food-borne pathogens in meat products (Ramos et al., 2013). In the swine industry, EO were also applied as AGP alternatives due to their antimicrobial, antioxidative and anti-inflammatory properties (Omonijo et al., 2018b). However, EO have lipophilic and volatile properties, which could be obstacles in the delivery of EO within the pig gut (Zhang et al., 2016). Most of the EO, without proper protection, are evaporated or oxidized during feed processing and delivery to the pig gut, and thus little amount of EO may be able to reach the lower gut of pigs where most pathogens reside and propagate (Zhang et al., 2014).

Encapsulation has become one of the most popular methods to deliver EO into the lower gut (Yang et al., 2016b). An ideal encapsulation should not only present the stability of EO but also release EO specifically in the target regions of the intestine (Chen et al., 2016). Many materials including polysaccharides (alginate and xanthan gum), proteins (whey protein and gelatin) and lipids (milk fat and hydrogenated oil) have been used to encapsulate EO for effective delivery in the gut (El Asbahani et al., 2015). Hydrogenated oil has been considered one of the most cost-effective materials for encapsulating EO in the feed because hydrogenated oil has low cytotoxicity (Müller et al., 2000) and higher stability (Souto and Müller, 2010). Therefore, lipid matrix micro-encapsulation) has been popularly used to deliver bioactive compounds (e.g., EO and vitamins) to the animal's gut. However, there is a lack of information on the stability of EO during feed processing and storage, and the intestinal release of EO from the lipid matrix microparticles in weaned piglets. More studies are still needed to comprehensively understand the mechanisms behind the protection of micro-encapsulated EO against pathogens in weaned piglets.

The following literature review summarizes the properties of gut ecosystem components during weaning, evaluation methods of AGP alternatives, and possible nutrients and feed additives to replace AGP.

2.0 CHAPTER 2 LITERATURE REVIEW

2.1 Gut ecosystem and its alteration during weaning phase

2.1.1 Gut morphology

Gut morphology is predominately related to the area, height, and density of the villus and crypts. Villus bulges into the lumen covered mainly with mature enterocytes and accompanied by occasional mucus-secreting goblet cells have functions of nutrient digestion and absorption, along with defending against pathogens and toxins (Hooper, 2015). Enterocytes are the major cell type in the intestinal epithelium in villus and play crucial roles in nutrient absorption and secretion of digestive enzymes and immunoglobulins (Kong et al., 2018). Goblet cells, which account for around 10% of intestinal epithelial cells, secrete mucus to protect the intestinal wall from pathogenic bacteria and toxins and to lubricate the passage of nutrients through the intestinal wall (Kim and Ho, 2010). Crypts are moat-like invaginations of the epithelium around the villus and are lined with largely younger epithelial cells that migrate to the villus tip as they mature (Brown et al., 2006). Increased villus height (VH) and decreased crypt depth (CD) represent a development in the digestion and absorption of nutrients (Hou et al., 2010). Increased VH means more surface for the absorption process and more epithelial cells in the small intestine, which have important roles in digestion and immunity; a decreased depth of crypts indicates that the epithelial cells in the small intestinal villus are growing rapidly (Zhang and Xu, 2006). The ratio of the VH/CD is a useful tool for estimating the digestive capacity in the small intestine because the decreased VH is less

detrimental when there is not also an increased CD (Hedemann et al., 2006). Weaned piglets undergo changes such as villus atrophy and crypts hyperplasia potentially due to post-weaning anorexia. From another perspective, villus atrophy and crypts hyperplasia can occur due to weaning stress because the morphological alteration can happen even in the presence of continuous nutrient supply (Kelly et al., 1991). The possible reasons for villus atrophy and crypts hyperplasia without post-weaning anorexia may be due to the increased concentration of blood glucagon, one of the stress-associated hormones and can decrease piglet's absorption ability (van Beers-Schreurs et al., 1992). The components of gut morphology, including villus and crypts, are closely related to nutrient digestion and absorption and protection from pathogenic bacteria and toxins, and the weaning process with or without post-weaning anorexia can damage the gut morphology of piglets.

2.1.2 Digestive enzymes and pH of gut

The nutrient digestive capacity of pigs is closely associated with the activities of digestive enzymes in the stomach, pancreas, and intestinal mucosa (Qian et al., 2016). Diverse enzymes are secreted in the stomach, pancreas and intestinal mucosa (Table 2.1). The activity of digestive enzymes can be altered at weaning due to complex interaction among the composition of the diet, feed intake, pH of the gut, and weaning stress (Hedemann and Jensen, 2004). The activity of pepsin, whose optimum is less than 2, can be decreased during the weaning phase (Lee et al., 2008). The possible explanation is that stomach pH can be increased during weaning possibly because acid secretion is reduced from parietal cells in the stomach at weaning accompanied by a reduction in lactic acid production from lactose (Efird et al., 1982). It is also vital to maintain low pH in the stomach of piglets for protection against the external environment to maintain an overall healthy gut ecosystem because low pH effectively reduces the passage of pathogenic bacteria into the small intestine (Heo et al., 2013). Gastric lipase

Table 2.1 Endogenous enzymes and their reaction in pigs.

Origin	Enzymes	Major enzyme reaction	References
Stomach	Pepsin	Polypeptides -> Polypeptide fragments	(Campos and Sancho, 2003)
	Gastric lipase	Triacylglycerol -> Diacylglycerol + Carboxylate	(Gargouri et al., 1986)
Pancreas	Lipase	Triacylglycerol r -> Diacylglycerol + Carboxylate	(Cera et al., 1990)
	Trypsin	Polypeptides -> Polypeptide fragments	(Makkink et al., 1994)
	Chymotrypsin	Polypeptides -> Polypeptide fragments	(Makkink et al., 1994)
	Amylase	Amylose -> Maltose and glucose	(Pandol et al., 1985)
Intestinal mucosa	Maltase-glucoamylase	Maltotriose and Maltose -> Glucose	(Van Beers et al., 1995)
	Sucrase-isomaltase	Sucrose -> Glucose and fructose; α -limit dextrin -> Glucose	(Van Beers et al., 1995)
	Lactase	Lactose -> Glucose + galactose	(Van Beers et al., 1995)
	Aminopeptidase	Polypeptides -> Amino acid + Polypeptide	(Maroux et al., 2018)
	Intestinal alkaline phosphate	Phosphate monoester -> Alcohol + Phosphate	(López-Canut et al., 2009)

plays a role in the digestion of triglycerides in piglets even though total pancreatic lipase activity is 600 times higher than the total gastric lipase activity (Newport and Howarth, 1985). The activity of gastric lipase dramatically decreased after weaning piglets and pancreatic lipase become the main enzyme to digest fat (DiPalma et al., 1991). However, there is a transition gap between the decrease of gastric lipase and the increase of pancreatic lipase, which results in lower fat digestibility during weaning (Jensen et al., 1997). Pancreatic enzymes play a crucial role in the digestion of the macronutrients and decreased the activity of pancreatic enzymes induces in the maldigestion of diet (Torres-Pitarch et al., 2017). Weaning stressors can dramatically decrease the activity and secretion of pancreatic enzymes (Lindemann et al., 1986). A study revealed that pancreatic enzyme activities were depressed in the first week after weaning probably for the adaption of enzyme levels in need, and were gradually recovered after weaning (Lindemann et al., 1986). Intestinal brush border enzymes are important to digest complex macronutrients to absorbable small nutrients (e.g. amino acids and glucose), which can be transported across the intestinal epithelium as a final step of digestion prior to absorption (Van Beers et al., 1995). During the weaning phase, lactase activity dramatically decreases due to the lack of lactose which was abundant in the sow milk (Miller et al., 1986). The activities of brush border enzymes can be affected the gut morphology because brush border enzymes are secreted from the enterocytes in the villus (Ma and Guo, 2008). A study by Zong et al. (2018) showed that brush border enzyme activities reached a minimum level between 3 and 5 d post-weaning and gradually recovered thereafter as an increase of substrate availability through feed intake and gut morphology were recovered. While enterocytes mainly secrete digestive enzymes, intestinal alkaline phosphatase (IAP), which has functions such as detoxification, maintenance of gut pH, modulation of gut inflammation, digestion of organic phosphate, and fat absorption can be secreted from enterocytes of pigs (Lackeyram et al., 2010).

In addition, because IAP is an intrinsic enzyme, which is more subtle to alterations in the brush border, the IAP activity may represent the gut maturation of pigs (Ghafoorunissa, 2001). Optimal digestive enzyme activities and pH are important for nutrient digestion and gut ecosystem of piglets and low enzyme activities can be accompanied by the alteration of gut pH and impaired gut morphology during weaning.

2.1.3 Nutrient transporters and sensors

Nutrient absorption can be divided into paracellular and transcellular pathways (Karasov, 2017). Nutrient transporters belong to transcellular absorption which represent either the uptake of small molecules by active (carrier-mediated) or passive (carrier-unmediated) transport (Wijtten et al., 2011). The expression of nutrient transporters is an important indicator for nutrient utilization capacity of animals (Moran et al., 2010b). Early weaning decreases the function of the Na⁺-glucose cotransporter 1 (SGLT1) and amino acid transport activities in the jejunum and ileum of piglets (Li et al., 2018). When pigs were weaned after 4 weeks, active absorption between 1 and 15 d after weaning was either similar to or higher than absorption before weaning, which showed that when pigs were weaned after 4 weeks, active absorption is not negatively affected by the weaning process (Lodemann et al., 2008). The activities of nutrient transporters can be recovered with the repair of intestinal architecture after the weaning phase (Lin et al., 2014). Regarding passive absorption, a study showed that 1 week after weaning, the absorption of D-xylose decreased to approximately 50% of the pre-weaning level (Kelly et al., 1990). Also, even 14 d after weaning, the absorption of D-xylose only reached 65% of the absorption level measured before weaning, which may imply that weaning can have a permanent effect on passive absorption (Miller et al., 1984). Wijtten et al. (2011) supposed that the decreased passive transcellular absorption after weaning is a defense mechanism to

protect the uncontrolled transport of potentially harmful agents from entering the body. Nutrient transporters are closely associated with the utilization of nutrients in pigs, and the weaning process can decrease the activities of nutrient transporters.

Nutrient sensors have been studied mainly to understand the dietary requirements and preferences of animals. However, a few studies found that nutrient sensors are also closely associated with the gut ecosystem of pigs (Lee et al., 2012a; Janssen and Depoortere, 2013). Not only do the porcine nutrient sensors exist in the oral cavity, but they also exist in different organs and act as a chemosensory system. The heterodimeric sweet taste receptors comprising Type 1 taste receptors 2 (T1R2) and Type 1 taste receptors 3 (T1R3) are expressed in intestinal enteroendocrine cells in pigs (Moran et al., 2010a). According to Daly et al. (2012), T1R2 and T1R3 has functions of intestinal glucose sensing, inducing GLP1 (glucagon like peptide 1), GLP-2 and GIP (glucose dependent insulinitropic peptide) release, which have been proved using endocrine cell lines, native intestinal tissue explants and knock out mice deficient in alpha-gustducin or T1R3. Shirazi-Beechey et al. (2014) showed that GLP1 and GIP improved insulin secretion and GLP2 improved intestinal growth and glucose absorption. Calcium sensing receptors (CaSR) also presented in the gastro-intestinal tract of pigs (Zhao et al., 2019). Fatty acid sensors 40 (GPR40), GPR 43, and GPR 120 were found in the gastrointestinal tract (Song et al., 2015). Nutrient sensory cells in the gut are known to be involved in the secretion of gut hormones and also other physiological functions (Roura et al., 2016). For instance, CaSR have functions of sensing nutrients, maintaining ion homeostasis, regulating the digestive process, controlling colonic fluid balance and inducing the growth of epithelial cells (Zhao et al., 2019). Most importantly, the activation of CaSR decreased the intestinal inflammatory response in weaned piglets (Huang et al., 2015). Not only do nutrient sensors have functions of nutrient sensing, but they are also involved in regulating the gut ecosystem of pigs.

2.1.4 Gut barrier integrity and tight junction proteins

Gut barrier integrity is maintained by a single layer of epithelium, mainly epithelial cells and tight junctions on the gastrointestinal tract of pigs (Wang et al., 2014). Tight junctions, multiprotein complexes located on the apical side of epithelial cells, play an important role in maintaining cell polarity and regulating barrier integrity that prevents pathogens and toxins from crossing the epithelial sheet between adjacent cells (Zhao et al., 2011). Tight junctions are constituted of transmembrane proteins including occludin (OCLN) and claudin (CLDN), junctional adhesion molecules, and peripheral membrane proteins such as zonula occludens 1 (ZO1), ZO2, ZO3, and the multi-PDZ domain protein 1 (MUPP1) (Moeser et al., 2017). The efficiency of cell-cell adhesion (e.g. gut barrier integrity) is determined by the quantity and distribution of the tight junctions (Li et al., 2012d). The weaning process impairs tight junction integrity and increases intestinal permeability which can induce the pathogenesis of numerous gastrointestinal diseases, such as inflammatory bowel disease, irritable bowel syndrome, celiac disease, and infectious enterocolitis (Odenwald and Turner, 2013). Compared with the preweaning stage (0 d), on 3 d, 7 d, and 14 d after weaning, jejunal transepithelial electrical resistance (TEER), which represents the intestinal mucosal barrier, was decreased, mRNA expression of OCLN, CLDN1 and ZO1 were reduced, and gut permeability (paracellular flux of dextran) was increased (Zhao et al., 2011). However, damaged gut barrier function and intestinal permeability in piglets recovered after 2 weeks of weaning (Peace et al., 2011). Gut barrier integrity is mainly maintained by tight junction proteins and can be damaged due to weaning stressors, resulting in diverse gastrointestinal diseases.

2.1.5 Immune system

Swine have a complex immune system which has functions of recognizing and attacking

pathogens and toxins. The immune system can be divided into two categories: innate immunity, which is general and non-specific and includes macrophages and cytokines; and adaptive immunity, which is specific and characterized by immunological memory, dendritic cells, and lymphocytes. Immune parameters to assess the activities of the immune system of pigs can be chosen based on the purpose of the experiment (Table 2.2). During the weaning period, the protective immunity shifts from passive maternal immunity to the active immunity of the piglet (Weiner et al., 2015). Due to the absence of necessary immune function during the weaning phase, piglets can have symptoms of diarrhea, inflammation, or even mortality (Han et al., 2016). Many studies documented that the immune system can be damaged, and an intestinal inflammatory response can be activated during the weaning phase. After 1 and 2 days of weaning, a decrease in jejunal expression of major histocompatibility complex (MHC) class 1 mRNA and an increase in the cluster of differentiation 4⁺ (CD 4⁺) T cells in jejunal villus were found in piglets weaned at 21 d of age (Heo et al., 2013). Moreover, compared to normal weaned piglets (20 d), early-weaned piglets (at 16 d and 18 d) challenged with Enterotoxigenic *Escherichia coli* (ETEC), had a lower number of mast cells and higher pro-inflammatory cytokines such as interleukin 6 (IL6) and IL8 in ileal mucosa (McLamb et al., 2013). Intestinal mast cells play an important role in the innate immune response to bacterial, parasitic and viral infections by releasing pro-inflammatory cytokines including tumor necrosis factor- α (TNF- α), IL6 and leukotriene B4 (LTB4) that mediate neutrophil recruitment into infected sites (Abraham and John, 2010). A study by Hu et al. (2013b) showed that after weaning, the expression of pro-inflammatory cytokines increased and returned to pre-weaning values within two weeks. The immune system, essential to piglets in protecting against pathogens and toxins, can be damaged during the weaning phase because of weaning stressors and a lack of protection derived from maternal milk.

Table 2.2 Parameters for evaluating the immune system of pigs.

Parameters	Category	Properties	References
CD3 ⁺ T cells (cluster of differentiation)	Adaptive immunity	Activated when antigens present	(Xiong et al., 2015b, a)
CD4 ⁺ T cells	Adaptive immunity	Activated when antigens present	(Shen et al., 2009; Xiong et al., 2015b, a)
CD8 ⁺ T cells	Adaptive immunity	Activated when antigens present	(Shen et al., 2009; Xiong et al., 2015b, a)
T-helper cell 17	Adaptive immunity	Effector memory T cells	(Luo et al., 2015)
Interferon- γ	Adaptive immunity	Pro-inflammatory cytokine	
Interleukin-1	Adaptive immunity	Pro-inflammatory cytokine	(Cavaillon, 2001; Lessard et al., 2015; Xiong et al., 2015b)
Interleukin-2	Adaptive immunity	Pro-inflammatory cytokine	(Sugiharto et al., 2015; Xiong et al., 2015b)
Interleukin-4	Adaptive immunity	Anti-inflammatory cytokine	(Cavaillon, 2001; Lessard et al., 2015)
Interleukin-6	Adaptive immunity	Pro-inflammatory cytokine	(Scheller et al., 2011; McLamb et al., 2013; Lessard et al., 2015; Wang et al., 2018a)
Interleukin-8	Adaptive immunity	Pro-inflammatory cytokine	(McLamb et al., 2013)
Interleukin-10	Adaptive immunity	Anti-inflammatory cytokine	(Cavaillon, 2001; Lessard et al., 2015; Sugiharto et al., 2015; Xiong et al., 2015b)
		Pro-inflammatory cytokine	(Shen et al., 2009)
Interleukin-12	Adaptive immunity	Pro-inflammatory cytokine	(Cavaillon, 2001; Lessard et al., 2015)
Interleukin-17A	Adaptive immunity	Pro-inflammatory cytokine	(Luo et al., 2015)

Interleukin-17F	Adaptive immunity	Pro-inflammatory cytokine	(Luo et al., 2015)
Interleukin-21	Adaptive immunity	Pro-inflammatory cytokine	(Luo et al., 2015)
Interleukin-22	Adaptive immunity	Pro-inflammatory cytokine	(Luo et al., 2015)
Tumor necrosis factor - α	Adaptive immunity	Pro-inflammatory cytokine	(Lessard et al., 2015; Wang et al., 2018a)
PGE ₂ (Prostaglandin)		Natural immunosuppressive molecule which reduces inflammatory responses.	(Kim et al., 2016)
Immunoglobulin G	Adaptive immunity	Antibody	(Takeyama et al., 2015)
Immunoglobulin A	Adaptive immunity	Antibody	(Sugiharto et al., 2015; Takeyama et al., 2015; Xiong et al., 2015b)
Immunoglobulin M	Adaptive immunity	Antibody	(Sugiharto et al., 2015; Rieckmann et al., 2018)
Natural killer cells	Innate immunity	Leukocyte	(Denyer et al., 2006; Annamalai et al., 2015)
Mast cells	Innate immunity	Leukocyte	(Pohl et al., 2017; Wang et al., 2018a)
Eosinophils	Innate immunity	Leukocyte	(Li et al., 2014)
Neutrophils	Innate immunity	Leukocyte	(Li et al., 2014)
Monocytes	Innate immunity	Leukocyte	(Li et al., 2014)

2.2 Gut microbiota

2.2.1 Understanding gut microbiota and its development

The lower gastrointestinal tract of swine is a natural shelter for diverse microbiota including bacteria, archaea, fungi, protozoans, and viruses, which have a symbiotic relationship with the animal (Barko et al., 2018). The microbiota has important roles in energy homeostasis, normal digestive functions, metabolism for vitamin synthesis, defense against pathogens, immunological activities such as catabolism of toxins, and neurodevelopment of pigs (Stanley et al., 2016). However, gut microbial populations such as *E. coli*, *Salmonella* spp., and *Clostridia* spp. can also induce diseases including post-weaning diarrhea (PWD) in the pig. There are both pros and cons of microbiota that can influence the animal's development and gut ecosystem (Fig. 2.1) (Pieper et al., 2006). During the weaning phase, weaning stress can result in a microbial imbalance because of the increased pathogenic bacteria including *E. coli* and *Salmonella* spp. and reduced beneficial bacteria such as lactic acid-producing bacteria including *Lactobacillus* spp. and *Bifidobacterium* spp. (Thu et al., 2011). While increased beneficial bacteria (e.g., lactic acid-producing bacteria) have a function of preventing diseases, an increase in pathogenic *E. coli* can account for PWD.

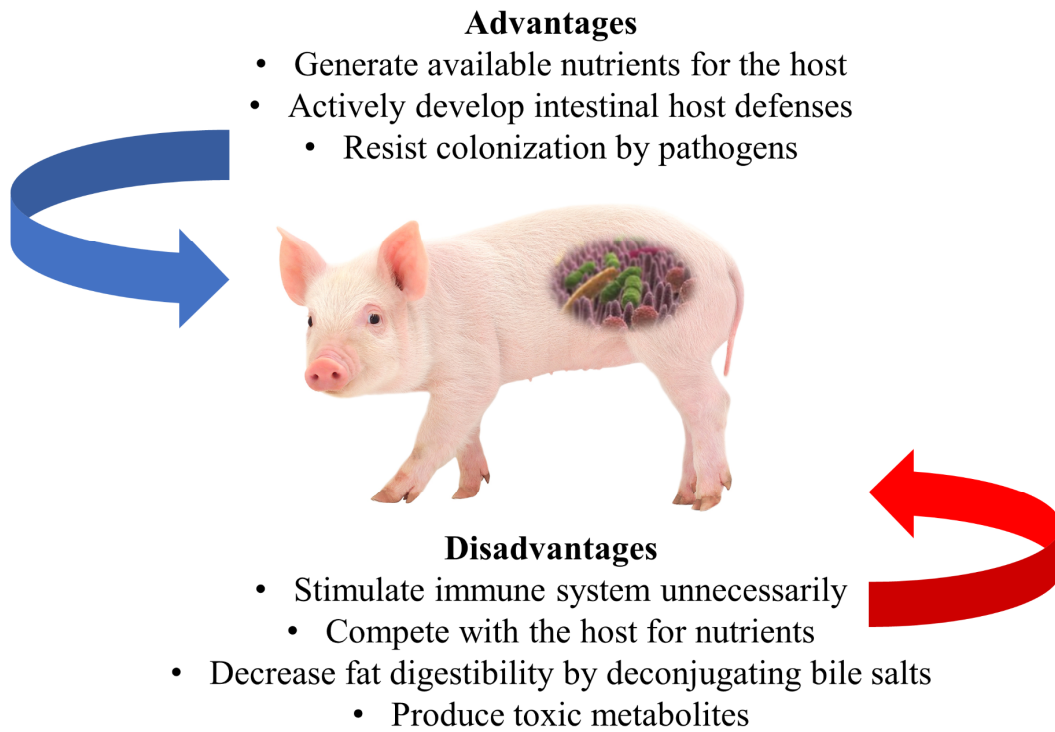


Figure 2.1 Advantages and disadvantages of gut microbiota in piglets. Gut microbiota can positively or negatively affect the host.

2.3 Assessment methods of gut health and gut barrier integrity in pigs

2.3.1 Considerations for *in vitro* and *in vivo* evaluation methods

The growing interest in identifying new antimicrobials has been accompanied by an equal interest in developing fast and reliable screening and evaluating methods. Traditionally, bioassays such as well diffusion, disk-diffusion, and agar or broth dilution have been among the commonly used techniques (Balouiri et al., 2016). Other novel and/or high-throughput assays such as the use of enterocyte cultures, *Caenorhabditis elegans* (*C. elegans*), and experimental animal models have started taking the lead in the past few years because these methods can provide a better understanding of the screened substance's impact on cellular viability.

2.3.2 *C. elegans* model

In the past four decades, many researchers have been extensively using the *C. elegans* model within the fields of biological research, including innate immunity and microbial pathogenesis studies. This system relies solely on the elicited innate immune defenses to cope with pathogen attacks as *C. elegans* lacks an adaptive immune system (Ewbank and Zugasti, 2011). The promise of this system is that many pathogenic microbes trigger specific mechanisms of innate immunity and lead to the overexpression of certain polypeptides (some have antibacterial activity) that only manifest during the course of pathogenesis. The use of the *C. elegans* system can aid in capturing such peptides and enhance the possibility for understanding the underlying mechanism(s) in large high-throughput *in vivo* screens of newly developed antimicrobials (Kong et al., 2016). This nematode system, if used correctly, offers inexpensive and robust screening platforms of antibiotics relying on a vast body of knowledge accumulated in regard to worm physiology and its bacterial/fungal pathogen interactions (Kong

et al., 2016). One of the drawbacks that pertain to this model is that this system does not denote the mode of action of bioactive compounds, hence generating the necessity for secondary studies that involve target-based screens such as the genetic knockdown of host or bacterial genes (Kim et al., 2017b). For innate immunity and microbial pathogenesis studies, the *C. elegans* model is a cost-effective and informative method that can be used with secondary studies to learn the mechanisms underlying the obtained results.

2.3.3 *In vitro* porcine intestinal cell model

Initially, *in vitro* experiments with porcine intestinal cell lines were conducted to study a number of bacterial infections (Schierack et al., 2006). However, porcine cell lines nowadays are used for pathogen studies and also for studying bioactive compounds to illustrate the interaction between the host cells and pathogens or bioactive compounds (Burt et al., 2016; Omonijo et al., 2018b). One of the most commonly used cell lines for study in pigs is porcine IPEC-J2, which are porcine intestinal columnar epithelial cells, isolated from the mid-jejunum of a neonatal piglet (Brosnahan and Brown, 2012). The factors that make this cell line unique include the fact that it is obtained from small intestinal tissue (compared to the common human colon-derived lines HT-29, T84, and Caco-2) and is not transformed (compared to the porcine small intestinal line, IPI-2I) (Brosnahan and Brown, 2012). There are diverse response criteria need in the experiments of *in vitro* porcine intestinal cell model including viability, TEER, verification of tight junction proteins by immunofluorescence, mucin production, proliferation assays, invasion assays, adhesion assays, and gene expression analysis of target genes including pro- and anti-inflammatory cytokines (Schierack et al., 2006). The cell line model provides diverse data for understanding the interactions between host cells and pathogens or bioactive compounds.

2.3.4 Ussing chamber system

Ussing chamber, an *ex vivo* method, is used to study the gut barrier function and the transport of ions, nutrients, and drugs across various epithelial tissues, especially intestine tissues of animals (He et al., 2013). An intestinal tissue sample can be mounted between the two chambers with Krebs Ringer buffers (KRB) and gas (5% CO₂ and 95% O₂) in the chamber keeping the tissue alive during the incubation period (Clarke, 2009). Electrophysiological properties including TEER, short circuit current (Isc), potential difference (mV) and epithelial conductance (*G*) (the inverse of resistance) can be measured with the Ussing chamber. Gut tightness, generated by tight junctions and mucus, can be expressed as TEER (Mardones et al., 2004). Increased TEER shows increased gut barrier function, and a reduced TEER represents decreased gut barrier function. The potential difference (mV) reflects the transmural potential difference that is generated by ion transport across the epithelium while short-circuit current (Isc) represents the net transmural ion transport (Woyengo, 2011). Increased electrophysiological response (Δ Isc) reflects either increased electrogenic anion secretion (e.g. Cl⁻ and HCO₃⁻) or increased electrogenic cation absorption (Na⁺). The transport activities of some nutrients (glucose and some amino acids) that are transported by sodium- or chloride-dependent transporters can be estimated by calculating Δ Isc after adding the nutrient in the mucosa side chamber. Also, the absorption of materials such as glucose, heparin, oligonucleotides, antibiotics, and amino acids can be determined by adding the nutrient to the mucosal area and calculating its concentration at the serosal side after a specific time (Tang et al., 2012). To study gastrointestinal epithelium permeability, the proportion of fluorescence-labeled substances such as fluorescein isothiocyanate-dextran and different isotope substances that can pass through the gastrointestinal epithelium can be calculated with exposed sample area and time (Cao et al., 2018). The Ussing chamber method is more accurate than cell culture-

based models, because of the presence of adequate paracellular permeability, an apical mucous layer, active transport proteins and drug-metabolizing enzymes (Castella et al., 2006). Advantages of employing Ussing chamber include the ability to study the regional differences along the intestine and bidirectional drug transport, and only a small amount of analytes are required for analysis (Balimane et al., 2000). Thus, Ussing chamber can provide the understanding of gut barrier integrity and transport of ions, nutrients, and drugs across epithelial tissues of a pig's intestine.

2.3.5 Experimental infection animal diseases models

The screening strategies to find AGP alternatives usually start with conventional bacteria-centered assays but in a later stage, it is pivotal to use experimental animal models to simulate the infection and treatment courses in order to investigate the effects of AGP alternatives on the gut ecosystem and growth performance of pigs. Experimental models can provide greater knowledge regarding different doses (concentrations in the feed) and mechanisms for new AGP alternatives to determine the role of this AGP alternative in the treatment of distinct infections.

The PWD induced by ETEC is an important worldwide disease in swine production because PWD usually results in weight loss, slow growth, more treatment costs, body weight heterogeneity, and even mortality (Lyutskanov, 2011). ETEC that expresses F4 (also designated K88) or F18 adhesive fimbriae facilitating the colonization of intestinal mucosa, produce toxins once they colonize in the intestine (Adewole et al., 2016). The experimental ETEC-challenged pig model has been well-established and used by many researchers. The oral gavage of ETEC to pigs is one of the most common methods to induce PWD in piglets (Bhandari et al., 2008). The dosage of oral administration can range approximately from 3 mL of 1×10^9 CFU·mL⁻¹ to

5 mL of 1×10^{12} CFU·mL⁻¹ and the experimental period differs depending on the experiment's purpose and conditions (Adewole et al., 2016). While some studies showed that growth performance was not influenced by the experimental ETEC challenge (Nyachoti et al., 2012), a number of other studies reported that growth performance was reduced due to the experimental ETEC challenge (Trevisi et al., 2009; Lee et al., 2012b). One of the possible reasons for this difference is that there is variance in terms of the responses in piglets to ETEC. Whether pigs are or are not susceptible to ETEC infection depends on the existence of a gene (Mucin 4 (MUC4) for ETEC F4; Fucosyltransferase 1 (FUT1) for ETEC F18) which is inherited as a simple Mendelian trait (there are two genotypes: resistant or susceptible) (Jensen et al., 2006). Once the pigs are gene screened to select resistant or susceptible genes towards ETEC F4 or F18-diarrhea in the ETEC challenge experiment, the experiment will be more accurate by decreasing the initial variance towards ETEC F4-diarrhea (Ren et al., 2012). In addition, it would be advantageous to select susceptible pigs to obtain diarrheic piglets to decrease the variation of the clinical symptoms from ETEC (Jensen et al., 2006). Besides ETEC, *Salmonella* spp. (a diarrhea and inflammation model), lipopolysaccharides (LPS) (an inflammation model), and also diquat (an oxidative stress model) can be used to investigate the effects of nutrients and AGP alternatives on challenged pigs (Boyen et al., 2008; Liu et al., 2012a; Cao et al., 2018).

2.3.6 “Omics” and molecular techniques for studying gut microbiota

The main purpose of “omics” technologies is the non-targeted identification of all gene products (transcripts, proteins, and metabolites) existing in a specific biological sample. Omics technologies include genomics, transcriptomics (gene expression profiling), proteomics, and metabolomics (Deusch et al., 2015). These advanced omics technologies serve to investigate

microbial communities as a whole and to explore more comprehensive studies of the composition and functionality of gut microbiota (Gong et al., 2018). With metagenomics, a collective way to study both the structure and function of microbiota, total community DNA is obtained from fecal or digesta samples and the microbiome is analyzed by whole-genome shotgun (Gong and Yang, 2012). In metatranscriptomics, total RNA extracted from complex microbial populations is used; and this method provides information about how the host and diet affect the microbiota (Sekiroy et al., 2010). Metabolomics can be used to determine the function of gut microbiota through the investigation of microbiota and host metabolite profiles with nuclear magnetic resonance (NMR), mass spectroscopy and other methods (Gong and Yang, 2012). Metaproteomics investigates the protein stock of a specific sample at a specific time point, which permits the identification of the active microbial fraction and their expressed metabolic pathways (Wilmes and Bond, 2006). Metagenomics (Looft et al., 2014; Mann et al., 2014), metabolomics (Hanhineva et al., 2013; Pieper et al., 2014) and metaproteomics (Tilocca et al., 2017) have all been used to study pig gut microbiota. There are no published metatranscriptomics studies on pig's gut microbiota.

In addition to omics techniques, there are still many other culture-independent techniques including the PCR-based DNA profiling, quantitative PCR (Q-PCR), fluorescent in situ hybridization (FISH), flow cytometry, DNA sequencing, and DNA microarray in the field. The PCR-based DNA profiling method including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), and terminal-restriction fragment length polymorphism (T-RFLP) have been widely used to characterize the gut microbiota (Gong et al., 2002; Li et al., 2003). The principle of all PCR-based DNA profiling techniques including DGGE, TGGE, SSCP and T-RFLP needs to employ PCR primers targeting 16S rRNA genes (or cpn60 genes) to amplify

16S rRNA (or cpn60) sequences from target bacteria (Gong and Yang, 2012). Q-PCR is a technique to study bacterial population size of gut microbiota by quantification based on primers with fluorescence-labeled group- or strain-specific probes or with a non-sequence specific DNA-binding dye (SYBR® green) during the PCR amplification procedure of a target gene (Feng et al., 2010). FISH can be utilized to investigate the microbial population and oligonucleotides labeled with fluorescence substances targeting 16S rRNA genes and are commonly used for the FISH analysis. The application of fluorescence *in situ* hybridization can be used for visualization and quantification of the human gastrointestinal microbiota. Flow cytometry is a technique used to count and assess mammalian cells and to study bacterial populations (Festin et al., 1987). In the analysis with flow cytometry, bacterial cells in a collected sample are fixed, and hybridized by using fluorescein-labeled antibodies (Festin et al., 1987) and then automatic analysis with flow cytometry to investigate microbial communities (Wallner et al., 1995). DNA sequencing can provide collective data on bacterial diversity and community structures by using full-length 16s rRNA gene sequences through the Sanger sequencing method (Xia et al., 2013). The DNA microarray has enabled researchers to analyze many genes in a single experiment (Schena et al., 1995). With this method, oligonucleotide probes obtained based on whole genomic DNA or 16S rRNA genes or cpn60 are produced and shown onto the array. Different fluorescence-labeled samples and a reference are merged, fragmented, and hybridized with the microarray and finally, the difference of fluorescence density can be determined to indicate the prevalence of target bacteria (Gong and Yang, 2012). Each omics and molecular technique have pros and cons, and several techniques should be utilized in a single experiment to supplement the results of the others.

2.4 Effects of dietary ingredients on gut microbiota, barrier integrity, and digestive physiology in pigs

2.4.1 Carbohydrates (Dietary fiber)

The carbohydrate fraction can be classified on the basis of the number of glycosidic linkages, into monosaccharides (sugar), oligosaccharides, and two broad classes of polysaccharides: starch and non-starch polysaccharides (NSP) (Lindberg, 2014). According to the cell wall structure, NSP and lignin have been defined as dietary fiber (DF) in the feed (Theander et al., 1994; Lindberg, 2014). Nonetheless, because non-digestible oligosaccharides such as raffinose, stachyose, and fructo-oligosaccharides, as well as resistant starch, are degraded by microbial enzymes and are further processed via similar metabolic pathways to produce short-chain fatty acids (SCFA), DF should include non-digestible oligosaccharides and resistant starch (Scott et al., 2013). In the swine industry, DF has been thought to be a feasible alternative to AGP because DF can improve growth performance, alleviate PWD, and modulate gut microbiota of pigs (Jha and Berrocoso, 2016). To be specific, DF has a great impact on both the mucosa and the microbiota and consequently has an important role in the regulation of the gut ecosystem (Heinritz et al., 2016). The DF resists host digestion and absorption, and is fermented by the microbiota, and selectively improves the abundance and activity of beneficial bacteria in the gut (Montagne et al., 2003). The classification was conducted on DF based on physicochemical properties because DF has different metabolic and physiological functions depends on viscosity, hydration, and fermentability (Agyekum and Nyachoti, 2017). Once beneficial bacteria colonize the gut, the abundance of pathogenic bacteria decreases because the colonizable areas of microbes are limited in the gut ecosystem of pigs (Cilieborg et al., 2016). In addition, through fermentation, OA including lactic acid and SCFA including formate, acetate, propionate, and butyrate, can be produced and have diverse

beneficial effects on the gut ecosystem. These OA and SCFA can constrain the growth of enteric bacterial pathogens including *Salmonella* spp., *E. coli*, and *Clostridium* spp. in the small and large intestine by providing an acidic environment (Wang et al., 2018b; He et al., 2019). Furthermore, some SCFA (e.g. acetate, butyrate, and propionate) influences the development of the gut ecosystem of piglets by activating epithelium cell proliferation (Montagne et al., 2003). However, a high concentration of DF in the feed can decrease the weight gain and feed intake of piglets because DF is indigestible by the host and DF can give a satiety, possibly decreasing feed intake, when bulky fibrous diets were provided; more studies are needed to find the appropriate concentration of DF in the piglet's diet (Wu et al., 2018). According to Agyekum and Nyachoti (2017), the feed containing a high concentration of DF can be possibly provided to the piglets by reducing particle size, pelleting, and using exogenous enzymes, which may increase nutrient utilization in the piglets. Hence, the appropriate amount of the DF with some treatments that increase nutrient utilization can improve the gut ecosystem of pigs without affecting growth performance by decreasing the abundance of pathogenic bacteria and developing the gut ecosystem of piglets by producing SCFA.

2.4.2 Proteins and functional amino acids

Spray-dried plasma, a protein source, can be used as an AGP alternative due to its immunoglobulin-rich property for piglets who may lack the ability to produce immunoglobulins themselves (Pérez-Bosque et al., 2016). Bosi et al. (2004) showed that a diet containing spray-dried plasma enhanced growth performance and improved gut barrier integrity, specific antibody defense and decreased inflammatory cytokine expression in piglets challenged with ETEC F4. Amino acids, the building blocks of proteins, are known to have diverse functions in the gut ecosystem of pigs (Wang et al., 2007). Every amino acid has

different gut health-promoting effects in piglets (Table 2.3). However, a high protein diet can increase the risk of inducing intestinal disorders because feeding a higher protein diet can increase the abundance of proteolytic microbes, including *E. coli* and *C. perfringens*, which damage intestinal integrity and increase protein fermentation (Lin and Visek, 1991; Nousiainen, 1991). The products of protein fermentation include toxic metabolites such as ammonia and amines as well as malodorous compounds including skatole and indole (Cone et al., 2005; Le et al., 2008). Furthermore, nitrogen excretion, which causes environmental pollution, can be induced by a high protein diet (Tous et al., 2016). Thus, while functional protein sources and amino acids can benefit the gut ecosystem of piglets, high protein diets should be avoided in the swine industry and exact levels of amino acids should be provided to meet the requirements.

2.4.3 Lipids (Fatty acids)

Lipids and fatty acids have a number of crucial biological functions such as serving as energy sources, acting as structural components of cell membranes, participating in signaling pathways, and modulating the immune system (Fahy et al., 2011). SCFA, fatty acids with a chain of less than six carbon atoms, include acetate, propionate, and butyrate (Liu, 2015). SCFA are the fermentation products of DF and probiotics in the lower gastrointestinal tract and play an important role in improving gut health and restricting inflammation in the small and large intestine of pigs (Rossi et al., 2010). Supplemental sodium butyrate improved jejunal gut morphology and intestinal barrier function in the jejunum which has shown to increase TEER, decrease paracellular flux of dextran (4kDa) and reduce the portion of degranulated mast cells and their inflammatory mediators content including histamine, tryptase, and mRNA expression of TNF- α and IL6 (Wang et al., 2018a). In addition, OCLN, one of the tight junction proteins, was increased in the duodenum and tended to be increased in the jejunum and colon when

Table 2.3 Effects of functional amino acids on pigs.

Functional Amino acids	Observations	References
Arginine	Improved the immune system including serum immunoglobulin G and immunoglobulin M in early weaned piglets	(Li et al., 2012b)
	Attenuated the effect of oxidative stress by improving gut morphology and relieving the expression of pro-inflammatory cytokines in piglets	(Zheng et al., 2017)
	Improved growth performance, gut morphology, barrier function (claudin1 mRNA expression), and antioxidant capacity (glutathione peroxidase mRNA expression) of low birth weight piglets.	(Zheng et al., 2018)
	Improved intestinal development and increased expression of vascular endothelial growth factor	(Yao et al., 2011)
Aspartate	Attenuated the lipopolysaccharide challenge effect by improving intestinal morphology and barrier function and reduced toll like receptor 4, tumor necrosis factor- α and ileal caspase-3 protein expression.	(Wang et al., 2017)
	Alleviated growth suppression and effects of oxidative stress when challenged by hydrogen peroxide.	(Duan et al., 2016)
Glutamate	Prevented intestinal epithelial damage (gut atrophy) after weaning	(Wu et al., 1996)
	Enhanced the expression tight junction protein and regulate corticotropin-releasing factor signaling in the jejunum.	(Wang et al., 2014)
	Stimulated the mTOR signaling and increases protein synthesis in enterocytes	(Wang et al., 2007)
	Enhanced the activities of lactase, maltase and sucrase in jejunum mucosa in weaned piglets	(He et al., 2016)
Leucine	Increased mucin production in the jejunal mucosa when challenged by Porcine Rotavirus	(Mao et al., 2015)
Lysine	Increased the population of <i>Lactobacillus</i> and <i>Bifidobacterium</i> in caecum and colon and raised the	(Zhou et al., 2018)

	expression of amino acid transporters	
Threonine	Increased intestinal mucin synthesis and immune status of intrauterine growth-retarded weanling piglets.	(Zhang et al., 2018a)

weaned piglets were fed with supplemental butyrate (Grilli et al., 2016). Sodium butyrate decreased the incidence of diarrhea in weaned piglets (Feng et al., 2018). MCFA, which are abundant in milk fat and various feed materials including coconut, palm, and cuphea seed oils, are saturated 6-12 carbon fatty acids. MCFA supplementation enhanced gut development through increased higher VH and improved growth performance in weaned piglets (Hanczakowska et al., 2011). In addition, the antimicrobial properties and gut health promoting-effects in pigs show that MCFA can be considered as AGP alternatives (Zentek et al., 2012). Long-chain fatty acids (LCFA) are fatty acids with a chain length of 14 or more carbons including omega-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid (EPA) [20:5(n-3)], and docosahexaenoic acid (DHA) [22:6(n-3)], are rich in fish oil. Fish oils have been shown to modulate the immune system by inhibiting the over-release of intestinal pro-inflammatory cytokines in piglets (Arnardottir et al., 2012). According to Liu et al. (2012b), supplementation of fish oil could improve intestinal morphology and gut integrity and reduce intestinal inflammation by decreasing TNF- α and toll-like receptors 4 (TLR4) expression in piglets challenged with LPS. Lipids and fatty acids have the ability to enhance gut health and modulate gut inflammation, whereas MCFA are known to have an antimicrobial effect.

2.4.4 Minerals

An appropriate amount of minerals must be supplied in swine rations to meet or exceed the requirement since minerals have functions in the digestive processes such as; metabolizing proteins, fats, and carbohydrates; and as the structure of chromosomes, enzymes, nerves, blood, skeleton, hair and milk. Minerals are an important factor in reproduction, growth, production, and resistance to parasites and diseases (Carlson and Boren, 2001). Calcium (Ca) and phosphorous (P) are essential minerals for pigs, particularly for the bone formulation, and are

also closely linked in the digestive processes and metabolism (Suttle, 2010). Many studies have demonstrated that Ca and P are related to the gut ecosystem and gut microbiota in weaned piglets. According to Metzler-Zebeli et al. (2012), a high Ca-P diet decreased the expression of IL1 β , a proinflammatory cytokine, in the duodenum and reduced cecal CD by 14% compared with low Ca-P diets. Furthermore, reduced dietary P altered transcription of phospholipase C, Ca signaling, and *nuclear factor of activated T-cells* signaling, which may indicate that P has an immunomodulatory function (Just et al., 2018). Since Ca and P are vital nutrients required for bacteria for a number of metabolic processes in the bacterial cells (Durand and Komisarczuk, 1988), differences in the intestinal availability and amount of Ca and P may alter the growth of certain bacterial species and genera. Furthermore, another study revealed that a high Ca-P diet increased *Lactobacillus* spp. by 1.4-fold in the stomach of pigs (Mann et al., 2014), however, a high concentration of P can increase the abundance of pathogens (Heyer et al., 2015).

Copper (Cu) and zinc oxide (ZnO) have an antimicrobial property, therefore they can inhibit pathogens and reduce the loss of nutrients due to fermentation. Pharmacological concentrations (above 3,000 mg/kg) of Cu and ZnO have been considered as effective growth promoters in the diets of weaned piglets (Højberg et al., 2005). Cu and ZnO could have an impact on reducing the population of *Clostridia* spp. and *E. coli* in weaned piglets (Song et al., 2013; Dębski, 2016). In addition to antimicrobial effects, ZnO is also known to have a role in anti-inflammation, antidiarrhea and restoration of mucosal barrier integrity (Jensen, 2016). This is because, zinc is essential in the maintenance of the gut barrier function by regulating the OCLN proteolysis and CLDN3 transcription and by upregulating protein kinase C zeta type (PKC ζ) via GPR39 (Miyoshi et al., 2016; Shao et al., 2017). Feeding diosmectite-ZnO composite alleviated PWD symptoms, improved gut morphology, improved gut integrity by

upregulating tight junction proteins (OCLN, CLDN1 and ZO1 in jejunal mucosa), and decreased mRNA expression level of pro-inflammatory cytokines (TNF- α , IL6, and interferon- γ) in weaned piglets (Hu et al., 2013a). Weaned piglets fed with dietary ZnO showed a higher abundance of transporters in the ileum (Yu et al., 2017). Supplementation of coated ZnO reduced the microbiota species richness and Shannon diversity index in the jejunal digesta and feces, indicating that ZnO has an impact on intestinal microbiota diversity (Shen et al., 2014). In contrast, Yu et al. (2017) have shown that both ZnO and antibiotics increased the microbiota diversity of ileal digesta, while they reduced the microbiota diversity of the colonic digesta. Since dietary Cu and/or ZnO can accumulate in pig feces and eventually increase the mineral content in the soil which negatively impacts the environment, there is now a sense that pharmacological concentrations of Cu and ZnO should be replaced with other bioactive compounds (Poulsen, 1998). This issue can probably be resolved with different encapsulating techniques decreasing the amount of ZnO supplementation and allowing a slower release of ZnO throughout the gut of piglets (Kim et al., 2015). Ciesinski et al. (2018) showed that a pharmacological concentration of ZnO can induce the presence of antimicrobial resistant *E. coli* in the swine gut. Therefore, a high concentration of ZnO can show beneficial effects to gut health of piglets, but the supplementation of high levels of ZnO can cause environmental issues and generation of resistant bacteria, which may decrease the potency of ZnO as an AGP alternative.

Enough dietary iron should be supplemented in the diet of pigs to prevent iron deficiency which can result in infection-related mortality and morbidity from diarrhea (Wayhs et al., 2004). However, a high concentration of iron should be avoided in pig nutrition because iron is the first limiting nutrient for some pathogens in mammals, therefore a high concentration of iron can induce the growth of some pathogens (Klasing, 2007). A high concentration of iron

has increased the growth and virulence of *Salmonella enterica* in *in vitro* model (Tan et al., 2019). Pigs fed with low and high dietary iron in the feed had increased gut permeability, and pigs fed high dietary iron had increased the expression of pro-inflammatory cytokines such as TNF- α , IL1 β and IL6 (Li et al., 2016). In summary, appropriate levels of Cu, ZnO and iron benefit piglets through antimicrobial and antidiarrheal effects, however, a high concentration of these minerals can cause a malfunction in piglets and result in environmental pollution.

2.4.5 Vitamins

An appropriate amount of vitamins should be supplemented in a piglet's diet to meet the requirement. However, to overcome oxidative stress, which is an imbalance between reactive oxygen species (ROS) and antioxidants which causes cellular damage and is related to weaning stress, higher concentrations of vitamins can be supplemented in the diets of piglets to relieve oxidative stress (Debier, 2007). Adding vitamins such as provitamin A carotenoids and vitamins C and E, which are natural antioxidants, can enhance the gut ecosystem of weaned piglets protecting it against oxidative stress-induced diseases (Deng et al., 2010). Alpha, beta, and gamma carotene are referred to as provitamins because they can be converted to vitamin A (Clemens et al., 1992). The benefits of provitamin A carotenoids are mainly related to their antioxidant properties (Britton, 2008). According to Kang et al. (2018), supplementation of the spontaneous oxidation of beta-carotene decreased necrotic enteritis in broiler chickens potentially because of its strong antioxidant property. In addition, fully oxidized beta-carotene functions to enhance the innate immune system and decrease inflammatory processes (Duquette et al., 2014). Rey et al. (2017) reported that supplementation of a high dose of vitamin E (250 mg·kg⁻¹) decreased muscle oxidation for piglets, and supplementation of a high dose of vitamin C (500 mg·kg⁻¹) increased the serum antioxidant power and serum IgG

concentration. Antioxidant blend including vitamins C and E could compensate for some of the harmful changes caused by weaned, by upregulating the gene expression of tumor protein 53 (p53) and peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1 α), which have roles in cell cycle arrest, DNA repair, apoptosis and senescence, and activate mitochondrial biogenesis and mitochondrial respiration (Zhu et al., 2012). However, a study showed that high concentrations of vitamin E (10 times of the dose recommended by NRC (2012) could also decrease gut morphology and function by inhibiting jejunal epithelial cell proliferation (Chen et al., 2019). An appropriate amount of vitamins (e.g. vitamins C and E) should be supplemented in the diets of piglets to attenuate oxidative stress, but an exceedingly high concentration of vitamins can cause toxicity to the host.

2.5 Antibiotics

Antibiotics have been used for 60 years in the swine industry due to their efficiency in promoting the growth rate, their ability to augment feed utilization, their cost-effectiveness, and their effectiveness in decreasing mortality from clinical diseases (Allen et al., 2013). There are four mechanisms by which are beneficial for swine production: (1) reducing sub-clinical infections, (2) decreasing growth-depressing microbial metabolites (3) suppressing microbial use of nutrients, and (4) increasing absorption and use of nutrients (Gaskins et al., 2002). However, because of the concerns of drug residues in meat products and the delivery of drug-resistant bacteria to humans, many countries have prohibited the use of in-feed antibiotics for swine as a regular means of growth promotion (Van der Fels-Klerx et al., 2011). In the past 20 years, a great deal of research has been conducted to find alternatives to AGP to sustain swine gut health and performance (Thacker, 2013). Each of the investigated replacements has different advantages and disadvantages (Table 2.4).

Table 2.4 Beneficial effects and shortcomings of each antibiotic alternative and feasible solutions.

Antibiotic alternatives	Beneficial effects	Shortcomings	Feasible solutions
Probiotics	Improve the intestinal development, host's immune regulation, gut microbiota modulation and alleviation of toxins effects (Barba-Vidal et al., 2018)	Storage and delivering to target site issues: survival of probiotics bacteria (Shah et al., 1995)	Microencapsulation of probiotics to increase stability in storage and to increase survivability in stomach (Martín et al., 2015)
Prebiotics	Modulate the balance of intestinal microbial population by increasing beneficial bacteria while decreasing pathogenic bacteria (Steed and Macfarlane, 2009; Herfel et al., 2011)	Whether prebiotics are beneficial to the immune system is still controversial (Pandey et al., 2015) Stimulate body's immune system (Drulis-Kawa et al., 2012)	Needs more research (Pandey et al., 2015)
Bacteriophages	Modulate the growth of specific harmful bacteria in pigs and do not harm useful bacteria unlike antibiotics (Kim et al., 2017a).	Possibility of producing resistant bacteria (León and Bastías, 2015) Sensitive to temperature, low pH (Smith et al., 1987)	Microencapsulation of bacteriophage for increasing storage stability and for improving passage rate in stomach (Johnson et al., 2008; Ma et al., 2008)
Antimicrobial peptides	Have broader range of antimicrobial function, rapidly killing bacteria, and highly selective toxicity (Hancock and Patrzykat, 2002; Zasloff, 2002)	Limited by the number of approved AMPs: poor selectivity, hemolytic activity and host toxicity, low stability to protease degradation <i>in vivo</i> , low	

		hydro solubility, and cost of production (Wang, 2017)	
Medium chain fatty acids	Show strong antibacterial activity (Skřivanová et al., 2009)	High inclusion of medium chain fatty acids decreased feed intake and body weight gain of weaned piglets (Li et al., 2015)	An appropriate amount of medium chain fatty acids should be supplemented to piglets
Exogenous enzymes	Increase digestibility and improve gut health (Torres-Pitarch et al., 2017)	Low enzyme activities, high cost, no appropriate standards, no antibacterial effects, stability issues (Cheng et al., 2014)	Coating enzymes to increase bioavailability (Liu et al., 2017a)
Phytochemicals (Essential oils)	Improve gut barrier integrity, augment the immune system, increase antioxidant activities and modulate intestinal microbiota (Omonijo et al., 2018c)	Storage issue (volatility), cost, and availability (Yang et al., 2015)	Microencapsulation for feeding can improve stability and passage rate in stomach (Omonijo et al., 2018a)
Phytochemicals (Plant extract)	Have antimicrobial, anti-inflammatory, and gene regulatory functions	Antinutritional factors including tannins and saponins which can decrease digestibility of nutrients and damage the gut ecosystem (Cheng et al., 2014)	An appropriate amount of medium chain fatty acids should be supplemented to piglets
Organic acids	Reduce gut pH, activate enzyme secretion, inhibit pathogenic bacteria, increase nutrient digestibility and retention (Papatsiros et al., 2012)	High inclusion level of organic acids can decrease feed intake due to strong odor and flavor (Partanen and Mroz, 1999)	An appropriate amount should be included (Partanen and Mroz, 1999)

2.6 Antibiotic alternatives

2.6.1 Probiotics

Probiotics are living microorganisms that can be beneficial for host health when appropriate amounts are provided (Hotel and Cordoba, 2001). Probiotics are beneficial for the host's gut ecosystem through four mechanisms: improvement of intestinal development; enhancement of the host's immune system; modulation of gut microbiota; and alleviation of the effects of toxins from harmful microbiota (Barba-Vidal et al., 2018). The gut health-promoting effects of probiotics are highly strain-specific, and due to synergetic effects, multi-strain mixtures may be more effective than a single strain (Timmerman et al., 2004). For instance, the most often used probiotics for swine are lactic acid bacteria including *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., and *Streptococcus* spp. (Ljungh and Wadstrom, 2006). According to Choi et al. (2016), the inclusion of *Lactobacillus acidophilus*, *Bifidobacterium subtilis* and *Streptococcus cerevisiae* complexes (4.0×10^8 ; 4.8×10^9 ; 1.0×10^4 CFU·g⁻¹ respectively) in the diets of weaned piglets enhanced VH in the duodenum, jejunum, and ileum. Probiotics have a role in modulating the host's intestinal balance by suppressing harmful microorganisms and supporting advantageous microorganisms (Fuller, 2012). For example, *Lactobacillus* spp. reduced the viability of pathogens by producing OA, hydrogen peroxide, and antimicrobial substances (Lidbeck and Nord, 1993). Moreover, *Bacillus* spp. and *Saccharomyces* spp. can produce antimicrobial substances (e.g. reuterin) and reduce the effect of pathogenic bacteria such as *E. coli* (Lidbeck and Nord, 1993). Certain probiotics can have an impact on the intestinal mucosal cell-cell interactions and cellular “stability” which improves the intestinal barrier via alteration of the phosphorylation of the

cytoskeletal and tight junction proteins (Willing et al., 2013). *Saccharomyces cerevisiae*, one of the probiotics, can metabolize or aid in the detoxification of certain inhibitory compounds such as amines or nitrates or scavenge for oxygen, which is critical in anaerobic conditions in the gut (Chaucheyras-Durand et al., 2008). To sum up, probiotics can improve the gut ecosystem by inhibiting the growth of pathogens and promoting the growth of beneficial bacteria throughout the gut.

2.6.2 Prebiotics

According to Gibson and Roberfroid (1995), a prebiotic is defined as a selectively fermented ingredient that makes specific changes both in the composition and/or activities in the gastrointestinal microbiota that may be beneficial to the host's health. The difference between DF and prebiotics is that DF is non-digestible plant-derived carbohydrates consisting of at least 3 units of individual sugars, while prebiotics is a substance obtained from plants, animals and microbial sources that can confer the health benefit. Examples of prebiotics include oligosaccharide carbohydrates such as fructo-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides, manno-oligosaccharides, and lactulose (Shokryazdan et al., 2017). Prebiotics modulate the balance of the intestinal microbial population by increasing beneficial bacteria such as *Bifidobacterial* spp. and *Lactobacillus* spp., and decreasing pathogenic bacteria in the gut (Herfel et al., 2011). In one study, chito-oligosaccharides reduced the population of *Staphylococcus aureus* (pathogenic) and increased the concentration of lactic acid bacteria, *Bifidobacteria* spp. and *Lactobacillus* spp. in the cecum compared to the control diet on d 7 post-weaning (Yang et al., 2012). The growth in the population of beneficial microbes in the gut augments the gut ecosystem by regulating the

expression of pro- and anti-inflammatory cytokines, particularly in intestinal cells (Kunavue and Lien, 2012). One experiment showed that the VH in the ileum and the concentration of total volatile fatty acids, produced by microbiota were higher in pigs fed with isomalto-oligosaccharides (Wu et al., 2017). Additionally, this study showed that prebiotics can be beneficial to the immune system by increasing glutathione peroxidase, catalase, and IgG in weaned piglets. There are synergetic effects when prebiotics and probiotics are used together in animal feeds, called the symbiotic effects (Gibson and Roberfroid, 1995). When *Lactobacillus paracasei* were blended with fructo-oligosaccharides, there were symbiotic effects in increasing beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. and decreasing *E. coli* and *Clostridium* spp. in weaned piglets (Nemcova et al., 1999). Prebiotics can benefit the gut ecosystem of piglets by being nutrients for beneficial bacteria, which decreases the population of pathogens once beneficial bacteria colonize in the gut.

2.6.3 Bacteriophages

Bacteriophages, which are live viruses only affecting and attacking targeted bacteria, are a recently developed technique to replace AGP in the livestock industry (Hussain et al., 2017). Bacteriophages show efficient antimicrobial effect by infecting and attacking bacteria and entering a lysogenic or lytic cycle within a bacterial cell, which replicates bacteriophages (Fig. 2.3) (Verstappen et al., 2016). Certain bacteriophages specifically modulate the harmful bacteria in pigs and do not harm useful bacteria, unlike AGP (Kim et al., 2017a). For efficiency, it is beneficial to use a blend of several kinds of bacteriophages in order to control the pathogenic bacteria including *E.coli* and *Listeria* spp. (Anany et al., 2011). Bacteriophages can not only be used for therapeutic purposes against diseases such as PWD, but may also be used

for improving growth performance, feed efficiency, and gut microbiota in pigs (Gebru et al., 2010; Cha et al., 2012; Kim et al., 2014). According to Lee et al. (2016), the supplementation of bacteriophages increased the population of total anaerobic bacteria including *Bifidobacterium spp.*, *Lactobacillus spp.*, and *Clostridium spp.* Additionally, VH of the duodenum and jejunum was increased. Dietary phage supplementation attenuated the symptoms of the ETEC-challenged infection by improving the gut structure, the immune system, and growth performance in weaned piglets (Sabouri et al., 2017). Likewise, supplementation of dietary ETEC F4 and F5 (K99) specific bacteriophages showed higher weight gain, lower fecal consistency score, and less fecal shedding and intestinal adhesion of ETEC F4 and F5 in piglets compared to piglets that did not get dietary bacteriophages (Lee et al., 2017). Not only can bacteriophages specifically modulate the growth of targeted bacteria, but they can also improve gut health and growth performance of piglets.

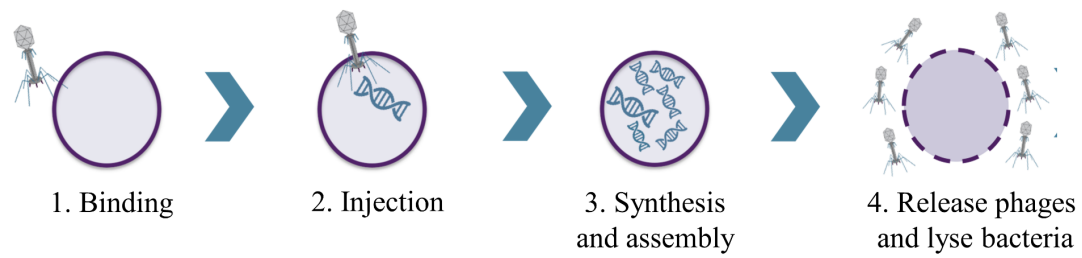


Figure 2.2 Mechanism of bacteriophage therapy. This schematic illustration represents the developmental cycle of lytic bacteriophage.

2.6.4 Antimicrobial peptides

Antimicrobial peptides (AMP), which are small gene-encoded peptides, act as part of the innate host defense mechanism and are shown to attack bacteria, regulate bacterial infections and manage host responses to infection (Yeung et al., 2011). In the swine industry, AMP have been considered as an alternative to AGP because of their benefits, including a broader range of antimicrobial function, rapidly killing bacteria, and highly selective toxicity (Hancock and Patrzykat, 2002). Most importantly, bacteria rarely develop resistance to AMP because of AMP's ability to disturb bacterial membranes through non-particular electrostatic interaction with the components of the lipid membrane (Chou et al., 2010). Supplementation of AMP attenuated the incidence of PWD and atrophy of gut structure and increased the population of *Lactobacillus* spp. in ETEC F4 challenged piglets (Wu et al., 2012). Likewise, supplementation of AMP to weaned piglets enhanced the gut structure and reduced fecal and intestinal coliforms compared to pigs fed with the control diet without AMP (Yoon et al., 2013). When AMP were provided to weaned piglets challenged with deoxynivalenol, a mycotoxin produced by some *Fusarium* spp., they were capable of improving intestinal morphology and activating epithelial cell proliferation and protein synthesis (Xiao et al., 2013). In the swine industry, AMP are considered a promising AGP replacement since AMP provide a lot of benefits to the gut ecosystem of piglets and rarely induce the growth of antibiotic-resistant bacteria in the gut.

2.6.5 Medium chain fatty acids (MCFA)

Medium chain fatty acids (MCFA) are saturated 6-12 carbon fatty acids and are considered as alternatives to AGP because of their strong antibacterial activity due to the

anionic part of the molecule acting against Gram-positive cocci (Bergsson et al., 2001) and *E. coli* (Skřivanová et al., 2009). The anionic part of fatty acids modifies the physicochemical properties of the digestive tract environment where the microorganisms exist and affect the expression of some genes of microorganisms and the host (Baltić et al., 2017). Zentek et al. (2011) showed that diet MCFA increased the population of eubacteria, Enterobacteriaceae, clostridial clusters I and IV, *Lactobacillus johnsonii*, and *Lactobacillus amylovorus*, which are considered beneficial bacteria in the gastric contents. The concentration of bacteria including *Salmonella* spp. and coliforms in the jejunum and cecum digesta were decreased by MCFA supplementation (Hanczakowska et al., 2016). Medium chain triglycerides, which are triglycerides with fatty acids and have similar functions with MCFA improved the gut immune system of piglets by modulating the recruitment and the maturation of immune cells (Hassan et al., 2018). Also, MCFA could have an impact on post-weaning gut development such as greater VH (Hanczakowska et al., 2016). With a focus on its antimicrobial properties, MCFA can improve the gut ecosystem of piglets, making it a promising AGP alternative.

2.6.6 Exogenous enzymes

Supplementation of exogenous enzymes, which can help the digestion of complex matrix of a piglet's diet, can bridge the gap during weaning until a piglet's endogenous enzymes are ready to develop (Torres-Pitarch et al., 2017). The addition of exogenous lipase may be an effective way to compensate for the shortage of endogenous lipolytic enzymes during the weaning phase (Zhang et al., 2018b). Proteases are also one of the most common enzymes used in piglets to increase protein utilization and to enhance the gut ecosystem. Zuo et al. (2015) noted that dietary protease enhanced the gut structure by increasing the ratio of VH to CD in

the duodenum, jejunum, and ileum and increased nutrient efficiency by increasing the mRNA abundance of amino acid transporters. The possible explanation would be that protease promotes the release of functional amino acids from the ingredients which can improve the gut ecosystem. Carbohydrases such as xylanase and β -mannase are mainly used to break down the structure of cereal non-starch polysaccharides (NSP) that have negative effects on nutrient digestibility (Van Kempen et al., 2006). Supplementation of xylanase could increase fecal *Lactobacillus* spp. and digestibility of dry matter, nitrogen, and energy digestibility (Lan et al., 2017). The mechanism by which non-starch polysaccharide-degrading enzymes affect intestinal microbiota is by breaking down the structure of undegraded substrates (Rajagopalan et al., 2013) and by releasing oligosaccharides, which have potential prebiotic effects, from cell wall NSP (Pluske et al., 2002). Because IAP can detoxify LPS, IAP can be added to the diet of piglets. According to Beumer et al. (2003), when IAP from calf intestine was added to the diet of piglets, IAP decreased LPS-mediated inflammation by reducing the expression of TNF- α in the serum. Phytase, phytic acid (an organic form of phosphorus in plants) degrading enzymes, become an essential feed additive for pigs because P availability in pigs is low while P is important in bone and cell membrane structure, energy metabolism and the other metabolic pathways (Lu et al., 2019). In addition, without phytase supplementation, pigs excrete 50 – 80% of P intake because of the low availability of plant P, which causes environmental pollution through soil contamination (Kornegay et al., 1997). Therefore, exogenous enzymes can be added to the diet of piglets to enhance feed efficiency, to improve the gut ecosystem and to minimize negative environmental effects.

2.6.7 Phytochemicals (EO and plant extracts)

Essential oils (EO) are natural bioactive compounds derived from plants and are known to possess antibacterial and antifungal properties and protect the host against infectious diseases (Perez-Roses et al., 2016). In addition, EO have been shown to improve gut integrity, and the immune system, to increase antioxidant activity and to modulate intestinal microbiota of animals (Fig. 2.4). There are many kinds of EO, and it is thought that each EO might have different effects in weaned piglets (Table 2.5). The addition of oregano EO to the diet of weaned piglets was shown to increase VH and expression of OCLN and ZO1 in the jejunum, which represented improved intestinal barrier functions (Zou et al., 2016b). A blend of cinnamaldehyde and thymol was able to increase the amount of IgG and IgM in a linear fashion in serum, which is indicative of an improved immune system (Su et al., 2018). Furthermore, in this research, plant EO supplementation could induce activation of antioxidant functions by decreasing malondialdehyde and increasing glutathione in serum, which indicates improved whole-body antioxidant status and a reduction in lipid peroxidation, respectively. The addition of a blend of thymol and cinnamaldehyde to the diets of piglets reduced the incidence of diarrhea and decreased *E.coli* counts in their feces (Li et al., 2012c). Also, the ratio of *Lactobacillus* spp. to *E.coli* increased while the total counts of aerobe number in the rectum decreased (Li et al., 2012a). A study by Omonijo et al. (2018b) showed that thymol enhanced barrier function, decreased ROS production, and reduced inflammatory response in the porcine intestinal epithelial cells during LPS-induced inflammation. However, issues of cost-effectiveness and feed palatability, as well as the lipophilic and volatile properties of EO, can be obstacles when applying EO to a pig's diet. However, these challenges can partially be

resolved through microencapsulation and nanotechnology to include a lower level of EO and to increase the stability of EO to be included in the pig diets.

Table 2.5 Effects of essential oils on piglets.

Essential oils	Observations	References
Essential oils blend: Fenugreek (40%), clove (12.5%), cinnamon (7.5%) and carrier (40%)	Enhanced growth performance, serum immunoglobulin G concentration and nitrogen digestibility and decreased noxious gas concentration	(Cho et al., 2005)
	Improved growth performance, immunity and microbiota	(Li et al., 2012c)
	Enhanced growth performance, digestibility of crude protein and dry matter, gut structure, plasma total antioxidant capacity, the number of beneficial bacteria and decreased pathogenic bacteria	(Li et al., 2012a)
	Enhanced growth performance, digestibility, gut structure, microbiota (lower <i>E. coli</i>), the immune system (immunoglobulin A, immunoglobulin G) and total antioxidant capacity when weaned piglets fed low protein diets.	(Zeng et al., 2015a)
Essential oils blend: 18% thymol and cinnamaldehyde	Reduced serum lipid peroxidation level	(Jiang et al., 2017)
	Increased gut <i>Lactobacillus</i> spp. counts and decreased the incidence of diarrhea	(Cairo et al., 2018)
Brazilian red pepper		

Plant extracts are known to have antimicrobial, anti-inflammatory, and gene regulatory functions, which make them promising for human and veterinary medicine (Windisch et al., 2008). There are diverse kinds of plant extracts, and their effects on animals differ depending on the types and parts of plants. When capsicum oleoresin, garlic botanical, and turmeric oleoresin were supplemented to weaned piglets, capsicum oleoresin and turmeric oleoresin increased the expression of the genes related to gut barrier function and immune response, indicating that feeding plant extracts can improve the gut ecosystem of weaned piglets (Liu et al., 2014). The extract of *Yucca schidigera*, native to North America and rich in saponin, is known to increase growth performance, improve feed efficiency, promote anti-protozoal and nematocidal activity, and modify the microbial population in the gut ecosystem of pigs (Cheeke, 2000). According to Cromwell et al. (1985), *Yucca schidigera* extract improved average daily gain (ADG) in weaned piglets. Seaweed extract contains mannitol, storage and structural polysaccharides, phlorotannins, amino acids, high level of minerals including Ca and iodine, and vitamins such as thiamin, ascorbic acid, tocopherols, and carotenoids (MacArtain et al., 2007). Phlorotannins in the brown seaweed extract are thought to be an antimicrobial substance against both Gram-positive and Gram-negative bacteria (Eom et al., 2012). Leonard et al. (2011) showed that supplementation of seaweed extract decreased the population of *E. coli* and Enterobacteriaceae in the colon digesta of weaned piglets. Dietary Chinese medicinal herbs (composed of *Panax ginseng*, *Dioscoreaceae opposita*, *Atractylodes macrocephala*, *Glycyrrhiza uralensis*, *Ziziphusjuzube* and *Platycodon grandiflorum*) increased the VH and Lactobacillus counts in the ileal digesta as well as decreased coliforms counts in the colon of weaned piglets (Huang et al., 2012). A number of secondary metabolites are produced by plants. Among these, the group of polyphenols might be the most promising ones due to their

antioxidative, anti-inflammatory, and gene regulatory properties (Gessner et al., 2017). Feeding grape seed and grape marc meal extract, which are rich in polyphenols, to weaned piglets, decreased the expression of pro-inflammatory genes and produced a higher ratio of VH and CD in the duodenum and jejunum than in the control pigs (Fiesel et al., 2014). Yang et al. (2019a) showed that Red-osier dogwood (native to North America), which is rich in phenolic compounds, improved cellular activity by directly reducing ROS and improving the cellular antioxidant system as well as by upregulating tight junction proteins. There are many kinds of phytochemical compounds; since each compound has different effects on the gut ecosystem, appropriate kinds and amounts of phytochemical compounds should be applied to piglets.

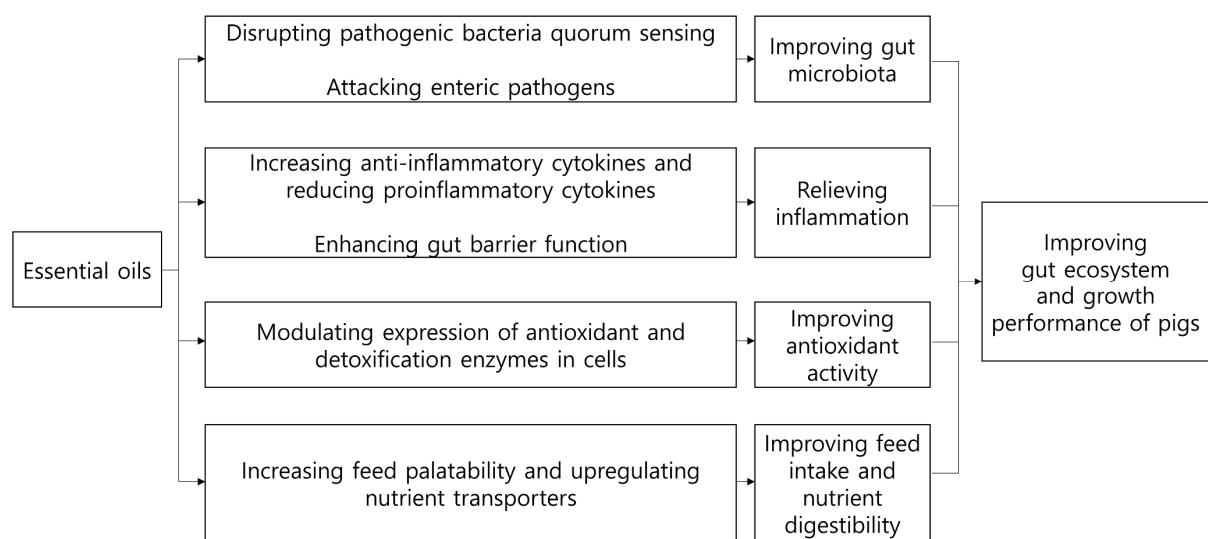


Figure 2.3 Schematic diagram illustrating the four different potential mechanisms by which essential oils improve the gut ecosystem and growth performance of piglets (Omonijo et al., 2018c).

2.6.8 Organic acids (OA)

In swine production, OA are considered promising AGP alternatives and a blend of diverse OA is known to improve gut health and growth performance of weaned piglets (Kil et al., 2011). The mode of actions for OA includes reducing gut pH, stimulating the secretion of enzymes, inhibiting the growth of pathogenic bacteria, and improving the growth and recovery of the intestinal morphology (Papatsiros et al., 2012). Supplementation of 1% lactic acid and 1% formic acid reduced pH in the stomach and decreased the population of Enterobacteriaceae in the stomach of weaned piglets (Hansen et al., 2007). The addition of citric acid in the weaned pig diet yielded a significant decrease in fecal counts of *Salmonella* and *E.coli* and an increase in *Lactobacillus* spp. and *Bacillus* spp. compared to the control diet without any OA (Ahmed et al., 2014). Coated OA (benzoic acid, calcium formate, and fumaric acid) increased VH in the duodenum and jejunum, attenuated diarrhea and improved growth performance in weaned piglets (Xu et al., 2018). Humic substances including humic acid, fulvic acid, and humin are organic residues generated from the decomposition of organic matter in the soil (Kaevska et al., 2016). These humic substances are known to have antidiarrheal, analgesic, immunostimulatory, and antimicrobial properties (Huck et al., 1991). Dietary supplementation with sodium humate and ZnO reduced the incidence of diarrhea and increased the population of beneficial bacteria in feces of experimental ETEC-challenged piglets compared to control ETEC F4 and F18 challenged piglets (Kaevska et al., 2016). A study by Weber et al. (2014) revealed that supplementation of humic acid and butyric acid improved the immune system in LPS challenged piglets. Taken together, available evidence shows that OA are shown to have diverse benefits to piglets, which includes decreasing the pH of the gut, activating enzyme

secretion, and having an antimicrobial effect on pathogenic bacteria.

2.7 Conclusion

The weaning period is the most challenging and demanding phase for pigs since piglets have a functionally and anatomically immature gut ecosystem, and there are diverse stress factors associated with weaning process. As a result, in the weaning phase, piglets undergo dramatic alterations in their gut ecosystem. In order to assess the gut ecosystem of pigs, several *in vitro*, *in vivo*, and *ex vivo* methods have been developed and utilized. After world-wide restrictions were put in place with regard to using AGP in feeds, a number of antibiotic alternatives have been introduced and evaluated in pursuit of replacing AGP. However, each AGP replacer candidate has pros and cons and more studies are needed to completely remove AGP from the swine industry.

3.0 CHAPTER 3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

The following hypotheses were tested in this thesis:

1. Lipid matrix microparticles can improve the stability of essential oils during feed pelleting and storage;
2. Lipid matrix microparticles can allow a slow release of essential oils in weaned pig gut; and
3. Microencapsulated essential oils and organic acids by a lipid matrix can improve gut health in weaned piglets with physiological challenges.

3.2 Objectives

The overall objective was to investigate the potential protective effects of essential oils and organic acids microencapsulated with a lipid matrix for improving gut health in weaned piglets. Specific objectives were to:

1. Evaluate the stability of thymol in the lipid matrix microparticles during feed pelleting and storage;
2. Validate and demonstrate the slow release of thymol in the lipid matrix microparticles with *in vitro* and *in vivo* approaches; and
3. To elucidate the molecular mechanisms of the function of microencapsulated OA and EO in experimentally infected weaned piglets with enterotoxigenic *E.coli* F4 by measuring nutrient absorption, immune responses, microbiota, and gut barrier function.

4.0 CHAPTER 4 MANUSCRIPT I

EVALUATION OF LIPID MATRIX MICRO-ENCAPSULATION FOR INTESTINAL DELIVERY OF THYMOL IN WEANED PIGLETS

4.1 Abstract

The EO are defined as plant-derived natural bioactive compounds, which can have positive effects on animal growth and health due to their antimicrobial and antioxidative properties. However, EO are volatile, can evaporate quickly and be rapidly absorbed in the upper gastrointestinal tract. Also, due to their labile nature, the stability of EO during feed processing is often questionable, leading to variations in the final concentration in feed. Encapsulation has become one of the most popular methods of stabilizing EO during feed processing, storage and delivery into the lower gut. The objectives of the present study were to 1) evaluate the stability of thymol microencapsulated in combination with OA in commercially available lipid matrix microparticles during the feed pelleting process and storage; 2) validate and demonstrate the slow release of thymol from the lipid matrix microparticles in a simulated pig gastric fluid (SGF) and a simulated pig intestinal fluid (SIF); and 3) evaluate *in vivo* release of thymol from the lipid matrix microparticles along the pig gut. The results showed that thymol concentration was not significantly different in the mash and pelleted feeds ($P > 0.05$). In the *in vitro* study, 26.04% thymol was released in SGF, and the rest of the thymol was progressively released in SIF until completion, which was achieved by 24 h. The *in vivo* study showed that 15.5% of thymol was released in the stomach, and 41.85% of thymol was delivered in the mid-jejunum section. Only 2.21% of thymol was recovered in feces. In conclusion, the lipid matrix microparticles were able to maintain the stability of thymol during a feed pelleting process and storage and allow a slow and progressive intestinal release of thymol in weaned

piglets.

Keywords: Essential oils; Micro-encapsulation; Pelleting; *In vitro* release; *In vivo* release; Pigs

4.2 Introduction

During the weaning phase, piglets frequently have diarrheic symptoms and other intestinal disturbances, which can result in decreased growth performance and mortality (Yang et al., 2015; Hassan et al., 2018). Traditionally, AGP were used to reduce the complications associated with weaning. However, there is a concern regarding the transmission and the proliferation of resistant bacteria via the food chain, which has induced regulations and restrictions of the use of AGP in animal feed in many countries (Zeng et al., 2015b). Various AGP alternatives have been developed and practically used in the swine industry (Cheng et al., 2014).

Essentials oils (EO), natural bioactive compounds obtained from plants, are known to have antibacterial, antiviral, antifungal and antioxidative properties, and have traditionally been used as complementary or alternative medicines to improve human health or cure human diseases (Kim et al., 2008; Brenes and Roura, 2010; Omonijo et al., 2018b). With the identification of active components in plant extracts and some progress in the mechanistic studies of these components in animals, there has been an increase of studies in pursuit of using EO to substitute AGP in animal diets (Li et al., 2012c). Many studies found that various EO (e.g., thymol, cinnamaldehyde, eugenol, etc.) could improve growth performance (Manzanilla et al., 2006; Nofrarias et al., 2006), gut immune system (Su et al., 2018), gut morphology (Xu et al., 2018), and gut microbiota (Zeng et al., 2015a). However, the lipophilic and volatile properties of EO are obstacles that must be considered when including EO in pig feed (Omonijo et al., 2018c). Due to their volatile properties, EO may be absorbed into feed components or air-dried and evaporated during feed processing (e.g., pelleting), leading to reduced potency

(Si et al., 2006). Several studies indicated that EO were mainly or almost entirely absorbed in the stomach and the proximal small intestine of piglets after oral intake (Michiels et al., 2008). Thus, the majority of the EO, without proper protection, will be lost during feed processing and delivery to the pig gut and may not be able to reach the lower gut of pigs where most pathogens reside and propagate (Chen et al., 2017), which will reduce the profitability of feed mills and become one of the major barriers for EO application in feed. Thus, it is crucial to develop an effective and practically feasible delivery method for the use of EO in the feed.

Encapsulation, which provides a physical barrier for bioactive compounds and separates the core material from the environment until release, is thought to improve the stability of bioactive compounds and enable the slow release of EO in animals (Vidhyalakshmi et al., 2009). Lipid matrix micro-encapsulation has been popularly used to deliver bioactive compounds (e.g., EO, OA and vitamins) to the animal's gut (Liu et al., 2017b; Gottschalk et al., 2018; Yang et al., 2018; Kaur et al., 2019; Yang et al., 2019b). However, there is a lack of information on the stability of EO during feed processing and storage and the intestinal release of EO from the lipid matrix microparticles in animals. This study hypothesized that EO embedded (micro-encapsulated) in a commercially available lipid matrix microparticles as a blend of EO and OA will maintain their stability during the pelleting process and storage and EO may be slowly released in the pigs' gut. Therefore, the objectives of this research were to evaluate the stability of thymol in the lipid matrix microparticles during feed pelleting and feed storage and to determine the intestinal release of thymol using *in vitro* and *in vivo* approaches.

4.3 Materials and Methods

4.3.1 Materials

Thymol ($\geq 98.5\%$), α -methyl-trans-cinnamaldehyde ($\geq 98\%$), hexane (HPLC grade, 95%), pepsin derived from porcine gastric mucosa (≥ 250 units $\cdot$ mg⁻¹), bile salts, pancreatin originated from porcine pancreas (≥ 3 USP), and titanium dioxide ($\geq 99\%$ trace metal basis) were purchased from Sigma-Aldrich (Oakville, ON, Canada). A blend of EO and OA was embedded in lipid matrix microparticles (Jefo Nutrition Inc., Saint-Hyacinthe, QC, Canada). The components of the lipid matrix microparticles were hydrogenated vegetable oil for the matrix material and fumaric acid, sorbic acid, malic acid, citric acid, soya lecithin, thymol, vanillin and eugenol as active ingredients embedded (microencapsulated) within the lipid matrix.

4.3.2 Thymol stability in the lipid matrix microparticles during feed pelleting process and storage

The stability of thymol in the lipid matrix microparticles was determined during the pelleting process. A wheat-soybean meal (SBM) basal diet was formulated as shown in Table 4.1. The treatments included: 1) a control mash basal diet (CM), 2) CM + 0.2% of the lipid matrix microparticles (EOM), 3) CM pelleted (CP) and 4) EOM pelleted (EOP). The lipid matrix microparticles were premixed with corn (approximately 8 kg) before being added to the whole diets. The pelleting process was conducted with a Master Model California Pellet Mill (California Pellet Mill Co., San Francisco, CA, USA) at the Glenlea Swine Research Unit at the University of Manitoba. The air temperature during conditioning and pelleting was measured with a non-contact infrared thermometer (Fluke 62 mini infrared thermometer, Fluke Corporation, Everett, WA, USA). Conditioning before pelleting was conducted at 69 - 74°C by

directly adding steam to a mixer where feed and steam were thoroughly mixed and after 4 seconds the first feed particles moved to the pelleting part. The steam and feed mixture was pressed with a pressor that has a 4 mm diameter and 10 mm length and the pelleting temperature reached to 61°C. The total pelleting time of each batch was less than 2 min to pellet 50 kg of feed. Six samples were obtained from mash feed, and 6 samples after pelleting were collected. Every batch, the mash feed mixing was followed by the pelleting procedure. The pelleting process was conducted independently three times. The samples were kept at -80°C until further analyses.

Table 4.1 The composition of a wheat-soybean meal basal diet for the feed pelleting experiment (kg, as-fed basis).

Ingredients	kg
Wheat	400
Barley	60
Corn	250
Soybean meal (480 g crude protein·kg ⁻¹)	215
Soybean oil	10
Fish meal	40
Limestone	10
Vitamin-Minerals premix ¹	14
L-Lysine HCl	1
Total	1,000
Calculated net energy and nutrient content (g·kg ⁻¹)	
Net energy (kcal·kg ⁻¹)	2,272
Crude protein (%)	22.0

¹Supplied the following per kilogram of diet: 2200 IU vitamin A, 220 IU D3, 16IU 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 copper sulphate, 100 mg ferrous sulfate, 4 mg manganese oxide, 0.3 mg sodium selenite, and 100 mg zinc oxide.

The stability of thymol in the lipid matrix microparticles during feed storage was measured for 12 weeks at room temperature. The feeds from the third batch of EOM and EOP were used in the experiment. Six samples (400 g of feed) were taken from the EOM and EOP, respectively. Each feed sample was placed in an opened zip bag, and a total of 12 bags were stored in two plastic containers (45 cm × 30 cm × 40 cm) with the closed lid. The plastic containers were stored at a temperature of 23°C - 24°C and a relative humidity of 25 to 30%. At the time points of 0, 1, 3, 6, 9, 12 weeks, 25 g of feed were obtained from each bag and then stored at -80°C to minimize thymol evaporation until further analyses.

4.3.3 *In vitro* release of thymol in simulated gastric and intestinal fluids

The *in vitro* release profile of thymol in the lipid matrix microparticles was determined using a simulated pig gastric fluid (SGF) and a simulated pig intestinal fluid (SIF). Both SGF and SIF were prepared according to the methods described by Minekus et al. (2014) with some modifications. The SGF contained 47.2 mmol·L⁻¹ NaCl, 25 mmol·L⁻¹ NaHCO₃, 6.9 mmol·L⁻¹ KCl, 0.9 mmol·L⁻¹ KH₂PO₄, 0.5 mmol·L⁻¹ (NH₄)₂CO₃, 0.1 mmol·L⁻¹ MgCl₂(H₂O)₆, 0.15 mmol·L⁻¹ CaCl₂(H₂O)₂ and 2,000 U·mL⁻¹ pepsin originated from porcine gastric mucosa. The SIF contained 85 mmol·L⁻¹ NaHCO₃, 38.4 mmol·L⁻¹ NaCl, 6.8 mmol·L⁻¹ KCl, 0.8 mmol·L⁻¹ KH₂PO₄, 0.33 mmol·L⁻¹ MgCl₂(H₂O)₆, 0.6 mmol·L⁻¹ CaCl₂(H₂O)₂, 10 mM bile salts and 1% (by vol.) pancreatin originated from porcine pancreas (Liu et al., 2017a). The pH of SGF and SIF was adjusted to 3.0 and 7.0, respectively, using HCl or NaOH. The mixture of 9.5 mL of pre-warmed SGF (39°C) and 0.5 g of the lipid matrix microparticles was added into each tube and then incubated at 200 rpm for 2 h at 39°C. After that, 18 mL of pre-warmed SIF (39°C) was added into the tubes and pH was adjusted to 7.0. Then the tubes were horizontally

incubated at 200 rpm for 24 h at 39°C using a forced-convection laboratory oven (Heratherm, Thermo Scientific Inc., Waltham, MA, USA) and a shaker (MaxQ 2508, Thermo Scientific Inc.). At SIF 0 (SGF 2h + SIF 0), 1 (SGF 2 h + SIF 1 h), 2, 3, 4, 6, 8, 12, and 24 h, two samples (i.e. two tubes) were taken out to represent each time point and the pH of each sample was adjusted to 5.0 to minimize enzyme activities and the samples were then stored at –20°C until further analyses (Fig. 4.1). The two tubes collected for each time point were considered as the technical replicates and the *in vitro* release profile experiment was conducted in triplicates.

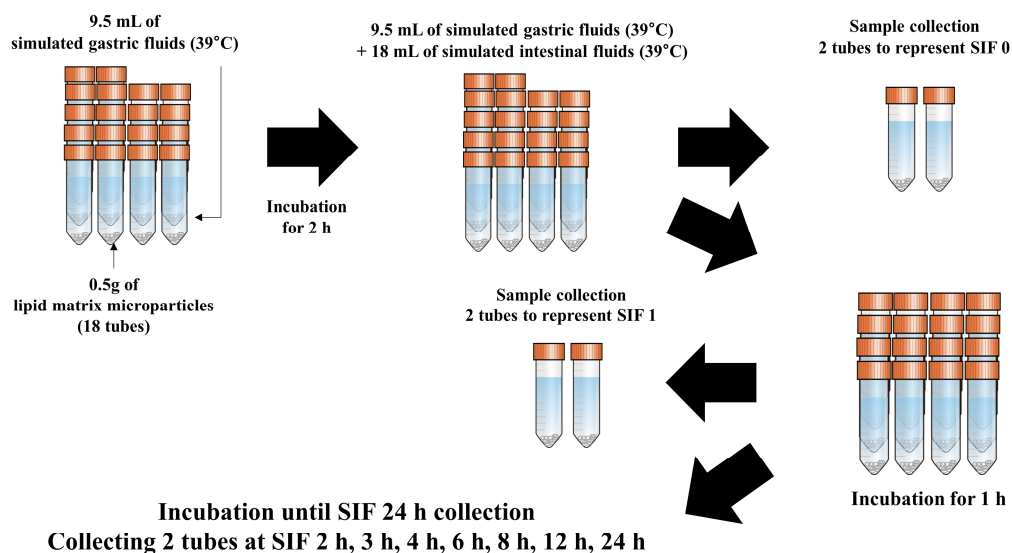


Figure 4.1 The flow diagram of the *in vitro* release profile study. The mixture of 9.5 mL of pre-warmed simulated gastric fluid (39°C) and 0.5 g of the lipid matrix microparticles was added into each tube (total 18 tubes) and then incubated for 2 h at 39°C. After that, 18 mL of pre-warmed simulated intestinal fluid (39°C) was added into the tubes. Two tubes were collected to represent SIF 0. After 1 h incubation, two tubes collected to represent SIF 1. The rest of the tubes were incubated until SIF 24 h collection and 2 tubes were collected to represent each time point.

4.3.4 *In vivo* recovery rate along the gut of weaned piglets

The experimental and animal care protocol (F17-018, AC11280) was reviewed and approved by the Animal Care Committee of the University of Manitoba and the pigs were cared for in accordance with the Canadian Council on Animal Care guidelines (CCAC, 2009). A total of 12 male piglets (TN Tempo $\times$ TN70; 9.86 ± 0.52 kg; 28 d) were obtained from the Glenlea Swine Research Unit at the University of Manitoba and housed individually after 4 d of group adaptation period (1 d – 5 d) in a temperature-controlled room in the T.K. Cheung Centre for Animal Science Research at the University of Manitoba. Room temperature was maintained at $29 \pm 1^\circ\text{C}$ during the first week and then reduced by 1.5°C for the rest of the experimental period. Piglets were randomly allotted to two treatments in a completely randomized design ($n = 6$): 1) a control corn-SBM basal diet (CF) and 2) a corn-SBM basal diet supplemented with $6 \text{ g}\cdot\text{kg}^{-1}$ lipid matrix microparticles (EOF). The corn-SBM basal diets were formulated to meet or exceed NRC (2012) nutrient specifications for pigs weighing 6-10 kg body weight (BW, Table 4.2). Zinc oxide was added in the diets to prevent diarrhea in pigs and titanium dioxide ($3 \text{ g}\cdot\text{kg}^{-1}$) was added as an inert marker to calculate the thymol recovery rate in the different gastrointestinal sections. All pigs had free access to water and feed during the whole experimental period and all experimental diets were provided in a mash form. On 8 d and 9d, feces were collected. On 9 d, final BW and feed intake were measured and thereafter, the pigs were anesthetized by an intramuscular injection of ketamine:xylazine ($20:2 \text{ mg}\cdot\text{kg BW}^{-1}$) and euthanized by intravenous injection of sodium pentobarbital ($110 \text{ mg}\cdot\text{kg BW}^{-1}$). The whole organs of the gastrointestinal tract were removed from the carcass and the digesta samples from the stomach, mid-jejunum (350 to 450 cm from the stomach-duodenum junction), ileum (upper

0 to 80 cm of the ileum-cecum junction), cecum and colon (lower 20 cm from the ileum-cecum junction) were collected into different sterilized containers (Adeola and King, 2006). The samples of collected digesta were kept at -20°C to be freeze-dried later. The individual pig was considered the experimental unit.

Table 4.2 The composition of diets used for the *in vivo* release experiment (kg, as-fed basis)¹.

Ingredients	Control diet	Microencapsulated essential oils diet
Corn	477.62	471.62
Soybean meal (480 g crude protein·kg ⁻¹)	160.00	160.00
Whey permeate	124.22	124.22
X-SOY600 ¹ (600 g crude protein·kg ⁻¹)	110.00	110.00
Fish meal	65.73	65.73
Soybean oil	15.00	15.00
Calcium (limestone)	14.32	14.32
Biofos 21% ²	5.73	5.73
Salt - bulk fine	5.00	5.00
Zinc oxide 72%	3.19	3.19
Vitamin-mineral premix ³ (1%)	10.00	10.00
L-lysine 78%	2.83	2.83
DL-methionine 99%	1.52	1.52
Threonine	1.32	1.32
L-tryptophan	0.51	0.51
Titanium dioxide (TiO ₂) ⁴	3.00	3.00
Microencapsulated essential oils ^{5,6}	0.00	6.00
Total	1000.00	1000.00
Calculated net energy and nutrient content (g·kg ⁻¹)		

Net energy (kcal·kg ⁻¹)	2475	2459
Crude protein (%)	22.4	22.3

¹Soy protein concentration (CJ Selecta, Goiania, State of Goiás, Brazil)

²Ca, 21%; P, 17% (The Mosaic Co., Plymouth, MN)

³Supplied the following per kilogram of diet: 2200 IU vitamin A, 220 IU D3, 16 IU 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 copper sulphate, 100 mg ferrous sulfate, 4 mg manganese oxide, 0.3 mg sodium selenite, and 100 mg zinc oxide.

⁴Titanium dioxide (TiO₂; Sigma-Aldrich, Oakville, Ontario, Canada)

⁵Lipid matrix microparticles including hydrogenated vegetable oil, fumaric acid, sorbic acid, malic acid, citric acid, soya lecithin, thymol, vanillin and eugenol (Jefo, Saint-Hyacinthe, Quebec, Canada)

⁶The lipid matrix microparticles were premixed in corn (approximately 8 kg) before being added to the whole diet.

4.3.5 Gas chromatographic determination of thymol

Thymol extraction from the feed or digesta samples was conducted according to the methods described by Folch et al. (1957) and Ndou et al. (2018) with some modifications. Samples were freeze-dried and finely ground with a coffee grinder (Applica Consumer Products Inc., Miami Lakes, FL, USA) and 1 g of sample was weighed and added to a 50 mL glass tube. Twenty milliliters of a mixture of chloroform/methanol (2:1, by vol.) and internal standard (α -methyl-trans-cinnamaldehyde) were added and shaken for 1 h to break down the lipid matrix microparticles and absorb thymol in the mixture. After shaking, 5 mL (25% by vol.) of water was added to separate the chloroform and methanol phase and the samples were centrifuged at $750 \times g$ for 15 min at 4°C. The chloroform phase was obtained with a Pasteur pipette (Fisher Scientific, Hampton, NH, USA) and was filtered with a filter paper (P5, Fisher Scientific) and dried under nitrogen gas (N_2) flux using an N-EVAP 112 evaporator (Organomation Associates Inc., Berlin, MA, USA) at 37°C. Methylation was done according to the method described by Ichihara and Fukubayashi (2010). Toluene (0.2 mL), methanol (1.5 mL), and 8% HCl (0.3 mL) were added sequentially and the mixture was vortexed and incubated at 45°C overnight. After the overnight incubation, the solution was evaporated under nitrogen gas (N_2) flux using an N-EVAP 112 evaporator (Organomation Associates Inc.). Hexane (2 mL) was added to dissolve thymol and water (2 mL) was added to wash hexane and then the tubes were vortexed and centrifuged at $750 \times g$ for 15 min at 4°C. Finally, the 2 mL of the hexane phase was obtained, and thymol content was analyzed by Gas chromatography – flame ionization detector (GC-FID).

Samples from the *in vitro* release experiment were thawed at room temperature and

centrifuged at $4700 \times g$ for 20 min at 4°C and the supernatant was filtered with a filter paper (P5, Fisher Scientific) and the filtered quantity of the supernatant was recorded. The filtered supernatant was mixed with 15 mL of hexane with internal standard using a rotator (Rotator AG, FINEPCR, Gunpo, Gyeonggi-do, Korea) for 1 h and centrifuged at $750 \times g$ for 10 min at 4°C and the hexane phase was obtained by a Pasteur pipette (Fisher Scientific). The obtained hexane was methylated as described above (Ichihara and Fukubayashi, 2010) and the samples were analyzed by GC-FID.

The amount of thymol was determined by GC-FID. The samples were separated on a CP Select Fames column ($100 \text{ m} \times 0.25 \text{ mm}$ diameter and $0.25 \mu\text{m}$ film thickness; Varian Canada, Mississauga, ON, Canada) using a Bruker 450 GC with FID (Varian Canada). The temperature program was 70°C for 2 min, the temperature was raised to 175°C at $25^{\circ}\text{C} \cdot \text{min}^{-1}$, held for 20 min, raised to 215°C at $1.5^{\circ}\text{C} \cdot \text{min}^{-1}$, held for 10 min, and raised to 250°C at $20^{\circ}\text{C} \cdot \text{min}^{-1}$ and held for 3 min and total run time was 67.62 min. Samples were run with a 20:1 split ratio and $0.8 \text{ mL} \cdot \text{min}^{-1}$ column flow. The temperature detector was 290°C and hydrogen was used as the carrier gas.

4.3.6 Calculation of thymol concentrations and recovery rates

The thymol concentration was calculated based on the peak area ratio between thymol (specific compound of interest) and α -methyl-trans-cinnamaldehyde (internal standard) as follows (FID, 2003):

$$\text{Thymol concentration (mg} \cdot \text{kg}^{-1}) = \frac{\text{AMOUNT}_{\text{IS}} \times \text{AREA}_{\text{SC}} \times \text{IRF}_{\text{SC}}}{\text{AREA}_{\text{IS}}}$$

(IS = internal standard, SC = specific compound of interest, IRF = internal response ratio)

between IS and SC).

According to Zhang et al. (2016), the recovery rate of thymol in the different gastrointestinal segments was calculated by analyzing thymol and titanium dioxide contents in feed or digesta. Samples for titanium analysis were prepared according to the method proposed by Lomer et al. (2000) and the titanium concentration was determined using an inductively coupled plasma spectrometer (Vista-MPX; Varian Canada). Thymol recovery rate was calculated based on the following equation (Zhang et al., 2016):

$$\text{RECOVERY}_{\text{THYMOL}}(\%) = \left[\frac{(\text{MARKER}_{\text{DET}} \times \text{THYMOL}_{\text{DIGESTA}})}{(\text{THYMOL}_{\text{DET}} \times \text{MARKER}_{\text{DIGESTA}})} \right] \times 100$$

4.3.7 Statistical analyses

GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA) was used to perform statistical analyses. In the pelleting experiment, the differences in thymol content between the EOM and EOP were analyzed by an unpaired *t*-test. In the storage stability experiments, total thymol contents were compared by one-way analysis of variance (ANOVA) followed by a Tukey's test. For the *in vitro* release experiment, a curve fitting program (*Padé* approximant) was used. In the *in vivo* release experiment, the mean and SEM were calculated. Data in all figures are shown as means $\pm$ SEM. For all statistical analyses, $P < 0.05$ was considered significant.

4.4 Results

The wheat-SBM basal diets either not supplemented or supplemented with thymol microencapsulated in the lipid matrix microparticles were pelleted at up to 74°C. Thymol was

not detectable in the non-supplemented diets (mash feed and pelleted feed). As shown in Fig. 4.2, there was no difference in the thymol content between EOM and EOP in the three different batches ($P > 0.05$) (Fig. 4.2). As shown in Fig. 4.3, the total amount of thymol in both EOM and EOP did not change during the studied periods (12 weeks) ($P > 0.05$).

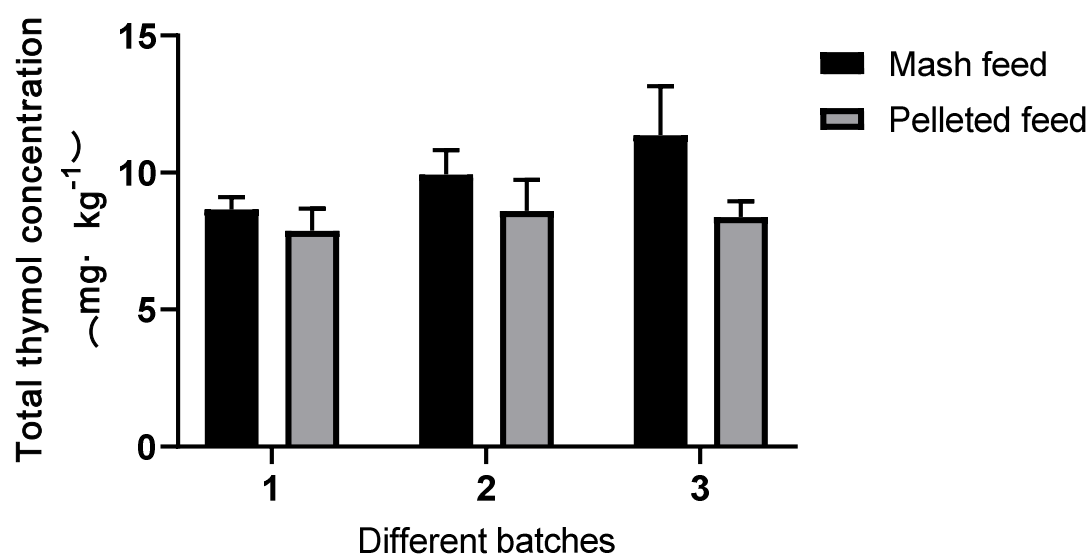


Figure 4.2 Effect of feed pelleting process on total thymol content in a diet either non-supplemented or supplemented with thymol microencapsulated in the lipid matrix microparticles. Total thymol content in the diets was analyzed by a gas chromatography-flame ionization detector. Thymol was not detectable in the diets not supplemented with thymol microencapsulated in the lipid matrix microparticles (both mash and pelleted feeds). Each value represents the mean $\pm$ SEM, $n = 6$. Three independent batches were conducted to check the variation of the feed pelleting process.

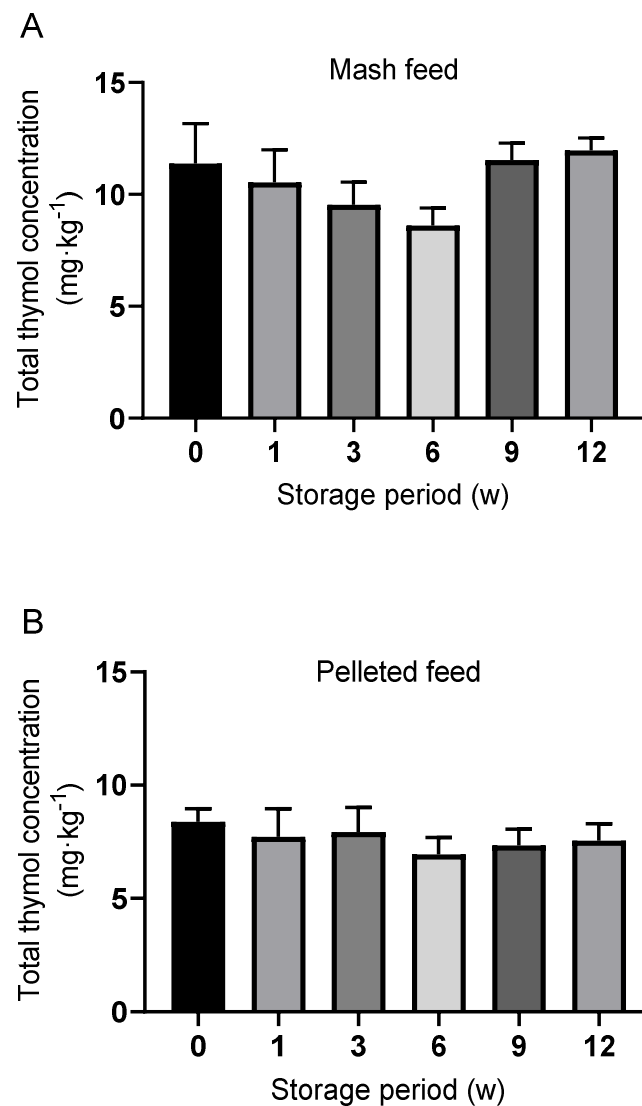


Figure 4.3 The stability of thymol microencapsulated in the lipid matrix microparticles in the mash feed (A) and pelleted feed (B) during storage. Mash and pelleted feeds supplemented with thymol microencapsulated in the lipid matrix microparticles were stored at room temperature (22-24°C) and 20-30% humidity for 12 weeks. Each value represents the mean $\pm$

SEM, n = 6.

In vitro release profile of thymol from the lipid matrix microparticles were investigated in SGF and SIF. As shown in Fig. 4.4, 26.04% thymol was released in SGF, and the rest of the loaded thymol was progressively released in SIF until completion, which was achieved by around 24 h. The recovery rate of thymol was determined along the gut of weaned piglets fed a diet either non-supplemented or supplemented with $6 \text{ g} \cdot \text{kg}^{-1}$ thymol microencapsulated in the lipid matrix microparticles. During the whole experiment period, all pigs were healthy and consumed the feed at the normal quantity. The average final BW of all the pigs was 11.5 ± 0.99 kg and daily feed intake (d 6 – d 9) was 0.45 ± 0.12 kg. There was no significant difference between the CF and EOF in the final BW and daily feed intake ($P > 0.05$). Thymol was not detectable along the gut of weaned piglets fed a diet non-supplemented with thymol microencapsulated in the lipid matrix microparticles. As shown in Fig. 4.5, 15.5% of thymol was released in the stomach, and 41.1% of thymol was delivered to the mid-jejunum section. The thymol was recovered in the ileum, cecum, and colon at 14.36%, 14.92%, and 14.35%, respectively. Only 2.21% of thymol was recovered in feces.

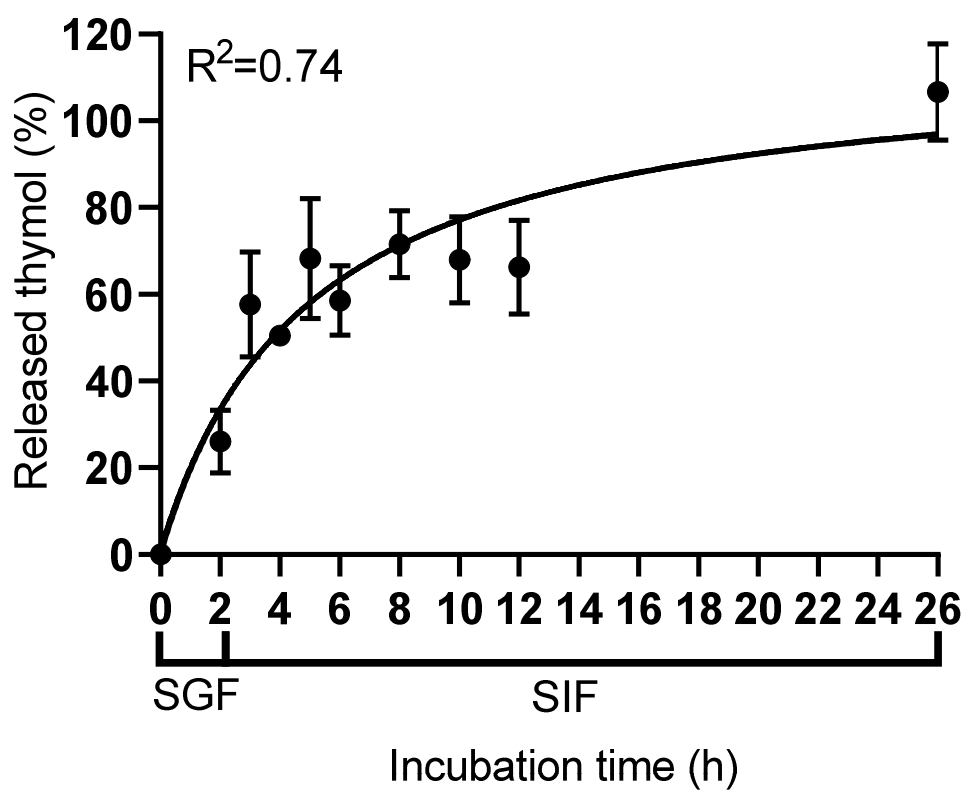


Figure 4.4 *In vitro* release profile of thymol from the lipid matrix microparticles in simulated pig gastric fluid (SGF) and simulated pig intestinal fluid (SIF). Each value represents the mean $\pm$ SEM, $n = 3$.

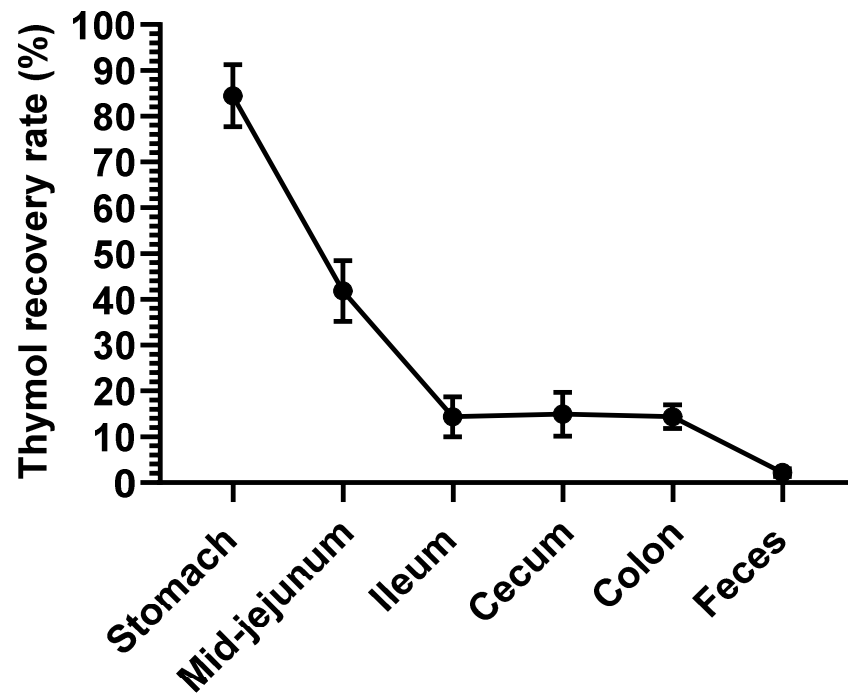


Figure 4.5 The recovery rate of thymol along the gut of weaned piglets fed a diet either non-supplemented or supplemented with thymol microencapsulated in the lipid matrix microparticles. Thymol was not detectable along the gut of weaned piglets fed a diet non-supplemented with thymol microencapsulated in the lipid matrix microparticles. Each value represents the mean $\pm$ SEM and $n = 6$.

4.5 Discussion

Encapsulation, defined as a process in which micro- or nano- particles or droplets are encircled by a coating, or embedded in a homogeneous or heterogeneous matrix, is a helpful method to improve the potency of feed additives (Gharsallaoui et al., 2007). The benefits of encapsulation are to 1) improve the storage stability of feed additives; 2) protect feed additives during feed processing including mixing, conditioning and pelleting; 3) mask unpleasant odor that can decrease feed intake; 4) improve the ease of handling of liquid feed additives (e.g., EO) by changing liquid to solid state; 5) slowly release bioactive compounds along the gut of animals; and 6) reduce the effective dosage of bioactive compounds that have high cost and environmental issues. A broad range of bioactive compounds such as probiotics (Liu et al., 2016), EO (Omonijo et al., 2018a), ZnO (Xie et al., 2011), OA (Grilli et al., 2010), bacteriophages (Huff et al., 2013) and enzymes (Chandrasekar et al., 2017) have been encapsulated for improving animal health.

An ideal encapsulation should not only increase the stability of EO, but also release them specifically in the target regions of the intestine (Chen et al., 2017). Wall or matrix materials are one of the most influential factors in controlling the release of bioactive compounds (Carneiro et al., 2013). Many wall or matrix materials, including polysaccharides (alginate xanthan gum), proteins (whey protein and gelatin) and lipids (milk fat and hydrogenated fat), have been used to encapsulate EO for effective delivery to the gut (Piva et al., 2007; Liu et al., 2016; Zhang et al., 2016; Chen et al., 2017). The benefits of encapsulated EO with hydrogenated fat are to facilitate slow release (Mehnert and Mäder, 2012) and to have high stability (Souto and Müller, 2010). Furthermore, solid lipid has been considered as the

most cost-effective material for encapsulating EO. Solid lipid has also been used for encapsulating probiotics (Okuro et al., 2013), ZnO (Jang et al., 2014), vitamin A (Jenning et al., 2000) and OA (Piva et al., 2007). However, the stability of EO during feed processing and storage and the intestinal release of EO in animals are still not clear. Therefore, this study evaluated the stability of thymol in lipid matrix microparticles during a feed pelleting process and feed storage and determined the intestinal release of thymol using *in vitro* and *in vivo* approaches.

In modern farming, pig diets are commonly provided in a pellet form (Fahrenholz, 2012). The pelleting process is mainly composed of conditioning and pelleting. Conditioning refers to adding steam and heat to improve the binding property, while the purpose of pelleting is to agglomerate small particles into large particles (Falk, 1985). It has been proven that pelleting pig' diets enhance palatability, growth performance, nutrient and energy digestibility, and feed utilization efficiency compared to mash feeding (Lahaye et al., 2008; Vukmirović et al., 2017). However, the side effects of pelleting, including the possibility of breaking down of nutrients and feed additives, should be considered (Lewis et al., 2015; Kiarie and Mills, 2019). The most negative effects of pelleting are from wet steam, fat addition and high energy input, which can decrease the stability of nutrients and feed additives (Broz et al., 1997). For example, Jongbloed and Kemme (1990) showed that when the pelleting temperature reached over 80°C, the activity of exogenous phytase was decreased in the animal feed. In this study, the pelleting process did not change total thymol in the feed. The melting point of hydrogenated vegetable oil (matrix materials of the lipid matrix microparticles) is between 50 – 54°C and the range of the measured temperature during the conditioning and pelleting process in this experiment

reached between 61°C - 74°C. However, the pelleting process did not break down the lipid matrix microparticles and evaporate thymol in lipid matrix microparticles. There are several potential reasons: 1) the conditioning and pelleting time (less 2 min for pelleting 50 kg) in this experiment was not long enough to break down the lipid matrix microparticles and to evaporate thymol; 2) feed ingredients possibly protected the lipid matrix microparticles during the conditioning part of the pelleting process in this experiment; and 3) after being melted during pelleting, lipids might still be with thymol together and then become solid particles again after pelleting. However, different pelleting conditions (e.g., higher temperature and longer time) may be able to break down the lipid matrix microparticles and evaporate EO. More studies are required to understand the effects of the pelleting process on the recovery rate of EO in lipid matrix microparticles with diverse pelleting conditions.

It was expected that there should be free thymol released from the lipid matrix microparticles but remained in the pelleted feed because pelleting aggregates the feed ingredients, which may inhibit the instant evaporation of thymol. The released thymol in the pelleted feed would be evaporated as when the pelleted feed was stored for 12 weeks. However, because the amount of thymol in the pelleted feed did not change, it can be deduced that lipid matrix microparticles remained intact during the commercial pelleting process. In the swine industry, compound feed is stored for up to 3 months before it is used. The free form of EO is vulnerable to oxidation by air and light (Moghaddam and Mehdizadeh, 2016). Furthermore, a study by Luo et al. (2005) showed that there are some mineral sources, including copper in the animal feed, which can accelerate the oxidation of bioactive compounds. In this experiment, EO encapsulated with hydrogenated vegetable oil maintained their stability during the storage

and after mixing with other ingredients and pelleting. A potential reason is that hydrogenated vegetable oil, used as a matrix material in the experiment, may be resistant to oxidation and can maintain solid because its melting point is between 50 - 54°C (Gupta, 2017). According to Mavromichalis and Baker (2000), harsh environmental conditions can be applied to feed in animal rooms where the temperature increases to more than 39°C and during storage in silos and normal storage areas during the summer months (Alabadan and Oyewo, 2005) indicating the need for more storage stability studies in high-temperature environments.

In this study, lipid matrix microparticles could maintain their stability in SGF (pH 3) and released most of the EO in SIF. This is because lipids cannot be digested by pepsin and only digested by lipase with emulsification by bile salts in intestinal pH (e.g., pH 6-7) (Hussain et al., 2015). The 26.04% of released thymol in SGF may include the solubilized EO that existed on the surface of the microparticles and released EO from the physical pressure of shaking 2 h in SGF. While lipase in SIF may have played a critical role to break down the lipid matrix particles in SIF, bile salts also may have played an important function by emulsifying the lipid matrix microparticles, which generated new surfaces of the lipid matrix microparticles and facilitated the digestion of the lipid matrix microparticles (Schonewille et al., 2016). In agreement with the *in vitro* release study, Hamoudi et al. (2011) showed that it took approximately 24 h to release lipophilic drugs (progesterone) from the lipid beads made of α -cyclodextrin and soybean oil in SGF and SIF. It is important to note that there was a difference in the release profile when EO were encapsulated with the different wall or matrix materials. A study by Zhang et al. (2016) showed that EO encapsulated with alginate-whey protein was released at approximately 20 – 30% in the SGF incubation after 2 h and completely released at

6 h (SGF 2 h + SIF 4 h). Omonijo et al. (2018a) showed that approximately 50% of thymol encapsulated with starch and alginate were released within 2 h of incubation in SGF and 100% release was observed following by incubation in SIF for an additional 2 h. These differences might be due to using different wall/matrix materials or differences in *in vitro* experimental conditions such as enzyme concentrations and incubation temperature.

In the *in vivo* study, pharmacological concentration of zinc oxide (above 3,000 mg/kg) were added to the diets in order to prevent PWD, which may be able to affect release profile along the pig gut. *In vivo* study showed that around 15.5% of thymol was released in the stomach and 41.85% of thymol was delivered to the mid-jejunum section and only 2.21% of thymol was recovered in the feces, which is considered a slow release. A slow-release can be defined as releasing minimal amounts of bioactive compounds in the stomach and delivering high amounts of such compounds to the mid-jejunum section and releasing most of the bioactive compounds before they are excreted. Zhang et al. (2014) showed that approximately 38%, 19% and 4% of the non-encapsulated form of carvacrol (e.g. EO) was recovered in the stomach, duodenum, and jejunum of weaned piglets, respectively, which indicated significant amount carvacrol disappeared in the stomach. Thus, as 84.5% of thymol was recovered in the stomach in the study, it can be inferred that only a minimal release occurred thus indicating a slow-release.

A nutrient with digestibility of more than 90% is considered as very digestible for pigs and thus 2.21% of remained thymol in the feces indicates that almost all of the thymol disappeared in the gut of pigs (Jørgensen et al., 2000). the *in vivo* study, 15.5% of the released thymol in the stomach may have included the solubilized thymol from the surface of the lipid

matrix microparticles and released thymol from the physical pressure of the segmentation movement of the stomach. Also, some of the lipid matrix microparticles may have been digested by gastric lipase, which is secreted from gastric chief cells in the fundic mucosa and plays an important role in the digestion of lipid, especially in piglets. The possible reason for the difference between released thymol in SGF (SIF 0, 26.04%) and the stomach in pigs (15.5%) would be that SIF 0 represents finished incubation in the SGF 2h, but a recovery rate of thymol in the stomach represents the released thymol during incubation in the stomach. Therefore, it would be more accurate to calculate the thymol recovery rate in the duodenum, but it was not feasible to collect duodenal digesta from piglets.

Most of the thymol from the lipid matrix microparticles were released in the jejunum, which can be estimated by subtracting the recovery rate of the ileum (14.36%) from the stomach (85.5%). Pancreatin enzymes, including lipase and bile salts, are secreted into the duodenum and most of the lipid sources are digested before they reach the ileum (Valette et al., 1992). However, the recovery rates of thymol in the ileum (14.36%), cecum (14.91%) and colon (14.35%) were similar. The potential explanation could be the 14.36% of thymol in the ileum existed as released form but thymol was not absorbed in the ileum, cecum and colon, and after digesta were excreted as feces, most of the thymol was evaporated. There have been a few *in vivo* studies that have investigated the release profile of EO in pigs. In one of those studies, when EO microparticles encapsulated with alginate-whey protein were supplemented to pig, roughly 75%, 68%, 51%, 17%, 5%, and 5% was recovered in the stomach, duodenum, jejunum, ileum, cecum, and colon, respectively (Zhang et al., 2014). According to Piva et al. (2007), encapsulated OA and natural identical flavors with hydrogenated vegetable lipids

showed a gradual decrease in the gastrointestinal tract (stomach, cranial jejunum, caudal jejunum, ileum and cecum) of growing pigs compared to the non-encapsulated form of OA and natural identical flavors. As the lipid matrix microparticles were digested, some of the released thymol possibly showed beneficial properties such as antimicrobial, antioxidative, and anti-inflammatory effects in the gastrointestinal tract of weaned piglets. Furthermore, some of the released thymol was most likely absorbed as secondary metabolites (thymol sulfate and thymol glucuronide) through the intestinal wall and transported by the blood to the liver (Pisarčíková et al., 2017). Therefore, the *in vivo* release experiment showed that the lipid matrix microparticles maintained their stability in the stomach and slowly released most of the thymol in the small intestine and delivered some thymol to the large intestine.

The current study shows that the lipid matrix microparticles can maintain stability during the pelleting process and storage. *In vitro* and *in vivo* release experiments demonstrated that the lipid matrix microparticles allowed for a slow release in simulated digestive fluids and along the gut of weaned piglets. The efficacy of lipid matrix microparticles *in vivo* has recently been validated by Xu et al. (2019) in weaned piglets challenged with ETEC F4 by measuring growth performance and gut barrier function. However, more research is needed to investigate the effects of lipid matrix microparticles on nutrient absorption, immune responses and microbiota in weaned piglets experimentally infected with *E. coli* F4.

4.6 Conclusion

The current study shows that the lipid matrix microparticles can maintain stability during the pelleting process and storage. *In vitro* and *in vivo* release experiments demonstrated that the lipid matrix microparticles allowed for a slow release in simulated digestive fluids and

along the gut of weaned piglets. The efficacy of lipid matrix microparticles *in vivo* has recently been validated by Xu et al. (2019) in weaned piglets challenged with ETEC F4 by measuring growth performance and gut barrier function. However, more research is needed to investigate the effects of lipid matrix microparticles on nutrient absorption, immune responses and microbiota in weaned piglets experimentally infected with ETEC F4.

Table 4.3 The composition of a wheat-soybean meal basal diet for the feed pelleting experiment (kg, as-fed basis).

Ingredients	kg
Wheat	400
Barley	60
Corn	250
Soybean meal (480 g crude protein·kg ⁻¹)	215
Soybean oil	10
Fish meal	40
Limestone	10
Vitamin-Minerals premix ¹	14
L-Lysine HCl	1
Total	1,000
Calculated net energy and nutrient content (g·kg ⁻¹)	
Net energy (kcal·kg ⁻¹)	2,272
Crude protein (%)	22.0

¹Supplied the following per kilogram of diet: 2200 IU vitamin A, 220 IU D3, 16IU 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 copper sulphate, 100 mg ferrous sulfate, 4 mg manganese oxide, 0.3 mg sodium selenite, and 100 mg zinc oxide.

Table 4.4 The composition of diets used for the *in vivo* release experiment (kg, as-fed basis)¹.

Ingredients	Control diet	Microencapsulated essential oils diet
Corn	477.62	471.62
Soybean meal (480 g crude protein·kg ⁻¹)	160.00	160.00
Whey permeate	124.22	124.22
X-SOY600 ¹ (600 g crude protein·kg ⁻¹)	110.00	110.00
Fish meal	65.73	65.73
Soybean oil	15.00	15.00
Calcium (limestone)	14.32	14.32
Biofos 21% ²	5.73	5.73
Salt - bulk fine	5.00	5.00
Zinc oxide 72%	3.19	3.19
Vitamin-mineral premix ³ (1%)	10.00	10.00
L-lysine 78%	2.83	2.83
DL-methionine 99%	1.52	1.52
Threonine	1.32	1.32
L-tryptophan	0.51	0.51
Titanium dioxide (TiO ₂) ⁴	3.00	3.00
Microencapsulated essential oils ^{5,6}	0.00	6.00
Total	1000.00	1000.00
Calculated net energy and nutrient content (g·kg ⁻¹)		

Net energy (kcal·kg ⁻¹)	2475	2459
Crude protein (%)	22.4	22.3

¹Soy protein concentration (CJ Selecta, Goiania, State of Goiás, Brazil)

²Ca, 21%; P, 17% (The Mosaic Co., Plymouth, MN)

³Supplied the following per kilogram of diet: 2200 IU vitamin A, 220 IU D3, 16 IU 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 copper sulphate, 100 mg ferrous sulfate, 4 mg manganese oxide, 0.3 mg sodium selenite, and 100 mg zinc oxide.

⁴Titanium dioxide (TiO₂; Sigma-Aldrich, Oakville, Ontario, Canada)

⁵Lipid matrix microparticles including hydrogenated vegetable oil, fumaric acid, sorbic acid, malic acid, citric acid, soya lecithin, thymol, vanillin and eugenol (Jefo, Saint-Hyacinthe, Quebec, Canada)

⁶The lipid matrix microparticles were premixed in corn (approximately 8 kg) before being added to the whole diet.

5.0 CHAPTER 5 MANUSCRIPT II
EFFECTS OF MICRO-ENCAPSULATED FORMULA OF ORGANIC ACIDS AND
ESSENTIAL OILS ON THE NUTRIENT ABSORPTION, IMMUNITY,
MICROBIOTA AND GUT BARRIER FUNCTION OF WEANED PIGLETS
CHALLENGED WITH ENTEROTOXIGENIC *Escherichia coli* F4

5.1 Abstract

The purpose of the study was to investigate the effects of micro-encapsulated OA and EO on growth performance, immune system, gut barrier function, nutrient absorption, and microbiota in the weaned piglets challenged with ETEC F4. Twenty-four ETEC F4 susceptible weaned piglets were randomly distributed to four treatments including: (1) non-challenged negative control (NNC; piglets fed a control diet and challenged with phosphate-buffered saline (PBS); (2) negative control (NC; piglets fed a control diet and challenged with ETEC F4); (3) positive control (PC; NC + 55 mg·kg⁻¹ of Aureomycin); and (4) micro-encapsulated OA and EO (P(OA+EO); (NC + 2 g·kg⁻¹ of micro-encapsulated OA and EO). On d 7, ETEC F4 (5 mL of 1 × 10⁷ CFU·mL⁻¹) or PBS was inoculated to piglets and piglets were euthanized on 5 day post-inoculum (dpi). ETEC F4 infection significantly decreased ADG during the post-challenge period ($P < 0.05$). The piglets fed micro-encapsulated OA and EO had significantly lower core body temperature ($P < 0.05$) at 3 hpi (hours post-inoculum) compared to the NC piglets ($P < 0.05$). Diarrhea was significantly induced at 8 hpi, 16 hpi, 28 hpi, 34 hpi, and 40 hpi ($P < 0.05$) and tended to increase diarrhea at the 3 hpi ($P = 0.10$) and 24 hpi ($P = 0.09$) due to the ETEC F4 infection. The supplementation of micro-encapsulated OA and EO relieved diarrhea at 28 and 40 hpi ($P < 0.05$) and tended to alleviate diarrhea at 34 hpi ($P = 0.07$).

Intestinal permeability as indicated by the flux of fluorescein isothiocyanate-dextran D4 (FITC D4) was significantly decreased in the P(OA+EO) piglets ($P < 0.05$). Decreased jejunal VH due to ETEC F4 infection ($P < 0.05$) was significantly attenuated in the P(OA+EO) pigs ($P < 0.05$). The number of goblet cells per 100 μm VH tended to decrease in the PC ($P = 0.10$) and P(OA+EO) piglets ($P = 0.09$) compared to the NC piglets. The supplementation of OA and EO tended to alleviate ($P = 0.10$) the decreased maltase activity due to ETEC F4 infection ($P = 0.07$). The activity of IAP and relative B⁰AT1 mRNA abundance was significantly increased in the PC piglets ($P < 0.05$). The ETEC F4 infection significantly increased the mRNA abundance of IL8 ($P < 0.05$), which was attenuated in the PC and P(OA+EO) piglets ($P < 0.05$). In summary, the data suggest that supplementation of micro-encapsulated OA and EO alleviated the induced diarrhea and inflammation response, the damaged gut barrier integrity, intestinal morphology, enzyme activities and nutrient transport from ETEC F4 infection in weaned piglets. Therefore, micro-encapsulated OA and EO could be used as an alternative to antibiotics for swine production.

Keywords Micro-encapsulation, essential oils, organic acids, weaned piglets, *Escherichia coli* F4

5.2 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, increased morbidity and mortality (Pan et al., 2017). ETEC F4 is one of the common pathogens associated with PWD. The fimbriae (F4) of ETEC F4 can attach to epithelial receptors and release toxins in the intestine of pigs (Jacobsen et al., 2011). Over the last half-century, AGP 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 restrict the use of antibiotics in the livestock industry (Bengtsson and Wierup, 2006; Murphy et al., 2017).

A number of AGP alternatives have been developed and practically used in the swine industry (Heo et al., 2013), among which EO are considered as one of the most promising AGP alternatives in the swine industry due to their benefits to gut health and growth performance of pigs (Omonijo et al., 2018c). Antimicrobial effects to both Gram-negative and Gram-positive bacteria of diverse EO are already well documented (Chouhan et al., 2017). An *in vitro* study conducted by Si et al. (2006) showed that EO efficiently controlled the growth of pathogens including *Salmonella Typhimurium* DT 104, ETEC O157:H7 and ETEC F4. In addition, an *in vitro* study showed that EO (e.g. thymol) improved barrier integrity and attenuated inflammatory responses in the porcine intestinal epithelial cells (IPEC-J2) challenged with LPS

(Omonijo et al., 2018b). In pigs, the supplementation of EO improved growth performance (Xu et al., 2018), gut morphology (Zou et al., 2016a), enzyme activities (Xu et al., 2018), intestinal barrier function (Zou et al., 2016b), immune system activation (Williams et al., 2017), antioxidative capacity (Cheng et al., 2017) and microbiota (Cairo et al., 2018). Even though EO have benefits of promoting the growth performance and gut health and of pigs, their stability in the feed and along the gut restrains their application to pig diets (Omonijo et al., 2018a). However, micro-encapsulation, which provides a physical barrier for EO from their environment until their release, is thought to improve the stability of EO and enable the slow release of EO along the pig gut (Vidhyalakshmi et al., 2009).

The supplementation of EO with OA showed the synergistic effects to improve the growth performance and gut health of pigs (Yang et al., 2015). Zhou et al. (2007) reported that EO with OA showed synergistic antimicrobial effects against *Salmonella Typhimurium*. The supplementation of OA and EO improved nutrient digestibility and digestive enzyme activities in weaned piglets (Xu et al., 2018). However, more studies are still needed to comprehensively understand the mechanisms behind the protection of micro-encapsulated OA and EO against pathogens in weaned piglets. Therefore, the purpose of the study was to investigate the effects of micro-encapsulated OA and EO on growth performance, immune system, gut barrier function, nutrient absorption, and microbiota in the weaned piglets challenged with ETEC F4.

5.3 Materials and Methods

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

5.3.1 Virulence factors of enterotoxigenic *Escherichia coli* (ETEC) 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 4 virulence genes associated with adhesion including *faeG* (F4 fimbriae) and enterotoxins including *estA* (St_a, heat-stable toxin) and *estB* (ST_b, heat-stable toxin), *elt* (LT, heat-labile toxin) in ETEC F4 were checked by PCR (polymerase chain reaction) according to the method previously described by Zhu et al. (2011) with some modifications (Table 5.1). The genomic DNA from cultured ETEC F4 (1×10^9 CFU) was extracted using PureLink® Genomic DNA Kits (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted using an Ambion RiboPure™ RNA isolation kit (Ambion Inc., Foster City, CA, USA) and first-strand cDNA was synthesized using oligo (dT) 20 primers and Superscript II reverse transcriptase (Invitrogen) 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 ($10 \mu\text{mol} \cdot \text{L}^{-1}$), 10 µL of DreamTaq Green PCR Master Mix (2×) (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µL genomic DNA or 1 µL cDNA. PCR thermocycler conditions were as follows: 50°C denature 2 min, 95°C denature 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). All 4 virulence genes (*estA*, *estB*, *faeG* and *elt*) were expressed in the ETEC F4 used in the study (Fig. 5.1).

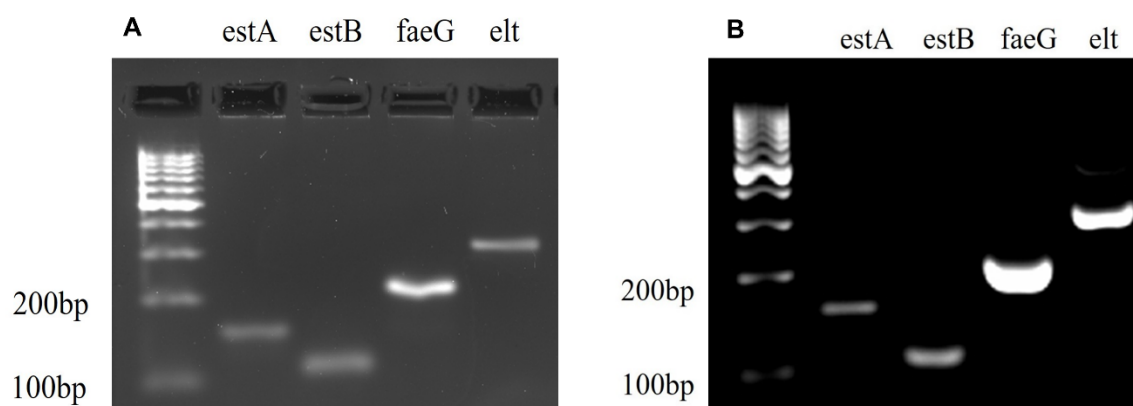


Figure 5.1 Agarose gel electrophoresis of the amplification products of virulence genes (Genomic DNA = A and RNA expression = B) in enterotoxigenic *E. coli* F4 used in the study. The four virulence genes were associated with adhesion (faeG), and enterotoxins (estA, estB, elt) of *E. coli* F4. The size of each gene was: estA = 158 bp; estB = 113 bp; faeG = 215 bp; elt = 322 bp. GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Waltham, MA, USA) was included in the first lane.

5.3.2 Genetic susceptibility screening and piglet selection

The ETEC F4 susceptible piglets were selected according to a previous publication (Jensen et al., 2006). Tails were collected when the pig's tails were docked on 3 d after farrowing. DNA was extracted using a method described by Truett et al. (2000). The PCR of MUC4 gene was performed using DreamTaq DNA polymerase (Thermo Fisher Scientific) with 2 mmol·L⁻¹ MgCl₂, 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°C subsequently 95°C for 30 s, annealing at 65°C for 30 s and extension at 72°C 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) at 37°C 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). 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 BW were selected.

5.3.3 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 and shaking 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. The culture was grown for 2.5 h. Necessary preliminary experiments such as a growth curve and standard curve

were generated first before preparing with 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, 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.

5.3.4 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 T.K. Cheung Centre for Animal Science Research at the University of Manitoba. Room temperature was maintained at 29 ± 1 °C during the first week and then reduced by 1.5 °C for the rest of the experiment period (8-12 d). The selected susceptible weaned piglets were randomly distributed to 4 treatments with 6 replicates for each treatment. A corn-SBM basal diet was formulated to meet or exceed the NRC (2012) recommendations for 6-10 kg piglets (Table 5.2). The four treatments were: 1) non-challenged negative control (NNC; piglets fed a control basal diet and challenged with PBS; 2) negative control (NC; piglets fed a control diet and challenged with ETEC F4; 3) positive control (PC; NC + 55 mg·kg⁻¹ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada)); and 4) micro-encapsulated formula of OA and EO (P(OA+EO)); NC + 2 g·kg⁻¹ of a selected formula of OA (fumaric, citric, malic and sorbic acids) and EO (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada). Piglets were housed in individual pens and allowed free access to mash feed and water during the whole experiment period. During the 7 d of pre-challenge (adaptation period) and 4 day post-inoculum (dpi),

individual pig's BW and pen feed disappearance were recorded to calculate ADG, average daily feed intake (ADFI) and feed conversion ratio (FCR). For implementing the ETEC F4 challenge model in piglets, 5 mL of 1×10^7 CFU·mL⁻¹ ETEC F4 was administered to piglets with a syringe attached to polyethylene tube held into the upper esophagus on 7 d (Koo et al., 2017, 2019). Before the inoculation, core body temperature was measured and at 3 hpi (hour post-inoculum), 24 hpi, and 48 hpi, core body temperature was measured and fecal consistency score (0 = normal, 1 = soft feces, 2 = mild diarrhea, and 3 = severe diarrhea) was measured at 0 hpi, 3 hpi, 8 hpi, 16 hpi, 24 hpi, 28 hpi, 34 hpi, 40 hpi, 48 hpi and 54 hpi according to a previously published method (Marquardt et al., 1999).

Table 5.1 The ingredient composition of the basal diet (kg, as-fed basis).

Ingredients, kg, as-fed basis	Basal diet
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
Calcium (limestone)	14.32
Biofos 21% ³	5.73
Salt - bulk fine	5
Vitamin-mineral premix ⁴ (1%)	10
L-lysine 78%	2.83
DL-methionine 99%	1.52
Threonine	1.32
L-tryptophan	0.51
Total	1,000.00
Calculated net energy and nutrient content (g·kg ⁻¹)	
Metabolizable energy (kcal·kg ⁻¹)	3,389.11
Net energy (kcal·kg ⁻¹)	2,475
Crude protein (%)	22.35
SID ⁵ Lysine	1.34
SID ⁵ Methionine	0.5
SID ⁵ Threonine	0.87
SID ⁵ Tryptophan	0.27

¹The diet for Positive control (PC) was prepared by adding 55 mg·kg⁻¹ of Aureomycin 220G (Zoetis Canada Inc., Kirkland, QC, Canada) into the basal diet. The diet for micro-encapsulated organic acids and essential oils (P(OA+EO)) was prepared by adding 2 g·kg⁻¹ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

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

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

⁴Supplied the following per kilogram of diet: 2,200 IU vitamin A, 220 IU vitamin D3, 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 copper sulphate, 100 mg ferrous sulfate, 4 mg manganese oxide, 0.3 mg sodium selenite, and 100 mg zinc oxide.

⁵Standardized ileal digestible amino acids.

5.3.5 *In vivo* gut permeability

On 4 dpi, 4 h after oral gavage of fluorescein isothiocyanate-dextran 70 kDa (10 mg per pig; FITC-D70; molecular weight 70 kDa; Sigma-Aldrich Co., St. Louis, MO, USA) in 5 mL PBS buffer, blood samples (serum) were collected from each piglets through jugular vein into heparinized vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) 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 $750 \times g$ for 15 min to separate serum from red blood cells and stored at -80°C until further analyses. The fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 528 nm using a Bio-Tek PowerWaveTM HT Microplate Scanning Spectrophotometer (BIO-TEK Instruments, Inc., Winooski, VT, USA) and the concentrations of FITC-D70 in the serum samples ($\text{ng}\cdot\text{mL}^{-1}$) were calculated based on a standard curve.

5.3.6 Sample collection

At the end of the experiment (on 5 dpi) all piglets were anesthetized by an intramuscular injection of ketamine:xylazine ($20:2 \text{ mg}\cdot\text{kg}^{-1} \text{ BW}$) and euthanized with a captive bolt gun. The abdomen was immediately opened and the whole gastrointestinal tract was removed from the carcass. Initially, the mid-jejunum (400 cm from the stomach-duodenum junction) was located and a 10 cm of the mid-jejunum was put in an ice-cold KRB and delivered to the laboratory for the Ussing chamber analysis. Another 10 cm of the mid-jejunum tissue was removed and immediately snap-frozen in liquid nitrogen. The samples were stored at -80°C until further analyses. Afterward, a 2 cm of the mid jejunum tissue was collected and fixed in a 10% formaldehyde solution for gut morphology measurement. The digesta of the colon (20 cm from the ileum-cecum junction) was collected and immediately frozen in liquid nitrogen. The

samples were stored at -80°C until further analyses.

5.3.7 Ussing chamber

The electrophysiological properties including short-circuit current and transepithelial electrical resistance (TEER) were determined using a modified Ussing chambers (VCC-MC8; Physiologic Instruments Inc., San Diego, CA, USA) containing pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes housed in 3% agar bridges and filled with KRB buffer (in $\text{mmol}\cdot\text{L}^{-1}$: 154 Na^{+} , 6.3 K^{+} , 137 Cl^{-} , $0.3\text{ H}_2\text{PO}_4$, 1.2 Ca^{2+} , 0.7 Mg^{2+} , 24 HCO_3^{-} - pH 7.4 with $1\mu\text{mol}\cdot\text{L}^{-1}$ of indomethacin). Five milliliters of the KRB buffer solution with $10\text{ mmol}\cdot\text{L}^{-1}$ D-glucose was added to the serosal chambers, and five milliliters of KRB buffer solution enriched with $10\text{ mmol}\cdot\text{L}^{-1}$ 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, $10\text{ mmol}\cdot\text{L}^{-1}$ D-glucose was added to the mucosal chamber to measure the sodium-dependent glucose transportation and $10\text{ mmol}\cdot\text{L}^{-1}$ 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 SGLT1 was determined by subtracting the short circuit current value before stimulation from the peak after stimulation. When D-glucose was added, $0.1\text{ mg}\cdot\text{mL}^{-1}$ of FITC-D4 (molecular weight 4 kDa; Sigma-Aldrich Co.) was added to the mucosal side and after 1 h,

the sample (1 mL) were obtained from the serosal side to measure intestinal permeability.

5.3.8 Intestinal morphology analysis

After fixation in 10% neutral-buffered formalin, samples were embedded in paraffin and 5 μ m 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 by water for 2 min and placed in the Schiff reagent for 10 min and washed by water for 10 min. Finally, the samples were counterstained in hematoxylin for 10 s and wash and dehydrated. For the quantification of Alcian blue/The periodic acid–Schiff (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). VH, CD and VH:CD were measured and the number of goblet cells per 100 μ m VH and 100 μ m CD was counted using Infinity Analyze software (version 6.5.4; Lumenera Corporation, Ottawa, ON, Canada). All measurable villus and crypts were measured, which was 50 to 150 measurements per each sample.

5.3.9 Total antioxidant capacity, total GSH and GSH/GSSG assays

Total antioxidant capacity (TAC) in the mid-jejunal was measured in duplicate by using the Colorimetric Microplate Assay Kits for Total Antioxidant Capacity (TA02, Oxford Biomedical Research, Oxford, MI, USA) (Yang, 2011). Briefly, 200 mg of liquid nitrogen pulverized samples were weighted 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 $3,600 \times g$ for 12 min at 4 °C. Aliquot of supernatant was taken for the analysis of their protein content using the Pierce™ BCA

Protein Assay Kit (Thermo Fisher Scientific). 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 was 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 $\text{mM} \cdot \text{mg protein}^{-1}$.

Total glutathione (GSH) and oxidized glutathione (GSSG) in the mid-jejunal tissues were measured in duplicate by using the Glutathione colorimetric detection kit (Invitrogen). Briefly, 30 mg of liquid nitrogen pulverized samples will be weighted 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 $3,600 \times g$ for 10 min at 4 °C. Aliquot of supernatant was taken for the analyses of protein content using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Afterward, 5-sulfo-salicylic acid dihydrate was added to the obtained supernatant to precipitate protein, and then centrifuged at $3,600 \times g$ for 10 min at 4 °C. After deproteinization, total GSH and GSSG levels in the resulting supernatant were measured according to the manufacture's protocol. Reduced GSH was calculated by the equation: $\text{Reduced GSH} = \text{Total GSH} - 2 \times \text{GSSG}$.

5.3.10 Digestive enzyme activity assays

The maximal enzyme activity (V_{max}) of intestinal digestive enzymes including aminopeptidase N (APN), IAP, maltase, maltase-glucoamylase (MGA), and sucrase was determined in the study. Specifically, about 200 mg of liquid nitrogen pulverized, and frozen intestinal tissue samples were thawed in an ice-cold homogenizing buffer (50 $\text{mmol} \cdot \text{L}^{-1}$ D-

mannitol and $0.1 \text{ mmol} \cdot \text{L}^{-1}$ 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). 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 by the procedure of Dahlqvist (1964). MGA (EC 3.2. 1.20) activity was analyzed according to the method of Lackeyram et al. (2012). The V_{max} was expressed $\text{nmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$.

5.3.11 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.). 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 $1 \mu\text{g}$ 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 shown in Table 5.1. The primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). Real-time PCR was carried out using an SYBR Green Supermix (Biorad) on a CFX Connect™ Real-Time PCR Detection System (Biorad) (Omonijo et al., 2018b). A total of $1 \mu\text{L}$ cDNA was added to a total volume of $20 \mu\text{L}$ containing $10 \mu\text{L}$ SYBR Green supermix, and $300 \text{ nmol} \cdot \text{L}^{-1}$ of each forward and reverse primers. Thermal condition for all reactions was: denaturation 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 $10^{(-1/\text{slope})}$ (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.

Table 5.2 Primer sequences for gene expression of virulence factors of *Escherichia coli* F4, *Escherichia coli* F4 receptor, tight junction proteins, nutrient transporters, inflammatory cytokines and digestive enzymes of pigs.

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 GGTTGCTTGCAAAGTGGTGT	
CLDN3	123	CTACGACCGCAAGGACTACG TAGCATCTGGGTGGACTGGT	(Omonijo et al., 2018a)
OCLN	93	CTGTGGATGTCCTGCGTGT GGTTGCTTGCAAAGTGGTGT	(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 ⁰ AT1	102	AGGCCCAGTACATGCTCAC CATAAATGCCCTCCACCGT	(Yang et al., 2016a)
EAAC1	168	CCAAGGTCCAGGTTTTGGGT GGGCAGCAACACCTGTAATC	(Omonijo et al., 2018a)
ASCT2	206	GCCAGCAAGATTGTGGAGAT GAGCTGGATGAGGTTCCAAA	(Yang et al., 2016a)
IL8	126	CACCTGTCTGTCCACGTTGT AGAGGTCTGCCTGGACCCCA	(Omonijo et al., 2018a)
IL10	220	CATCCACTTCCCAACCAGCC CTCCCCATCACTCTCTGCCTTC	(Lee and Kang, 2017)
IL1 β	91	TGGCTAACTACGGTGACAACA	

TLR2	109	CCAAGGTCCAGGTTTTGGGT 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	

¹Note: 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; IL1 β : Interleukin 1 β ; 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; B⁰AT1: Neutral amino acid transporter; MGA: Maltase-glucoamylase; SI: Sucrase-isomaltase; APN: Aminopeptidase N.

5.3.12 Western blotting

Relative protein abundance of ZO1, OCLN, and neutral amino acid transporter (B⁰AT1) 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.) containing a complete cocktail of proteinase inhibitors and protein concentration was analyzed by a BCA protein detection kit (Thermo Fisher Scientific) following the manufacturer's instructions. Protein samples were then denatured in 1× Laemmli buffer with mercaptoethanol at 95°C for 5 min and loaded into the wells of 4-12% gradient pre-made SDS-PAGE gel (Biorad) 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). 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), rabbit anti-OCLN (1:500 dilution, Thermo Fisher Scientific), and rabbit anti-B⁰AT1 (1:2,000 dilution, provide by Dr. François Verrey at University of Zurich, Switzerland) (Romeo et al., 2006) at 4°C 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) 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) to the membranes and images were captured using a ChemiDoc MP imaging system (Biorad). The intensity of the bands was quantified using Image Lab 6.0 software (Biorad). β -actin (from mouse, Thermal Fisher Scientific) was used as the 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 $\pm$ SEM (n=4).

5.3.13 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 manufacture's instruction. 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 system (Biorad). Briefly, 25 μ l 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 $\times$ Evagreen Supermix (Biorad) was prepared and 20 μ l of the mixture was transferred into a sample well of the droplet generator cartridge (DG8 cartridges; Biorad). Droplet Generation Oil (70 μ l) (Biorad) was added to the oil wells of DG8 cartridges. Droplets were generated using a droplet generator (Biorad) and were gently transferred onto the 96-well PCR plate (Biorad). The faeG gene in the droplets was amplified on the C1000 Touch thermal cycler (Biorad) 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) and data were analyzed by QuantaSoft (Biorad). Data were presented as log₁₀(faeG gene copies· μ g DNA⁻¹).

5.3.14 Statistical analyses

All data were analyzed using the PROC MIXED of SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA) with an individual animal used as the experimental unit. The LSMEANS statement with the Tukey-adjusted PDIFF option was employed to calculate and split the treatment mean value for each treatment. The NC piglets were compared by preplanned

contrasts with either the NNC piglets to determine the ETEC F4 inoculation effects, or the PC piglets or the P(OA+EO) piglets to determine the effects of Aureomycin or the micro-encapsulated OA and EO, respectively. Results in tables were shown as least-square means and pooled standard errors of the means and results in figures shown as mean $\pm$ SEM. Differences were considered significant at $P < 0.05$, and trends ($0.05 \leq P \leq 0.10$) were also presented.

5.4 Results

5.4.1 Growth performance, rectal temperature and diarrhea score

As shown in Table 5.3, there was no significant difference in the ADG, ADFI and FCR observed among all treatment groups during the pre-challenge period ($P > 0.05$). During the post-challenge period (0 – 11 d), ETEC F4 infection significantly decreased ADG of the NC piglets when compared to the NNC piglets ($P < 0.05$). There was no significant difference in the ADG observed among the PC, P(OA+EO) and NC piglets had similar ADG with the NC piglets ($P > 0.05$) although it was numerously higher in the P(OA+EO) piglets. However, there was no significant difference in the ADFI among all treatment groups during the post-challenge period ($P > 0.05$). During the whole period (0 – 11 d), there was no significant difference in the ADG, ADFI and FCR observed among all treatment groups ($P > 0.05$).

Table 5.3 Effects of micro-encapsulated organic acids and essential oils on the growth performance of weaned piglets during the pre-challenge period (0-7 d), post-challenge period (0 – 4 days post-inoculum) and whole period (0-11 d).

Items	NNC	ETEC F4-challenged			SEM	<i>P</i> value ¹			
		NC	PC	P(OA+EO)		TRT	1	2	3

Pre-challenge									
ADG (g·d ⁻¹)	202	257	219	243	30.6	0.60	0.22	0.39	0.76
ADFI (g·d ⁻¹)	298	362	317	370	28.1	0.24	0.12	0.27	0.86
FCR (g·g ⁻¹)	1.73	1.42	1.55	1.58	0.20	0.76	0.29	0.66	0.59
Post-challenge ²									
ADG (g·d ⁻¹)	446 ^a	240 ^{ab}	183 ^b	354 ^{ab}	53.2	0.02	0.02	0.51	0.16
ADFI (g·d ⁻¹)	635	538	477	608	58.1	0.29	0.27	0.51	0.43
Whole period									
ADG (g·d ⁻¹)	291	251	195	284	34.2	0.26	0.44	0.31	0.52
ADFI (g·d ⁻¹)	420	422	364	456	34.7	0.39	0.97	0.29	0.52
FCR (g·g ⁻¹)	1.50	1.82	2.08	1.62	0.18	0.17	0.23	0.36	0.46

NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 mg·kg⁻¹ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 g·kg⁻¹ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada); ADG: average daily gain; ADFI: average daily feed intake; FCR: feed conversion ratio (Feed to gain ratio).

¹TRT: treatment; Contrast: (1) NNC v. NC; (2) NC v. PC; (3) NC v. P(OA+EO).

²The FCR during the post-challenge period was unable to calculate due to the minus ADG during the post-challenge period.

^{a,b}Values within a row with different superscripts differ significantly at $P < 0.05$.

As shown in Fig. 5.2, the core body temperature was significantly increased in the NC piglets when compared to the NNC piglets ($P < 0.05$) and the piglets fed P(OA+EO) tended to have lower core body temperature when compared to the NC piglets ($P = 0.06$). At 24 hpi, the P(OA+EO) piglets tended to have a lower core body temperature when compared to the NNC piglets ($P = 0.09$).

As shown in Fig. 5.3, inoculation of ETEC F4 significantly induced diarrhea at 8 hpi, 16 hpi, 28 hpi, 34 hpi, 40 hpi ($P < 0.05$) and tended to increase diarrhea at the 3 hpi ($P = 0.10$) and 24 hpi ($P = 0.09$) in the NC piglets when compared to the NNC piglets. The supplementation of micro-encapsulated OA and EO significantly relieved diarrhea at 28 hpi and 40 hpi ($P < 0.05$) and showed a tendency to alleviate diarrhea at 34 hpi ($P = 0.07$) when compared to the NC piglets. At 48 hpi and 54 hpi, there was no significant difference in the diarrhea index observed among all treatment groups during the pre-challenge period ($P > 0.05$).

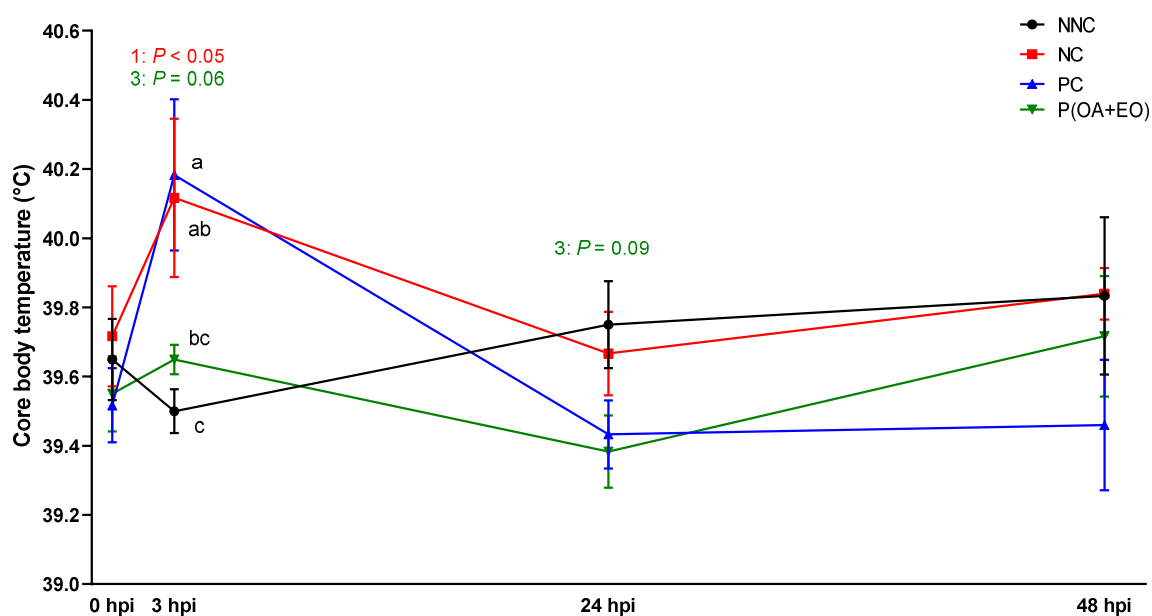


Figure 5.2 Effects of micro-encapsulated organic acids and essential oils on the core body temperature in weaned piglets. Core body temperature of weaned piglets was measured in the NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate-buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 mg·kg⁻¹ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 g·kg⁻¹ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada) groups during 48 hpi (hour post-inoculation). Each value represents the mean ± SEM. Bars with different letters are significantly different ($P < 0.05$). At each time point, the pre-planned contrasts were designed to compare NNC vs NC (1), NC vs PC (2), and NC vs P(OA+EO) (3). The contrasts were presented when $P \leq 0.10$. hpi: hour post-inoculation.

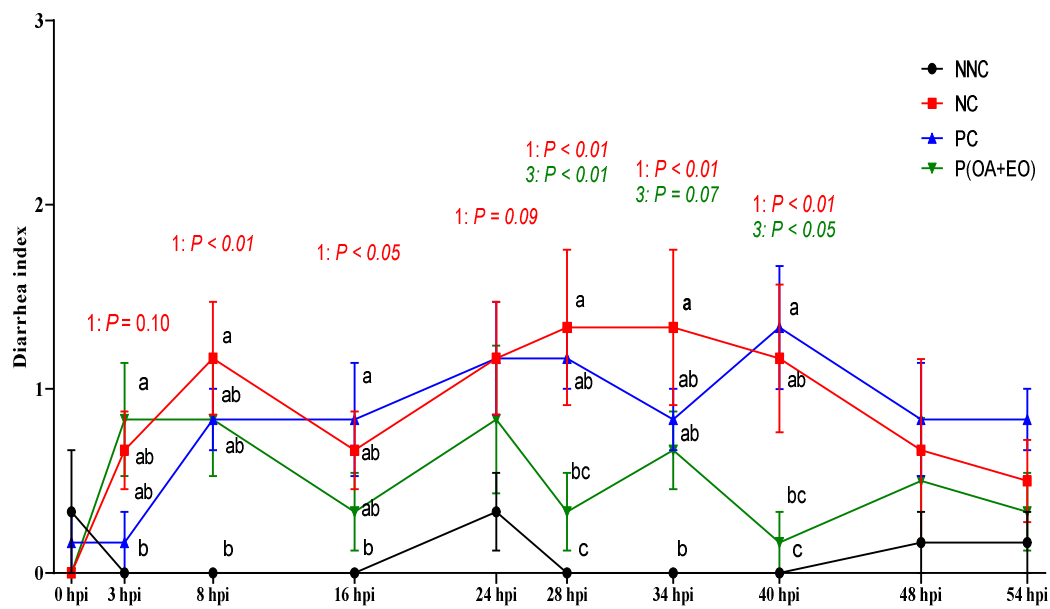


Figure 5.3 Effects of micro-encapsulated organic acids and essential oils on diarrhea score in weaned piglets. Diarrhea score of weaned piglets was measured in the NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate-buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 mg·kg⁻¹ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 g·kg⁻¹ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated 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 are significantly different ($P < 0.05$). At each time point, the pre-planned contrasts were designed to compare NNC vs NC (1), NC vs PC (2), and NC vs P(OA+EO) (3). The contrasts were presented when $P \leq 0.10$.

5.4.2 Gut permeability and glucose transport

As shown in Table 5.4, there was no significant difference in TEER and SGLT1 dependent short-circuit current measured by the Ussing chamber ($P > 0.05$). However, the P(OA+EO) piglets tended to have a lower FITC-D4 concentration in the serosal chamber when compared to the NC piglets ($P = 0.05$). There was no significant difference observed in *in vivo* gut permeability measured by the oral gavaging FITC-D70 assay among the NNC, NC and P(OA+EO) piglets ($P > 0.05$). However, the PC piglets had a higher concentration of FITC-D70 in blood when compared to the NNC and P(OA+EO) piglets ($P < 0.05$).

Table 5.4 Effects of micro-encapsulated organic acids and essential oils on electrophysiological properties including transepithelial electrical resistance (TEER, $\Omega \cdot \text{cm}^2$) and SGLT1 dependent short-circuit current ($\mu\text{A} \cdot \text{cm}^{-2}$) and flux of fluorescein isothiocyanate–dextran 4 kDa (FITC-D4, $\mu\text{g} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) of weaned piglets jejunum mounted in Ussing chambers (*Ex vivo*) and flux of fluorescein isothiocyanate–dextran 70 kDa (FITC-D70, $\mu\text{g} \cdot \text{mL}^{-1}$) in weaned piglets (*In vivo*)

Items	NNC	ETEC F4-challenged			SEM	<i>P</i> value ¹			
		NC	PC	P(OA+EO)		TRT	1	2	3
<i>Ex Vivo</i>									
TEER	41.77	50.00	42.19	54.70	6.35	0.44	0.39	0.44	0.62
SGLT1									
dependent short-circuit current	80.85	48.84	42.10	54.57	12.82	0.26	0.11	0.77	0.78
FITC-D4 flux	45.11	55.21	46.88	31.82	7.55	0.24	0.38	0.49	0.05
<i>In vivo</i>									
FITC-D70 flux	1,032 ^b	1,357 ^{ab}	1,682 ^a	1,006 ^b	151.2	0.02	0.17	0.18	0.14

NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 mg·kg⁻¹ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 g·kg⁻¹ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada); SGLT1: Na⁺-glucose cotransporter 1; FITC-D4 and FITC-D70: fluorescein isothiocyanate–dextran 4 kDa and 70 kDa; TEER: transepithelial electrical resistance.

^lTRT: treatment; Contrast: (1) NNC v. NC; (2) NC v. PC; (3) NC v. P(OA+EO).

^{a,b}Values within a row with different superscripts differ significantly at $P < 0.05$.

5.4.3 Intestinal morphology and goblet cells

As shown in Table 5.5, ETEC F4 inoculation significantly decreased mid-jejunal VH in the NC piglets when compared to the NNC piglets ($P < 0.05$). The piglets supplemented with micro-encapsulated OA and EO significantly increased VH in the jejunum ($P < 0.05$) when compared to the NC piglets. However, no significant difference was found in the CD and VH:CD among all treatment groups ($P > 0.05$). ETEC F4 inoculation numerically increased the number of goblet cells per 100 μm VH when compared to the NNC piglets ($P = 0.12$). There was a tendency in the number of goblet cells per 100 μm VH with the supplementation of Aureomycin ($P = 0.10$) and micro-encapsulated OA and EO ($P = 0.09$) when compared to the NC piglets. The number of goblet cells per 100 μm CD was not affected by treatments ($P > 0.05$).

Table 5.5 Effects of micro-encapsulated organic acids and essential oils on morphology including villus height (VH, μm), crypt depth (CD, μm), VH:CD and the number of goblet cells per 100 μm VH and 100 μm CD in the mid-jejunum of weaned piglets

Items	ETEC F4-challenged				SEM	<i>P</i> value ¹			
	NNC	NC	PC	P(OA+EO)		TRT	1	2	3
VH	478 ^{ab}	364 ^b	441 ^{ab}	512 ^a	31.89	0.04	0.03	0.13	<0.01
CD	278	250	250	270	14.67	0.49	0.23	0.97	0.39
VH:CD	1.96	1.90	1.94	2.11	0.19	0.89	0.86	0.90	0.48
Number of goblet cells per 100 μm VH	2.58	3.64	2.46	2.47	0.44	0.26	0.12	0.10	0.09
Number of goblet cell per 100 μm CD	5.71	6.04	5.62	5.91	0.34	0.85	0.53	0.44	0.82

NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 $\text{mg}\cdot\text{kg}^{-1}$ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 $\text{g}\cdot\text{kg}^{-1}$ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

¹TRT: treatment; Contrast: (1) NNC v. NC; (2) NC v. PC; (3) NC v. P(OA+EO).

^{a,b}Values within a row with different superscripts differ significantly at $P < 0.05$.

5.4.4 Digestive enzyme maximal activities

As shown in Table 5.6, no significant difference was found in the V_{max} of APN, MGA, sucrase among all treatment groups ($P > 0.05$). The PC piglets had significantly higher V_{max} of IAP when compared to the NC piglets ($P < 0.05$). The V_{max} of maltase tended to decrease due to the ETEC F4 inoculation in the NC piglets when compared to the NNC piglets ($P = 0.07$) and the piglets supplemented with micro-encapsulated OA and EO tended to have higher V_{max} for maltase when compared to the NC piglets ($P = 0.10$).

Table 5.6 Effects of micro-encapsulated organic acids and essential oils on the activities ($\text{nmol}\cdot\text{L}^{-1}\cdot\text{mg protein}^{-1}\cdot\text{min}^{-1}$) of brush border digestive enzymes in the mid-jejunum of weaned piglets

Items	NNC	ETEC F4-challenged			SEM	<i>P</i> value ¹			
		NC	PC	P(OA+EO)		TRT	1	2	3
Aminopeptidase N	0.13	0.10	0.11	0.14	0.02	0.39	0.34	0.84	0.13
Intestinal alkaline phosphatase	0.45	0.40	0.52	0.46	0.04	0.22	0.42	0.04	0.25
Maltase	78.63	54.04	70.64	76.11	8.71	0.27	0.07	0.24	0.10
Maltase-glucoamylase	4.36	3.95	4.02	4.49	0.50	0.87	0.61	0.93	0.50
Sucrase	14.43	11.57	12.90	15.93	3.02	0.81	0.56	0.79	0.37

NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 $\text{mg}\cdot\text{kg}^{-1}$ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 $\text{g}\cdot\text{kg}^{-1}$ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

¹ TRT: treatment; Contrast: (1) NNC v. NC; (2) NC v. PC; (3) NC v. P(OA+EO).

5.4.5 Total antioxidant capacity (TAC), total GSH and GSH/GSSG

As shown in Table 5.7, there was no significant difference in TAC, total GSH, GSSG, reduced GSH and reduced GSH:GSSG observed among all treatment groups ($P > 0.05$).

Table 5.7 Effects of micro-encapsulated organic acids and essential oils on the total antioxidant capacity (TAC, $\text{mmol}\cdot\text{L}^{-1}\cdot\text{mg protein}^{-1}$), total glutathione (GSH, $\text{nmol}\cdot\text{L}^{-1}\cdot\text{mg protein}^{-1}$), oxidized glutathione (GSSG, $\text{nmol}\cdot\text{L}^{-1}\cdot\text{mg protein}^{-1}$), and reduced GSH:GSSG in the mid-jejunum of weaned piglets

Items	NNC	ETEC F4-challenged			SEM	<i>P</i> value ¹			
		NC	PC	P(OA+EO)		TRT	1	2	3
TAC	84.88	79.80	82.18	85.51	3.61	0.78	0.35	0.68	0.30
Total GSH	3.26	3.09	2.98	2.95	0.21	0.32	0.62	0.76	0.68
GSSG	0.44	0.42	0.50	0.55	0.05	0.42	0.79	0.31	0.10
Reduced GSH ²	2.37	2.24	1.99	1.85	0.22	0.23	0.71	0.47	0.28
Reduced GSH:GSSG	5.46	5.69	4.06	3.81	0.71	0.70	0.83	0.16	0.11

NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 $\text{mg}\cdot\text{kg}^{-1}$ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 $\text{g}\cdot\text{kg}^{-1}$ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

¹ TRT: treatment; Contrast: (1) NNC v. NC; (2) NC v. PC; (3) NC v. P(OA+EO).

² Reduced GSH = Total GSH – 2 × GSSG.

5.4.6 Relative mRNA abundance in the jejunum

The relative mRNA abundance of genes associated with gut barrier function, immune system, nutrient transport, digestive enzymes was analyzed by real-time PCR (Table 5.8). There was no significant difference in the relative mRNA abundance of zonulna occludens 1 (ZO1) and OCLN among all treatment groups ($P > 0.05$). ETEC F4 inoculation significantly decreased the relative mRNA abundance of CLDN1 and mucin 2 (MUC2) in the NC piglets when compared to the NNC piglets ($P < 0.05$). The PC piglets supplemented with Aureomycin had a higher level of relative mRNA abundance of CLDN3 when compared to the NNC piglets ($P < 0.05$). There was no significant difference 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 all treatment groups ($P > 0.05$). The relative mRNA abundance of SGLT1 and B⁰AT1 was significantly decreased due to ETEC F4 inoculation when compared to the NNC piglets ($P < 0.05$). The PC piglets had a higher relative mRNA abundance of B⁰AT1 when compared to the NC piglets ($P < 0.05$). Among the genes related to the immune system including IL8, IL10, IL1 β , toll-like receptor 2 (TLR2), TLR5 and TLR7, only the relative mRNA abundance of IL8 was significantly increased due to ETEC F4 inoculation ($P < 0.05$) in the NC piglets when compared to the NNC piglets. The PC and P(OA+EO) piglets significantly decreased the relative mRNA abundance of IL8 when compared to the NC piglets ($P < 0.05$). Compared to the NNC piglets, ETEC F4 inoculation significantly decreased relative mRNA abundance of MGA, sucrase-isomaltase (SI), APN ($P < 0.05$) in the NC piglets, however, the relative mRNA abundance of MGA, SI, and APN in the PC and P(OA+EO) piglets was not different from those in the NC piglets ($P > 0.05$).

Table 5.8 Effects of micro-encapsulated 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 piglets¹.

ETEC F4-challenged					<i>P</i> value ²				
Items	NNC	NC	PC	P(OA+EO)	SEM	TRT	1	2	3
<u><i>Gut barrier integrity</i></u>									
ZO1	1.02	0.78	0.87	0.72	0.12	0.33	0.20	0.62	0.75
CLDN1	1.04 ^a	0.61 ^b	0.50 ^b	0.41 ^b	0.09	<0.01	0.01	0.46	0.23
CLDN3	1.01	0.75	1.15	0.92	0.10	0.23	0.15	0.05	0.36
OCLN	1.03	0.89	0.86	0.81	0.13	0.65	0.48	0.86	0.67
MUC2	1.01 ^a	0.40 ^{ab}	0.49 ^{ab}	0.33 ^b	0.15	0.02	0.02	0.68	0.74
<u><i>Nutrient transporters</i></u>									
SGLT1	1.01 ^a	0.50 ^b	0.69 ^{ab}	0.52 ^b	0.09	<0.01	<0.01	0.16	0.88
PepT1	1.05	0.64	0.80	0.73	0.16	0.37	0.11	0.53	0.73
B ⁰ AT1	1.02 ^a	0.36 ^b	0.84 ^{ab}	0.49 ^b	0.09	<0.01	<0.01	0.01	0.41
EAAC1	1.06	0.76	0.81	0.96	0.17	0.64	0.25	0.86	0.47
ASCT2	1.03	1.18	1.11	1.02	0.35	0.99	0.78	0.90	0.79
<u><i>Immune system</i></u>									
IL8	1.02	1.92	1.03	1.02	0.27	0.07	0.03	0.03	0.02
IL10	1.09	1.04	0.73	1.01	0.19	0.61	0.31	0.94	0.19
IL1β	1.02	0.99	0.78	0.72	0.13	0.32	0.86	0.31	0.19
TLR2	1.04	1.35	0.82	1.12	0.21	0.41	0.32	0.11	0.51
TLR5	1.02	0.86	0.52	0.55	0.18	0.18	0.54	0.20	0.24
TLR7	1.05	0.75	0.70	0.65	0.12	0.12	0.11	0.77	0.59
<u><i>Digestive</i></u>									

enzymes

MGA	1.01 ^a	0.41 ^b	0.61 ^{ab}	0.53 ^{ab}	0.11	0.02	<0.01	0.27	0.51
SI	1.04	0.56	0.65	0.63	0.15	0.15	0.04	0.71	0.76
APN	1.00	0.49	0.64	0.57	0.13	0.08	0.02	0.47	0.69

NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 mg·kg⁻¹ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 g·kg⁻¹ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada).

¹Note: 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; B⁰AT1: Neutral amino acid transporter; 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-isomaltase; APN: Aminopeptidase N.

²TRT: treatment; Contrast: (1) NNC v. NC; (2) NC v. PC; (3) NC v. P(OA+EO).

^{a,b}Values within a row with different superscripts differ significantly at $P < 0.05$.

5.4.7 Relative protein abundance of tight junction proteins and nutrient transporter

As shown in Fig. 5.4, ETEC F4 inoculation tended to increase the protein abundance of OCLN ($P = 0.09$) and significantly decreased ZO1 ($P < 0.05$) in the NC piglets when compared to the NNC piglets. The supplementation of Aureomycin significantly decreased the relative protein abundance of OCLN ($P < 0.05$) when compared to the NC piglets ($P < 0.05$). However, the relative protein abundance of ZO1 and OCLN in the P(OA+EO) piglets was not significantly different from those in the NC piglets ($P > 0.05$). There was no significant difference in the relative protein abundance of B⁰AT1 among all treatment groups ($P > 0.05$).

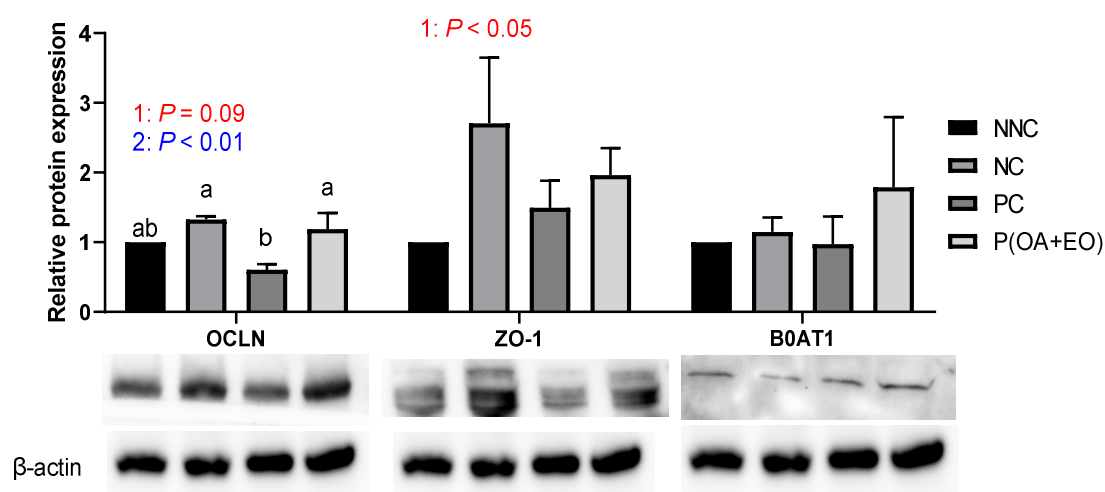


Figure 5.4 Effects of micro-encapsulated organic acids and essential oils on the relative abundance of proteins associated with gut barrier integrity and nutrient transporters in weaned piglets. Mid-jejunal relative protein abundance of zonulna occludens 1 (ZO1), occludin (OCLN), and B⁰AT1 (neutral amino acid transporter) was measured in the NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate-buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 mg·kg⁻¹ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 g·kg⁻¹ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada) groups. Each value represents the mean ± SEM. Bars with different letters are significantly different ($P < 0.05$). The pre-planned contrasts were designed to compare NNC vs NC (1), NC vs PC (2), and NC vs P(OA+EO) (3). The contrasts were presented when $P \leq 0.10$.

5.4.8 ETEC F4 abundance in the colon digesta

As shown in Fig. 5.5, ETEC F4 inoculation significantly increased the ETEC F4 gene (faeG) ($P < 0.05$) in the colon digesta in the NC piglets when compared to the NNC piglets. However, there was no significant difference in the copy number of faeG observed among the piglets challenged with ETEC F4 ($P > 0.05$) although it was numerous lower in the P(OA+EO) piglets.

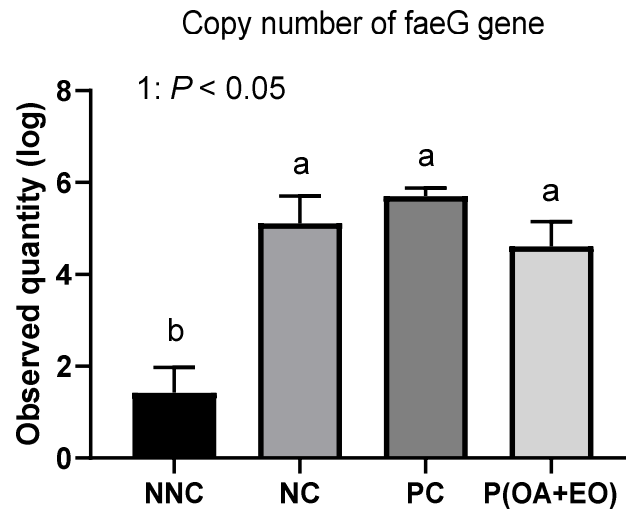


Figure 5.5 Effects of micro-encapsulated organic acids and essential oils on DNA abundance of faeG (F4 fimbriae) in the colon digesta in weaned piglets. DNA abundance of faeG (F4 fimbriae) in the colon digesta (20cm from the ileum-cecum junction) was measured in the NNC (non-challenged negative control): pigs fed a control diet and challenged with phosphate-buffered saline; NC (negative control): pigs fed a control diet and challenged with enterotoxigenic *Escherichia coli* F4; PC (positive control): NC + 55 mg·kg⁻¹ of Aureomycin (Zoetis Canada Inc., Kirkland, QC, Canada); P(OA+EO) (micro-encapsulated organic acids and essential oils): NC + 2 g·kg⁻¹ of a selected formula of organic acids (fumaric, citric, malic and sorbic acids) and essential oils (thymol, vanillin and eugenol) micro-encapsulated in a matrix of triglycerides (Jefo Nutrition Inc., QC, Canada) groups. Data were presented as log₁₀(faeG gene copies·μg DNA⁻¹). Each value represents the mean ± SEM. Bars with different letters are significantly different ($P < 0.05$). The pre-planned contrasts were designed to compare NNC vs NC (1), NC vs PC (2), and NC vs P(OA+EO) (3). The contrasts were presented when $P \leq 0.10$.

5.5 Discussion

This study was to investigate whether the supplementation of micro-encapsulated OA and EO could alleviate the responses to bacterial 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 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, 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 checked 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 checking 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). ETEC F4 induces more clinical symptoms if piglets have susceptible alleles of the MUC4 gene (Fairbrother et al., 2005). So it is necessary to choose susceptible piglets for this challenge study in order to successfully induce diarrhea and minimize variations among piglets (Trevisi et al., 2015; Sterndale et al., 2019). In the study, the symptoms of bacterial infection were successfully achieved, which can be indicated by increased diarrhea index and core body temperature and

compromised gut health.

The purpose of this study was to evaluate dietary strategies as AGP alternatives. The AGP (low dosage of medicine) 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, an appropriate amount of ETEC F4 that may show only mild diarrhea should be inoculated to piglets. 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 selected in this study.

In this study, during the pre-challenge period, the supplementation of micro-encapsulated OA and EO did not affect the growth performance. The ETEC F4 infection significantly decreased the ADG, 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) inflammation (Kim et al., 2016); 3) increased diarrhea (Cho et al., 2012); and 4) decreased available nutrients for pigs due to the inoculation of ETEC F4, which may compete for nutrients with the host (Richards et al., 2005). In this study, the supplementation of micro-encapsulated OA and EO numerically increased ADG although statistically difference was not achieved in this study ($P = 0.16$) and the possible reason might be that the microencapsulated OA and EO attenuated bacterial infection symptoms of piglets. Similarly,

Devi et al. (2015) showed that the supplementation of a blend of EO including cinnamon, fenugreek, clove improved ADG when compared to the control group but 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. Kwak et al. (2019) showed that micro-encapsulated 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 may come from different kinds of OA and EO, challenging inoculum or experimental designs (e.g. experimental period and replicates).

In this study, the supplementation of micro-encapsulated OA and EO attenuated the increase of core body temperature by ETEC F4 infection. An increase in core body temperature may imply inflammatory reactions (Kwak et al., 2019). Similarly, the relative mRNA abundance of IL8 (pro-inflammatory cytokines) was increased due to ETEC F4 infection and attenuated by the supplementation of microencapsulated OA and EO in this study. Pro-inflammatory cytokines (IL8), produced by various cell types such as macrophages, endothelial cells, B cells and mast cells, are one of the important markers for inflammation (Akira et al., 1993). The alleviated inflammation by the supplementation microencapsulated OA and EO could be explained by enhanced gut barrier function indicated by decreased FITC-D4 uptake in the jejunum. Gut barrier function, the first defense line against the hostile environment, protects noxious antigens and pathogens from permeating into the body (Wijten et al., 2011). It is already well documented that supplementation of OA and EO can enhance the gut barrier

function of pigs (Grilli et al., 2015; Zou et al., 2016b; Omonijo et al., 2018b). Alleviated core body temperature and enhanced gut barrier function, which are achieved by the supplementation of microencapsulated OA and EO, possibly explain the mitigated diarrhea in the present study, which supports the fact that OA and EO have an antidiarrheal effect (Tsiloianis et al., 2001; Suiryanrayna and Ramana, 2015; Omonijo et al., 2018c). The supplementation of Aureomycin decreased mRNA expression of IL8, which is consistent with a previous study (Koo et al., 2019). This also could be explained by the enhanced gut barrier function indicated by the up-regulated relative abundance of mRNA of CLDN3 in the PC piglets, one of the tight junction proteins, which are vital in the maintenance of gut barrier function (Li et al., 2019). However, the antidiarrheal effect of Aureomycin supplementation was not shown in the current study.

To study gut permeability of piglets, in addition to FITC-D4 assay in the Ussing chamber, FITC-D70 in PBS ($2 \text{ mg} \cdot \text{mL}^{-1}$) was orally gavaged to the piglets. Briefly, under normal (health) conditions, FITC-D should not cross the epithelial barrier and digested by digestive enzymes, however, once tight junctions proteins are damaged due to inflammation, pathogens and toxins, the FITC-D molecule can enter blood circulation (Yan et al., 2009; Baxter et al., 2017). Ussing chamber is specifically measuring intestinal permeability of the mid-jejunum (1 cm^2 in the study) but *in vivo* gut permeability assay may measure gut permeability starting from the esophagus to possibly up to ileum (Baxter et al., 2017). This may explain why the different results from *in vivo* assay and Ussing chamber analysis were obtained. In the analysis, a higher molecule of FITC-D has a lower conjugated fluorescence substances compared to a lower molecule of FITC-D, therefore there was no need to dilute the

samples.

Enterocytes in the villus play crucial roles in nutrient digestion and absorption, therefore the increased VH may imply better nutrient digestion and absorption (Kong et al., 2018). In this study, ETEC F4 infection decreased the VH, which is consistent with a study by Yi et al. (2005). The possible reason is that toxins produced from ETEC F4 could induce villous atrophy in pigs (Rong et al., 2015). In this study, the supplementation of micro-encapsulated OA and EO significantly increased VH in the weaned piglets. Similarly, a micro-encapsulated OA and EO product also improved VH in the broiler chickens (Liu et al., 2017b). The increased VH in this study may partially explain the enhanced maltase activity in piglets fed P(OA+EO). Brush border digestive enzymes, including APN, IAP, maltase, sucrose, are expressed from enterocytes in villus. Therefore, the activities of digestive enzymes are closely related to the gut morphology (Hedemann et al., 2006). The activities of brush border digestive enzymes, which are responsible for the final stage of digestion before absorption, are one of the indicators of the gut health of piglets (He et al., 2016). According to Platel and Srinivasan (2000), EO including curcumin, capsaicin and piperine have been documented to enhance the brush border enzyme activities in animals. A study by Diao et al. (2015) reported that the dietary supplementation of 2,000 mg·kg⁻¹ benzoic acid and 100 mg·kg⁻¹ of thymol increased activities of brush border enzymes in the jejunal digesta of piglets. In the study, the piglets fed PC treatment showed significantly higher IAP activity when compared to the NC piglets. IAP is a gut health-related enzyme because it plays an important role in regulation of gut inflammation, digestion of organic phosphate and fat in pigs, and also IAP is an intrinsic enzyme, which its increased activity may represent the matured gut morphology and gut functions of piglets

(Ghafoorunissa, 2001; Lackeyram et al., 2010). The ETEC F4 infection reduced relative mRNA abundance of digestive enzymes including MGA, SI, APN, which may be closely related to the decreased VH due to ETEC infection in this study.

Goblets cells produce mucus that provides a physical barrier to inhibit the penetration of pathogenic bacteria and to protect epithelial cells from digestive enzymes secreted by microbiota (Neutra, 1987). In addition, mucus provides lubrication for nutrients to be transported across enterocytes (Specian and Oliver, 1991). According to Brown et al. (2006), bacterial infection may stimulate the production and release of mucus from goblet cells as a defense system. Furthermore, an increased number of goblet cells in the villus was thought to be a marker of diarrhea in piglets (Claus et al., 2001), and the increased number of the goblet cells per 100 μm VH may have decreased the number of enterocytes, which is crucial for nutrient digestion and absorption in the villus. In this study, the NC piglets had the highest number of goblet cells per 100 μm VH, and the supplementation of micro-encapsulated OA and EO decreased goblet cells per 100 μm VH. Potentially, the absence of the antimicrobials (e.g. ZnO) in the NC diets stimulated the increase of goblet cells as a defense mechanism and the possible protective effect of micro-encapsulated OA and EO may have decreased the number of goblet cells per 100 μm VH.

The inoculation of ETEC F4 significantly decreased the relative mRNA abundance of mid-jejunal SGLT1 and B⁰AT1 in the present study. The down-regulated mRNA expression of SGLT1 due to ETEC F4 infection showed a similar pattern in SGLT1 activity in the Ussing chamber but statistical significance was not achieved ($P = 0.11$). The SGLT1 is the main sugar transport system in pigs and B⁰AT1 transports leucine, valine and isoleucine, methionine and

proline (Hwang et al., 1991; Yang et al., 2016a). In this study, decreased nutrient transporters and their reduced activities may be possibly associated with villous atrophy and secreted toxins from ETEC F4 (Wu et al., 2015). The other explanation for decreased B⁰AT1 would be the decreased available proteins in the gut because inoculated ETEC F4 possibly competed for available proteins with the host (Jha and Berrocoso, 2016). Decreased available nutrients can decrease the expression of apical membrane nutrient transporters (Zhang et al., 2013).

The ETEC F4 infection increased the expression of OCLN and ZO1. These findings show different numerical patterns with relative mRNA expression of OCLN and ZO1 and are contradictory to the results of the gut and intestinal permeability assays in this study. In addition, it has already shown that ETEC infection decreased the protein expression of tight junctions (Yang et al., 2014; Wu et al., 2016). However, the current data can be supported by Wu and Su (2018) reporting that ETEC infection increased the expression of tight junction proteins via myosin light chain kinase (MLCK)-myosin II regulatory light chain (MLC20) pathways. These differences may be associated with the difference in ETEC strains, infectious models and analytical methods used.

The droplet digital PCR (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 NNC piglets and ETEC F4 inoculation significantly increased the number of ETEC F4 in the colon digesta, but the supplementation of Aureomycin or micro-encapsulated EO an OA did not decrease the number of ETEC F4 in the colon digesta of piglets.

In summary, overall data suggest that the supplementation of micro-encapsulated OA and EO alleviated the induced diarrhea and inflammatory responses, the compromised gut barrier integrity, intestinal morphology, enzyme activities and nutrient transport from ETEC F4 infection in weaned piglets. Therefore, micro-encapsulated OA and EO could be used as an alternative to antibiotics for swine production.

6.0 CHAPTER 6 GENERAL DISCUSSION AND CONCLUSION

6.1 General discussion

In this study, the lipid matrix microparticles were able to maintain the stability of thymol in feeds during the pelleting process and storage. Moreover, *in vitro* and *in vivo* release experiments suggested that the lipid matrix microparticles allowed for a slow release of thymol in simulated digestive fluids and along the gut of weaned piglets. Therefore, we could assume that the lipid matrix microparticles are able to maintain the stability of other compounds in the product (e.g., vanillin, eugenol, fumaric acid, and sorbic acid) in feeds during the pelleting process and storage, and allow for a slow release of these compounds in simulated digestive fluids and along the gut of weaned piglets. However, this assumption can be true only when these compounds have the same behavior with thymol in the lipid matrix microparticles, feeds and gut. It is also not clear if these compounds could interfere with each other on the release along the gut of weaned piglets. It is necessary to further investigate the stability and release of other compounds in the lipid matrix microparticles during feed process, storage and in simulated digestive fluids and along the gut of weaned piglets. Furthermore, the physicochemical and molecular properties (e.g., the distribution of encapsulated bioactive ingredients) of the lipid matrix microparticles are required to be investigated, which will provide the mechanisms underlying the phenomenon of stability or release of bioactive ingredients and will help to further optimize the lipid matrix microparticles to better protect and deliver bioactive ingredients.

In vitro and *in vivo* release studies were conducted to investigate the release profile of thymol in the lipid matrix microparticles in weaned piglets. Microencapsulated OA and EO also can be used for growing and finishing pigs to improve growth performance and nutrient

digestibility (Cho et al., 2014). There could be slight changes in the amount of released thymol from the lipid matrix microparticles in the stomach because gastric lipase activity decreases, the physical pressure of the segmentation movement and transit time can be altered as pigs grow (Jensen et al., 1997; Snoeck et al., 2004). Possibly, more thymol can be released from the lipid matrix microparticles in the intestine because pancreatic enzyme activity increases as pigs grow (Hedemann and Jensen, 2004). Thus, the release profile of thymol from the lipid matrix microparticles should be investigated in growing or finishing pigs.

The microencapsulated OA and EO showed the attenuating effects from ETEC F4 infection by improving gut barrier function, intestinal morphology, immune system, nutrient absorption, enzyme activities, and showing anti-diarrhea effect in weaned piglets. However, ddPCR assays showed that microencapsulated OA and EO could not reduce the DNA abundance of ETEC F4 in the study. However, it is thought that encapsulated OA and EO would have shown that antimicrobial effect to piglets in the study because gut health-promoting effects are closely associated with gut microbiota (Dowarah et al., 2017). Although ddPCR is a powerful method for absolute quantification of bacterial populations within the gut microbiota (Gong et al., 2018), more microbiota analyses (e.g. 16s rRNA gene sequence or microbiome) are required to investigate the effects of microencapsulated OA and EO on the abundance of all bacterial species including pathogenic and commensal bacteria in the colon digesta. Moreover, it is necessary to further elucidate the molecular mechanisms through the determination of host serum metabolite profiles in weaned piglets fed microencapsulated OA and EO using metabolomics.

There is growing advocacy for antibiotic-free pig production that leaves pigs at considerable risk of exposure to disease (Dee et al., 2018). The use of micro-encapsulated OA

and EO may improve animal health based on our study. However, there is a need to use a large scale study to investigate the effects of micro-encapsulated OA and EO on health status, nutrient utilization, and growth performance in weaned piglets in antibiotic-free pig production. It was reported that the use of EO can increase 3 to 19% of feed intake (Zeng et al., 2015b). Several studies also investigated the effects of EO on growth performance and carcass merit in growing-finishing pigs (Janz et al., 2007; Yan et al., 2010). Although there are concerns if the concentration of EO within diets could alter the flavor of pork product, sensory panelists were unable to detect a flavor or aroma differences between the conventional-fed and essential oil diets (Janz et al., 2007). Moreover, carcass and meat quality attributes were not affected in the finisher pigs fed with oregano EO diets when compared with conventional diets (Janz et al., 2007). The cost-effectiveness of using antibiotic alternatives is an important factor for producers to consider (Yang et al., 2015). Therefore, more research and information are needed to understand the effects of micro-encapsulated OA and EO on health status, nutrient utilization, growth performance and pork quality in growing-finishing pigs in antibiotic-free pig production.

The development of antibiotic-resistant microorganisms is one of the main reasons why AGP is banned and restricted in the swine industry (Manso et al., 2011). The mechanisms of inducing the growth of antibiotic-resistant bacteria include 1) reducing permeability by modulating outer membrane of porin proteins 2) increasing efflux of antibiotics by changing bacterial efflux pumps 3) altering the antibiotic target by mutating the target structure 4) protecting target structure by synthesizing the target site binding protein 5) and directly inactivating of antibiotics by hydrolysis (Blair et al., 2015). Those resistant mechanisms of microorganisms are closely related to modulation of genes because the microorganisms that

had resistant genes to translate vital proteins for microorganisms (e.g. porin proteins, efflux pump proteins, target site proteins) against antibiotics can be naturally selected, and also the microorganisms directly mutate genes to modulate protein expression as a defense system against antibiotics (Liu et al., 2010). These naturally selected or mutated genes in bacteria can be delivered intrinsically and transferred horizontally to other bacteria easily (Blair et al., 2015). Thus, ideally, alternatives for AGP should optimize antibiotic alternative effectiveness as well as to minimize the development of resistance mechanisms.

The development of the microencapsulated OA and EO is to reduce antibiotic use. However, relatively little information or consideration has been given to the development of resistance to alternatives (e.g., OA and EO) (Willing et al., 2018). EO may not induce instantly the growth of resistant bacteria because EO mostly show the antibacterial effect by modulating membrane structure and/or composition including LPS and lipoprotein of Gram-negative bacteria due to lipophilic property and peptidoglycan layer of Gram-positive bacteria because of hydrophobicity of EO and peptidoglycan layer, which may be hard to develop instant resistant system for microorganisms (Hurdle et al., 2011; Langeveld et al., 2014). However, according to Becerril et al. (2012), after bacteria exposed to oregano EO, *Serratia marcescens*, *Morganella morganii*, and *Proteus mirabilis* changed their antibiotic resistance profile and/or increased their resistance to oregano essential oils while cinnamon oils did not induce the development of resistant bacteria. In addition, resistant bacteria can be also generated because of OA because pH less-sensitive pathogens can be naturally selected (Ricke, 2003). However, the use of diverse antimicrobial substances (e.g., a blend of OA and EO) 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). It is also not clear if the microencapsulated OA and EO could support or reduce the development of antibiotic resistance. Therefore, it is necessary to investigate the effects of microencapsulated OA and EO used in weaned piglets on the development of antibiotic resistance and the development of resistance to OA and EO themselves.

6.2 General conclusion

The lipid matrix microparticles were able to maintain the stability of thymol during a feed pelleting process and storage (12 weeks) and allow a slow and progressive intestinal release of thymol in the weaned piglets. Moreover, the supplementation of micro-encapsulated OA and EO alleviated diarrhea and inflammation response, and improved gut barrier integrity, intestinal morphology, enzyme activities, and nutrient transport in the weaned piglets experimentally infected with ETEC F4. In conclusion, micro-encapsulated OA and EO can improve gut health in weaned piglets with physiological challenges and can be used as an alternative to antibiotics for swine production.

7.0 CHAPTER 7 FUTURE DIRECTIONS

Future directions include:

1. To investigate the physicochemical and molecular properties (e.g., the distribution of encapsulated bioactive ingredients) of the lipid matrix microparticles in order to provide the mechanisms underlying the phenomenon of stability or release of bioactive ingredients;
2. To further elucidate the molecular mechanisms of the function of micro-encapsulated OA and EO in experimentally infected weaned piglets by microbiome and metabolomics analyses;
3. To evaluate the effects of micro-encapsulated OA and EO on health status, nutrient utilization, and growth performance in weaned piglets in antibiotic-free pig production; and
4. To evaluate the effects of micro-encapsulated OA and EO on health status, nutrient utilization, growth performance and pork quality in growing-finishing pigs in antibiotic-free pig production.

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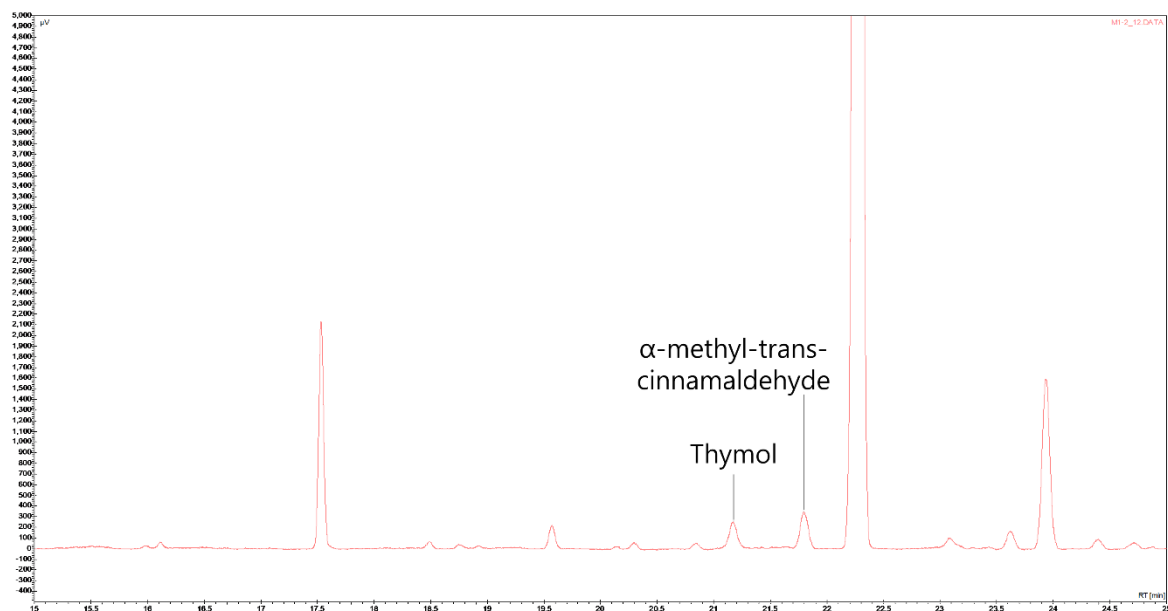
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Appendix 1. Partial gas chromatography-flame ionization detector (GC-FID) chromatogram of thymol (compounds of interested) in the feed and α -methyl-trans-cinnamaldehyde (internal standard)