MICROENCAPSULATION FOR EFFECTIVE DELIVERY OF ESSENTIAL OILS TO IMPROVE GUT HEALTH IN PIGS

By

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ABSTRACT

Antibiotics have been widely supplemented in feeds at sub-therapeutic concentrations to prevent post-weaning diarrhea and increase the overall productivity of pigs. However, the current trend worldwide is to minimize the use of in-feed antibiotics. The development of promising alternatives to in-feed antibiotics is important for maintaining the sustainability of swine production. This study was to investigate the anti-inflammatory functions of thymol that is considered as a potential antibiotic alternative with *in vitro* porcine intestinal epithelial cells (IPEC-J2) cells and develop novel microparticles to deliver thymol and lauric acid to pig intestinal tract effectively. The results suggest that thymol can enhance barrier function and reduce ROS production and pro-inflammatory cytokine gene expression in the epithelial cells during inflammation induced with lipopolysaccharide (LPS). The regulation of barrier function by thymol and LPS may be at post-transcriptional or post-translational levels. The incorporation of starch and alginate produced novel microparticles containing thymol and lauric acid through a melt-granulation process. The release of thymol and lauric acid from the microparticles was *in vitro* determined using simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), consecutively. The microparticles prepared with 2% alginate solution displayed a slow release of thymol and lauric acid in the SSF (21.2 ± 2.3%; 36 ± 1.1%), SGF (73.7 ± 6.9%; 54.8 ± 1.7%) and SIF (99.1 ± 1.2%; 99.1 ± 0.6%), respectively, whereas, the microparticles without alginate showed a rapid release of thymol and lauric acid from the SSF (79.9 ± 11.8%; 84.9 ± 9.4%), SGF (92.5 ± 3.5%; 75.8 ± 5.9%) and SIF (93.3 ± 9.4%; 93.3 ± 4.6%), respectively. In conclusion, the novel method developed in this study can be potentially used to deliver thymol and lauric acid effectively to pig intestinal tract, and the microparticles containing thymol may improve gut health by enhancing barrier function and reducing inflammatory cytokines secretion in pigs under physiological challenges.

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DEDICATION

This thesis is dedicated to God, my parents, and siblings who nurtured me to be what I am today.

FOREWORD

Part of this thesis has been presented as an oral presentation at the ASAS-CSAS Annual Meeting & Trade Show in Vancouver, Canada on July 6-12, 2018. This thesis was written in manuscript format, and it is made up of three manuscripts published or submitted in Animal Nutrition and Journal of Agricultural and Food Chemistry. All manuscripts have been listed as follows:

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LIST OF ABBREVIATIONS

AGP	Antibiotic Growth Promoters
AHL	Acyl-Homoserine Lactones
AKT	Protein Kinase B
AP-1	Activator Protein-1
ARE	Antioxidant Response Element
ASCT2	System ASC Amino Acid Transporter 2
ATP	Adenosine Triphosphate
A1-2	Auto Inducer 2
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
B^0AT1	Broad Neutral Amino Acid Transporters
CaSR	Calcium Sensing Receptor
CAT	Catalase
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbondioxide
COX-2	Cyclooxygenase-2
CycA	Cyclophilin-A
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DCFDA	Dichlorofluorescein Diacetate
DCF	Dichlorofluorescein

DMEM	Dulbecco Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DSC	Differential Scanning Calorimetry
EAAC1	Excitatory Amino Acid Transporter 1
EFSA	European Food Safety Authority
ELISA	Enzyme-linked Immunosorbent Assay
ESR-2	Millicell Electrical Resistance System
EXPI/EXPR	ExpiCHO Expression
FA	Fatty Acid
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FID	Flame Ionization Detector
FITC-Dextran	Fluorescein Isothiocyanate-Dextran
γ-GCL	Gamma-glutamyl Cysteine Ligase
γ-GCS	Gamma-glutamyl Cysteine Synthetase
G^-	Gram-Negative
G^+	Gram-Positive
GC	Gas Chromatography
GC-MS	Gas chromatography- Mass Spectrometry
GLP-1 and 2	Glucagon-Like Peptides 1 and 2
GPCR	G Protein-Coupled Receptors

GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GRAS	Generally Recognized as Safe
GSH	Glutathione
GST	Glutathione S-transferase
H_2O_2	Hydrogen Peroxide
HO1	Heme Oxygenase 1
HPLC	High Performance Liquid Chromatography
HPRT	Hypoxanthine Phosphoribosyl Transferase
IEC	Intestinal Epithelial Cells
IBD	Inflammatory Bowel Diseases
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL-1β	Infection-Interleukin 1 Beta
IL-8	Interleukin 8
IL-6	Interleukin 6
iNOS	Inducible Nitric Oxide Synthase
IPEC-1	Intestinal Porcine Epithelial Cells 1
IPEC-J2	Intestinal Porcine Epithelial Cells J2
IUPAC	International Union of Pure and Applied Chemists
Keap1	Kelch-Like ECH-Associated Protein 1

LA	Lauric Acid
LPS	Lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MDA	Malondialdehyde
MIC	Minimum Inhibitory Concentration
mRNA	Messenger Ribonucleic Acid
NF-κB	Nuclear Factor Kappa B
NO	Nitrogen Oxide
NRF2	Nuclear Factor-Erythroid 2-Related Factor-2
NQO1	NADH(P)H-Quinone-Oxidoreductase 1
OA	Organic Acid
OEO	Oregano Essential Oil
OCLDN3	Occludin- 3
O/W	Oil in Water Emulsion
PA	Palmitic Acid
PAMs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PepT1	Peptide Transporter 1
PFA	Paraformaldehyde
PGE_2	Prostaglandin
PRR	Pattern Recognition Receptors 1

PTMs	Post Translational Modifications
PTV	Programmable Temperature Vaporizer
QS	Quorum Sensing
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SA	Stearic Acid
SGF	Simulated Gastric Fluid
SGLT1	Sodium-glucose Transporter 1
SIF	Simulated Intestinal Fluid
SOD	Superoxide Dismutase
SSF	Simulated Salivary Fluid
T1R1+T1R2	Sweet Taste Receptor
T1R1+T1R3	Umami Taste Receptor
T2Rs	Bitter Taste-Sensing Type 2 Receptors
TEER	Transepithelial Electrical Resistance
TLRs	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor – Alpha
USFDA	U. S. Food and Drug Administration
WST-1	Water-soluble Tetrazolium Salts
ZO-1	Zonula Occludens

CHAPTER 1 GENERAL INTRODUCTION

Young piglets have a high susceptibility to various stressors, including bacterial pathogens, oxidative stress, and inflammation which leads to reduced growth performance, high mortality and morbidity rates and compromised animal welfare (Yang et al., 2015a; Hassan et al., 2018). Antibiotic growth promoters (AGP) have been widely used in pig diets, especially in nursery diets, to control incidences of post-weaning diarrhea and to improve growth performance. Total consumption of antimicrobials in animal food production worldwide was estimated at 63,151 tons in 2010, with an increasing trend; the annual consumption of antimicrobials per kilogram body weight was 148 mg/kg for pigs (Van Boeckel et al., 2015). This practice may lead to the spread of antimicrobial-resistant bacterial pathogens in both pigs and humans, posing a significant public health threat (Yang et al., 2015a). The use of AGP in food animal production has been banned in the European Union since 2006 (Bengtsson and Wierup, 2006). The U.S. Food and Drug Administration placed restrictions on antibiotic use in animals in December 2016. Canada will curtail AGP usage in livestock production starting from December 2018 with more countries expected to follow. Several challenges are associated with the withdrawal of antibiotics from feeds (Zhao et al., 2007). Therefore, it is critical to developing cost-effective antibiotic alternatives for ensuring the long-term sustainability of pig production (Yang et al., 2015a; Valenzuela-Grijalva et al., 2017). Organic acids (Eckel et al., 1992; De Lange et al., 2010), enzymes (Bedford and Cowieson, 2012; Kiarie et al., 2013), probiotics (Heo et al., 2004; Musa et al., 2009; Heo et al., 2013), antimicrobial peptides (Choi et al., 2013), medium chain fatty acids (Boyen et al., 2008) and essential oils (Windisch et al., 2008; Randrianarivelo et al., 2010; Gong et al., 2013) have been widely recognized as promising alternatives to antibiotics in feeds.

Essential oils are natural bioactive compounds derived from plants and have positive effects on animal growth and health (Puvača et al., 2013). Because of the antimicrobial, anti-

inflammatory and antioxidative properties, essential oils have been widely used as traditional medicines to improve health or cure diseases in humans (Brenes and Roura, 2010; Kim et al., 2008). The bioactive components in essential oils have been identified and some progress has been made to elucidate the mechanisms underlying the function of these compounds in animals, leading to increased research efforts to use essential oils to replace antibiotics in animal feeds (Li et al., 2012a). However, the application of essential oils in the feeds has been mainly based on the antimicrobial effects. Moreover, the minimum inhibitory concentration (MIC) of most essential oils are much higher than the acceptable levels in the animal industry in terms of cost-effectiveness and feed palatability (Yang et al., 2015a). Other than the varied results and unclear mechanisms, there are still several other challenges in using essential oils in animal feeds, including toxic effects, regulatory concerns, and high inclusion costs. Therefore, it is vital to investigate the specific effects and target sites (either animal host or its microbiome) of individual compounds in essential oils to facilitate the application of essential oils in swine production. An improved understanding of the mechanisms underlying the functions of essential oils, including the effects on the three components in the gut ecosystem: gut microbiota, gut physiology and immunology, will allow us to make the best use of essential oils in swine production. Microencapsulation and nanotechnology provide promising tools to effectively deliver essential oils to the animal gut and improve the efficacy of essential oils in swine production. The following literature review summarizes the efficacy, feasibility, potential mechanisms of the application of essential oils as antibiotic alternatives and considerations of the use of essential oils in swine production.

CHAPTER 2 LITERATURE REVIEW [1]

2.1 ESSENTIAL OILS

Essential oils are aromatic, volatile and oily liquids extracted from plant materials such as seeds, flowers, leaves, buds, twigs, herbs, bark, wood, fruits, and roots (Brenes and Roura, 2010). The word "essential" is postulated by Paracelsus in the theory of 'quinta essentia', which means that the quintessence can be useful medically due to its effectiveness (Oyen and Dung, 1999). Essential oils are a mixture of complex compounds which may vary in their individual chemical compositions and concentrations. For instance, the predominant components thymol and carvacrol found in thyme, researchers have found that they can be as low as 3% to as high as 60% of the total essential oils in thyme (Lawrence and Reynolds, 1983). Also, it was discovered that a major component of the cinnamon essential oils, cinnamaldehyde ranges from 60 to 75% of the total oil (Duke, 1986). These constituents of essential oils such as carvacrol and thymol present in thyme provide broad-spectrum antimicrobial activities against gram-negative and gram-positive bacteria, fungi and yeast (Roller and Seedhar, 2002; Abbaszadeh et al., 2014) Essential oils have greater effect against gram-positive (G^+) than gram-negative (G^-) bacterial pathogens because the entrance of hydrophobic compounds through the lipopolysaccharide structures of G^+ bacteria are limited due to their outer membrane coating the cell wall (Vaara, 1992). Various researchers have proven essential oils as alternatives to antibiotics because they have antimicrobial, anti-inflammatory, antioxidative, and coccidiostatic properties. They enhance digestibility (Chitprasert and Sutaphanit, 2014) and immunity (Brenes and Roura, 2010), promote gut health by minimizing the effect of the pathogenic bacteria (Chitprasert and Sutaphanit, 2014), and control odor and ammonia emission (Varel, 2002). Essential oils have two major classes of compounds, terpenes (e.g.

[1] The material presented in Chapter two of this thesis has been published in Animal Nutrition. The Authors are Faith A. Omonijo, Liju Ni, Joshua Gong, Qi Wang, Ludovic Lahaye, Chengbo Yang. Anim Nutr. 2018. 4(2):126-136

carvacrol and thymol) and phenylpropenes (e.g. cinnamaldehyde and eugenol). Terpenes are sub-divided in respect to the numbers of 5 – carbon building blocks known as isoprene units with mono ($C_{10}H_6$), sesqui ($C_{15}H_{24}$) and diterpenes ($C_{20}H_{32}$). There are some different sources of terpenes represented by the existence or non-existence of the ring structures, double bonds, and the addition of oxygen or the presence of stereochemistry (Lee et al., 2004). It is estimated that there are more than 1000 monoterpenes and more than 3000 sesquiterpenes based on various researchers (Cooke et al., 1998). There are just 50 phenylpropenes discovered (Lee et al., 2004). The commonly used essential oils in animals are carvacrol, thymol, citral, eugenol and cinnamaldehyde and their chemical properties and stability are described in Table 1.

2.2 ANTIMICROBIAL EFFECTS OF ESSENTIAL OILS

2.2.1 Mode of Action of Essential Oils

Although carvacrol and thymol have several target sites in bacterial cells, the biosynthetic machinery of bacterial cell walls is their main target site (Faleiro, 2011; Yap et al., 2014). First, carvacrol and thymol can sensitize the cell walls (including membranes) and cause significant membrane damages, leading to integrity collapse of the bacterial cytoplasmic membrane, leakage of vital intracellular contents and eventually death of the bacterial cells. The leakage often happens through cell wall damage, cytoplasmic membrane damage, cytoplasm coagulation and membrane protein destruction (Conner and Beuchat, 1984; Cox et al., 1998; Helander et al., 1998; Ultee et al., 2002) as well as reduction of proton motive force (Nazzaro et al., 2013). Secondly, with their lipophilic structure, carvacrol and thymol can easily get into the bacterial membranes among the fatty acid chains and cause the membranes to expand and become more fluidity. With these properties, carvacrol and thymol are regarded as promising alternatives to antibiotics in swine production systems (Kim et al., 1995; Lambert et al., 2001; Si et al., 2006b; Delaquis et al., 2002). The position of functional groups (e.g. hydroxyl or alkyl) in essential oils plays very important roles in the antimicrobial activities of

essential oils. Although thymol and carvacrol have similar antimicrobial effects, they have different effects on G^+ or G^- bacteria based on the positions of one or more functional groups in thymol and carvacrol. Their antimicrobial action highly depends on the hydroxyl group of the phenolic terpenoids and the presence of delocalized electrons (Lambert et al., 2001; Ultee et al., 2002), which often determine the level of their antimicrobial activity on different bacteria. Helander et al. (1998) reported that thymol and carvacrol were able to damage the outer membrane of *Salmonella typhimurium* and *Escherichia coli* O157: H7 because of their enhanced ability to release lipopolysaccharides and sensitize membrane. Both carvacrol and thymol have lipopolysaccharide releasing properties that make them have superior antimicrobial properties against some G^- bacteria when compared to other essential oils. Another hypothesis is the proton exchanger model and carvacrol can act as a trans-membrane carrier by exchanging its hydroxyl proton for a potassium ion resulting in dissipation of the pH gradient and electrical potential over the membrane, reduced proton motive force and depletion of intracellular adenosine triphosphate (ATP) pools. Loss of potassium can also cause problems, since it plays very important roles in the activation of a number of cytoplasmatic enzymes, in maintaining osmotic pressure and in the regulation of intracellular pH.

Generally speaking, bacteria can use ionic pumps to counter these effects and cell death does not always happen, but large amounts of energy are needed for this function and bacterial growth is compromised (Ultee et al., 1999; Ultee et al., 2002). Eugenol and cinnamaldehyde also have a phenolic functional group and their antimicrobial activities related to membrane effects and energy generation have been reported (Gill and Holley, 2004, Gill and Holley, 2006). A study has been reported that cinnamaldehyde and eugenol can effectively inhibit histidine decarboxylase activity of *Enterococcus aerogene*s at sublethal levels (Wendakoon and Sakaguchi, 1995). The hydroxyl group of eugenol and the carboxyl group of cinnamaldehyde are believed to bind to proteins, inhibiting the action of amino acid

decarboxylases in *E. aerogenes*. Therefore, the primary mechanism of action for thymol, carvacrol, eugenol, and cinnamaldehyde are related to their effect on the cytoplasmic membranes and energy metabolism.

2.2.2 Minimum Inhibitory Concentration (MIC) of Essential Oils

The definition of MIC is the minimal concentration of an antimicrobial compound that inhibits the growth of a microorganism. The MIC of an antimicrobial compound has been widely used in the laboratory to measure the activity of an antimicrobial compound against a microorganism. Individual essential oil's MIC can be different from bacterium to bacterium and from strain to strain. Laboratory conditions for MIC assays may also affect results. Some popularly used essential oils and their MIC values on several bacterial pathogens are described in Table 2.

2.2.3 Synergy of Essential Oils and Organic Acids

Most G^+ bacterial cell wall (approximately 90–95%) is comprised of peptidoglycan. This feature can allow hydrophobic compounds to easily penetrate the cells and then act on both cell wall and cytoplasm (Karatzas et al., 2001; Trombetta et al., 2005). After entering the cell, these compounds can not only affect several enzymes involved in energy production at lower concentrations but also denature proteins at higher concentrations. While G^- bacteria only have a 2–3 mm peptidoglycan layer comprising about 20% of the dry weight of bacterial cells. An outer-membrane is located in the outside of this peptidoglycan layer and is composed of a double layer of phospholipids firmly linked to the inner membrane by Braun's lipoprotein. Basically, G^- bacteria are more resistant to essential oils when compared with G^+ bacteria. The thick outer membrane in G^- bacteria reduces permeability and provides an extra layer to protect cells from essential oils.

Organic acids (OA) have a better efficacy against G^- bacteria than essential oils (Zhou et al., 2007; Souza et al., 2009; Mahmoud, 2014). A study has shown that grape seed extract

had a much higher MIC value at 10 mg/mL against *Vibrio parahaemolyticus* in sucked oysters when compared to citric acids (5 mg/mL) and lactic acids (1 mg/mL). Small hydrophilic organic acids are believed to be able to pass through the membrane via porin protein but not the hydrophobic polyphenol compounds. There are several factors contributing to the microorganism inhibition by organic acids, including a reduction in pH, the ratio of the non-disassociated form of organic acids, chain length, level of branching and cell physiology/ metabolism (Booth, 1985). The lipophilic nature of weak organic acids allows them to easily enter plasma membrane and thus reduce the pH of cell's interior, eventually leading to the death of bacterium (Wang et al., 2013).

There are several studies demonstrating the additive effects of some essential oils and organic acids (Zhou et al., 2007, Souza et al., 2009, Hulánková and Bořilová, 2011). Zhou et al. (2007) reported that an essential oil (carvacrol or thymol) in combination with an acetic acid or citric acid but not with lactic acid had a better efficacy against G^- bacteria (*Salmonella typhimurium*) when compared with individual essential oil or organic acids alone. The mechanisms underlying this potential synergism between some essential oils and organic acids are still not clear. However, it is well-known that phenols in essential oil can change the structure and function of the bacterial cell membrane, leading to swelling and thus increased membrane permeability. The compromised cell membrane might explain the observed synergism, since the phenolic compounds could cause significant damages to the cell membranes, increasing the susceptibility of the bacteria to OA. Moreover, the hydrophobicity of an essential oil is increased at low pH, enabling it to more easily pass through the lipids of the bacterial cell membrane (Karatzas et al., 2001). In recent studies, the results have clearly shown *in vivo* efficacy of such synergistic dietary strategies in pigs and poultry (Diao et al., 2015; Balasubramanian et al., 2016; Walia et al., 2017; Liu et al., 2017). The inclusion of

essential oils blends with formic acid and citric acid in pig diets before slaughter can hinder Salmonella shedding and seroprevalence (Walia et al., 2017).

2.2.4 Synergy of Essential Oils and Medium Chain Fatty Acids

As shown in Table 3, medium chain fatty acids (MCFAs) include lauric acid (C12), capric acid (C10), caprylic acid (C8), carboxylic acids (C7 and C9) and caproic acid (C6) and their derivatives are also another of the alternatives to antibiotics for animals (Boyen et al., 2008; Zentek et al., 2011; Zentek et al., 2012; Hanczakowska et al., 2013; Zentek et al., 2013; De Smet et al., 2016). MCFAs have the capacity to fight against the microbial activity of Salmonella and *E. coli* (Dierick et al., 2002; Rossi et al., 2010). Research carried out by Han et al. (2011) shows that the performance of pigs fed eucalyptus MCFA blend was the same as antibiotics. MCFAs are shown to have a good effect on G^- and G^+ bacteria. The effectiveness of the antimicrobial activity of MCFAs towards some groups of bacteria is different based on their chain length (Rossi et al., 2010). Caprylic acid may have a similar mode of action with short-chain fatty acids because MCFAs may inactivate bacteria by creating an acidic environment or by a direct impact on the expression of virulence factors necessary for Salmonella colonization. At low dietary levels, MCFAs may be regarded as modulators of the gastric microbiota in weaned piglets. The additive effects were observed with multiple strains of Salmonella, *Listeria monocytogenes, E. coli,* and *Streptococcus aureaus* when treated with a combination of oregano oil and caprylic acid (Hulánková and Bořilová, 2011). Similar effects of cinnamaldehyde and lauric acid against *Brachyspira hyodysenteriae*, the causative pathogen of swine dysentery, were observed *in vitro* (Maele et al., 2016). MCFAs are generally recognized as safe (GRAS) by the Food and Drug Administration (De Los Santos et al., 2008). Some MCFAs and their derivatives have strong and unpleasant smells that can reduce feed palatability and feed intake of pigs (Zentek et al., 2011). These may be overcome by using the

combination of essential oils and MCFAs. However, there is no information on the *in vivo* application of the combination of essential oils and MCFAs in swine production.

2.3 EFFECT OF ESSENTIAL OILS ON INTESTINAL INFLAMMATION

The gut has various important function including absorption of nutrient, electrolytes and water, secretion of water, electrolytes, immunobulins, cytokines and mucin, and selective barrier protection against harmful antigens, toxins and pathogens (Lallès et al., 2004). However, it has been believed that except its absorption, secretion and barrier properties, the gut also plays a dynamic role in organ integrity, immunity and body defense (Eckmann et al., 1995; Pitman and Blumberg, 2000). Gut epithelial cells play an important role in the immune system as "watch dogs" and they can detect the onset of immune responses or inflammation through cytokines. These cytokines are vital for recruitment and activation of different types of immune cells including neutrophils, macrophages, T and B cells and dendritic cells (Eckmann et al., 1995, Pitman and Blumberg, 2000). The gut immune system is dramatically different from the systemic immune system. The gut immune system must balance two opposite function: not only mounting an immune response to pathogens but also maintaining tolerance to antigens derived from commensal bacteria and compounds from feeds (Pitman and Blumberg, 2000).

The balance of those two opposite functions could cause feed intolerance, inflammation, and diseases (Yang et al., 2015a). Gut inflammation is associated with compromised gut growth and development and reduced efficiency of nutrient utilization. It has been reported that gut acute and chronic inflammatory diseases often lead to gut morphological changes, mucosa damage, increased mucosal permeability, compromised gut development and poor nutrient absorption capacity (Waters et al., 1999; Nagura et al., 2001; Podolsky, 2002; Strober et al., 2002). Generally, three types of gut inflammation have been observed in pigs related to pathogens, nutrition and management and they are pathogen infection-associated,

diet allergen-associated and weaning-associated gut inflammation (Yang et al., 2015a). Although the inflammation does not cause full-blown clinical symptoms, it leads to a significant reduction of growth performance and causes a considerable economic loss in pig production.

When the immune response is initiated, macrophages are recruited in the tissues to produce an inflammatory reaction, and then T cells are also involved in the inflammation in the later stages of immune response. Nuclear factor kappa B (NF-κB) is a transcriptional and plays very important roles during the above process (Rogler et al., 1998). After activated by several inducers such as pro-inflammatory cytokines, reactive oxygen species (ROS) and lipopolysaccharides, NF-kB is translocated from the cytoplasma to the nucleus and then induces the expression of numerous pro-inflammatory proteins including cytokines, chemokines, adhesion molecules and enzymes that are involved in inflammation, cell apoptosis and proliferation (Barnes and Karin, 1997). It has been evidenced that there is potential cross-talks between the Nrf2, and NF-κB pathways and nuclear factor-erythroid 2-related factor-2 (Nrf2) gene dysfunction could lead to increased susceptibility to inflammatory stresses (Khor et al., 2006). Given that gut inflammation in pigs does not only impair the function and integrity of the gut but also affect growth performance, dietary strategies are necessary to inhibit the inflammatory process in the gut. Essential oils have been shown to manipulate both Nrf2 and NF-κB transcription factors to suppress inflammation (Kroismayr et al., 2008; Wondrak et al., 2010; Zou et al., 2016a; Fang et al., 2017). Essential oils and avilamycin significantly reduced the expression of the NFκB, tumor necrosis factor-α **(**TNF-α) and the size of Peyer's patches in the intestine of weaned piglets, as well as the cyclin D1 in the colon, mesenteric lymph nodes and spleen (Kroismayr et al., 2008). Several studies have demonstrated that essential oils including cinnamaldehyde (Wondrak et al., 2010) and oregano oil (Zou et al., 2016a) increased the expression and translocation of Nrf2 and prevented the activation of NF-κB. These results

suggest that these essential oils can reduce inflammation, and eventually lead to the improvement of pig health and growth performance by modifying the Nrf2 and NF-κB pathways. The supplementation of cinnamon oil in feeds attenuated lipopolysaccharide (LPS)-induced injury by suppressing inflammation (Wang et al., 2015). There is experimental evidence showing that oral administration of essential oils significantly reduces and limits the severity and development of experimental autoimmune encephalomyelitis, mainly through the modulation of Th1/Treg immune balance (Alberti et al., 2014). Therefore, it is clearly demonstrated that essential oils can modulate pig immune responses through different cell signaling pathways to enhance pig health.

2.4 EFFECT OF ESSENTIAL OILS ON OXIDATIVE STRESS

Oxidative stress represents an important chemical mechanism that leads to biological damage, which in turn can affect growth performance and health in pigs, especially in modern high-performance swine production systems. Pigs are frequently exposed to several stressors including weaning, malnutrition, disease challenge, heat stress, in-feed mycotoxin contaminations, transportation, and overcrowding. These stressors are known to increase the production of reactive oxygen species (ROS) and when the antioxidant system is overwhelmed by the production of ROS, oxidative stress occurs. The oxidative stress might be associated with a drop in performance, compromised immunity, muscle degeneration, increased risk of stroke in fast-growing pigs, mulberry heart disease, reduced appetite, diarrhea, destruction of liver tissue, and increased risk of abortion of gestation sows.

As an important counterpart of NF-κB, Nrf2 is a redox-sensitive transcription factor and can be sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) under normal conditions. Nrf2 can be dissociated from Keap1 and translocated into the nucleus, activating the expression of genes containing an antioxidant response element (ARE) (Nair et al., 2008). The Nrf2-ARE pathway positively regulates the expression of antioxidant and

detoxification enzymes in cells such as glutathione peroxidase (GPx), catalase (CAT), glutathione S-transferase (GST), glutathione reductase (GR), superoxide dismutase (SOD), NADH(P)H-Quinone-Oxidoreductase 1 (NQO1), Heme oxygenase (HO1) and the glutathione (GSH) precursor gamma-glutamyl cysteine synthetase (γ-GCS) and these enzymes can help to re-establish cellular redox homeostasis (Dhakshinamoorthy et al., 2001; Lee and Johnson, 2004; Nguyen et al., 2009; Mine et al., 2015).

Synthetic antioxidants such as ethoxyquin, BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are commonly used as effective feed additives in pig diets in order to increase the stability of feed and protect nutrients (e.g. fat and vitamins) from oxidation. However, such synthetic antioxidants do not have biological effects *in vivo*, and their toxicological safety is also a concern. This has driven the search for natural compounds that could not only replace synthetic antioxidants in pig feed but also provide additional zootechnical benefits (Yang et al., 2015a). It is well known that essential oils and plant extracts have anti-oxidative effects (Baschieri et al., 2017) and they have been used successfully in animal diets (Mueller et al., 2012; Akbarian et al., 2014; Placha et al., 2014; Tan et al., 2015; Zou et al., 2016a; Liu et al., 2017). Zou et al. (2016a) investigated the antioxidative effects of oregano essential oil in pig small intestinal epithelial cells (IPEC-J2) and demonstrated that reactive oxygen species (ROS) and malondialdehyde (MDA) induced by hydrogen-peroxide (H_2O_2) were dramatically suppressed by oregano essential oil by inducing Nrf2 and several antioxidant enzymes (superoxide dismutase and g-glutamylcysteine ligase). Kang et al. (2015) also found that schisandrae semen essential oil attenuated cell damage induced by oxidative stress in C2C12 murine skeletal muscle cells through the upregulation of Hemy-oxygenase-1 (HO-1) mediated by Nrf2. Another *in vitro* study also indicated that lemongrass essential oil reduced DNA damage and oxidative stress induced by benzo(a)pyrene in human embryonic lung fibroblast cells (Jiang et al., 2017). Zeng et al. (2015) indicated that pigs fed an essential

oil diet had higher levels of albumin, immunoglobulin A (IgA), immunoglobulin G (IgG), total antioxidant capacity and lower fecal score when compared to pigs fed control diets. A recent *in vivo* pig study has shown that dietary supplementation with 100 mg/kg mixture of carvacrol and thymol (ratio: 1: 1) decreased the weaning associated intestinal oxidative stress through decreasing tumor necrosis factor α (TNF-α) and messenger ribonucleic acid (mRNA) (Wei et al., 2017). Increased oxidative stress leads to impaired milk production, reproductive performance, and longevity of sows (Berchieri-Ronchi et al., 2011; Zhao et al., 2013). Compromised ability to produce milk could directly impair the health and growth of nursing piglets. It may also have a long-term effect on health and growth throughout the life of pigs. An increase in systemic oxidative stress was observed in late gestation and early lactation of sows and the supplementation of oregano essential oil improved performance of their piglets through reducing oxidative stress (Tan et al., 2015; Tan, 2015). Therefore, supplementation of essential oils is a promising method to ameliorate the negative effects of oxidative stress induced by different stressors in modern high-performance swine production systems.

2.5 EFFECT OF ESSENTIAL OIL ON QUORUM SENSING

2.5.1 Bacterial Cell Signaling

Quorum sensing (QS) is referred to a regulatory system depending on population density in bacteria and it regulates gene expression in response to cell density through the accumulation of diffusible signaling molecules (De Kievit and Iglewski, 2000). QS has an important role to regulate various important physiological processes, particularly the expression of virulence factors which play important roles during the process of pathogenic microbe-host interactions (Greenberg, 2003; Xavier and Bassler, 2003; Vendeville et al., 2005; Defoirdt et al., 2013; Kantas et al., 2015; Joshi et al., 2016). There are three major types of signaling components that have been used for QS, including acyl-homoserine lactones (AHL), small polypeptides and autoinducer-2 (AI-2). AHLs are biosynthesized by the LuxI family

members of AHL synthases and mainly used in G^- bacteria (Parsek et al., 1999). However G^+ bacteria utilize modified oligopeptides as autoinducer molecules because G^+ bacteria do not have LuxI or LuxR homologues (Rocha-Estrada et al., 2010). G^- and G^+ bacteria can share AI-2 and the gene, LuxS that is responsible for the synthesis of AI-2 in bacteria (Schauder et al., 2001). It has been reported that QS plays significant roles in the regulation of virulence factors expression in numerous enteric pathogens (Ohtani et al., 2002; Vendeville et al., 2005; Khan et al., 2009; Zhu et al., 2011). Therefore, it can be a promising tool to control enteric pathogens in pig production through interfering QS by small molecules or QS quenching enzymes. However, identification of QS inhibitors for pathogens and studies on their mechanisms and applications in swine production are still required.

2.5.2 Disrupting Quorum Sensing

Each QS circuit used by a specific bacterium is different. However, all kinds of QS systems share a common mechanism comprised of signal synthesis, signal accumulation and signal detection (LaSarre and Federle, 2013). Therefore, there are three steps that QS inhibitors can target: QS signal biosynthesis, QS signal degradation and inactivation, and signal detection (Czajkowski and Jafra, 2009; LaSarre and Federle, 2013). Extensive studies have been investigating the application of small molecules to disrupt the expression of virulence genes that are regulated by QS and numinous natural and synthetic small molecules that can inhibit QS have been identified (Galloway et al., 2010). Eugenol and carvacrol are the most intensely studied QS-disrupting essential oils (Zhou et al., 2013; Burt et al., 2014; Mith et al., 2015). Except for the use of small molecules, another strategy for inhibiting QS is to degrade QS autoinducers enzymatically (Czajkowski and Jafra, 2009). There are three known classes of enzymes including lactonases, acylases, and oxidoreductases, hydrolyzing AHLs to produce products that are no longer active signaling agents (Hong et al., 2011). It has been recently reported that *Ruminococcus obeum* could decrease *Vibrio cholerae* growth and colonization by

increasing LuxS/AI-2-based QS of *Ruminococcus obeum* (Hsiao et al., 2014). In conclusion, it may be feasible to disrupt QS by using synthesized or natural small molecules, QS quenching enzymes and probiotics bacteria, which could limit the growth and colonization of enteric pathogens in swine production.

Numerous studies have shown that various essential oils can disrupt the QS of pathogenic bacteria (Bjarnsholt et al., 2005; Choo et al., 2006; Zhou et al., 2013). However, all evidence has been come from *in vitro* studies and studies on food microbiology (Kerekes et al., 2013; Alvarez et al., 2014). It is an unexplored strategy to control bacterial infections in animals by using inhibition of QS (Defoirdt et al., 2004). However, it is an exception in aquaculture. It has been reported that Cinnamaldehyde and its derivatives are effective against *V. harveyi* in brine shrimp (Niu et al., 2006, Brackman et al., 2008) and the potential mechanism is to disrupt protein-DNA interactions of the QS-responsive master regulatory protein LuxR. A most recent study strongly support that carvacrol and eugenol inhibit specific virulence determinants in pectobacteria through disputing the QS machinery and these two volatiles directly inhibit AHL production, potentially via direct interaction with ExpiCHO Expression (ExpI/ExpR) proteins (Joshi et al., 2016). Similar results were also found in a study indicating that essential oil (carvacrol and thymol) suppresses biofilm (Oh et al., 2017). Although the application of QS inhibition in pig pathogens are still lacking, it is expected that this strategy will receive significant attention in swine production in the coming years.

2.6 EFFECT OF ESSENTIAL OILS ON INTESTINAL MICROBIOTA AND MICROBIOME

The pig gut is generally considered to be sterile prior to birth, but rapidly becomes colonized with microbes from the environment, diet, and parents (Jensen, 1998; Kim and Isaacson, 2015). The colonizing microbes subsequently develop into a highly diverse microbiota with varying microbial density and composition among different gut compartments.

Our understanding of gut microbiota composition and function has been significantly improved following the application of molecular and "omics" methodologies in combination with bioinformatics and statistical tools. For example, Firmicutes and Bacteroidetes were shown to be the most dominant phyla in pigs regardless of age, followed by Proteobacteria, Actinobacteria and Spirochaetes (Kim et al., 2012; Lu et al., 2014; Kim and Isaacson, 2015; Slifierz et al., 2015; Zhao et al., 2015). Nevertheless, there are still some dynamic shifts in the composition of gut microbiota with age. The phylum Proteobacteria was found to be more abundant in the pig gut prior to weaning (Zhao et al., 2015). In general, the gut microbiota becomes increasingly stable during animal growth and consequently, it becomes more resistant to dietary perturbations (Kim and Isaacson, 2015). This explains why piglets are more susceptible to pathogen infection than adult pigs. It also demonstrates the importance of modulating the gut microbiota of young animals in order to have a healthy microbiota developed for better animal performance. Several *in vivo* studies indicated that essential oils increased the *lactobacillus* group and decreased *E. coli* or total coliforms in piglets (Namkung et al., 2004; Castillo et al., 2006; Li et al., 2012b; Zeng et al., 2015; Wei et al., 2017). These results were consistent with the results observed in several poultry studies with the supplementation of essential oils (Oviedo-Rondón et al., 2006; Tiihonen et al., 2010; Amerah et al., 2011; Basmacioğlu-Malayoğlu et al., 2016; Cetin et al., 2016; Liu et al., 2017), suggesting that essential oil treatment led to some fundamental changes within gut microbiota mainly in the number of observed *Lactobacillus* species. However, studying the effects of essential oils on the gut microbiome of pigs with integrated approaches (e.g. various molecular and "omics" technologies as well as bioinformatics and statistical analyses) is still needed in order to comprehensively monitor the shifts in composition and functionality of the microbiota in response to dietary essential oils treatments.

2.7 EFFECT OF ESSENTIAL OILS ON FEED PALATABILITY AND DIGESTIBILITY AND NUTRIENT METABOLISM

Essential oils can increase feed palatability and intake with the enhanced flavor and odor (Kroismayr et al., 2006). However, the observed effect of supplemented essential oils to pig diets on feed intake is not consistent (Neill et al., 2006; Stelter et al., 2013; Zeng et al., 2015). It is believed that the increased feed palatability associated with the supplementation of essential oils could also be due to their antioxidative properties that can preserve the qualities of diets and prevent the release of unfavorable odors from the diets (Franz et al., 2010, Solà-Oriol et al., 2011). Therefore, it might be interesting to replace chemical antioxidants (e.g. ethoxyquin and butylated hydroxytoluene) commonly used in the animal diet with enough amounts of essential oils (natural antioxidants), particularly when chemical antioxidants are prohibited (Yang et al., 2015a).

The gastrointestinal tract is the largest and most vulnerable surface to the outside world in the body and it not only absorbs nutrients but also senses luminal nutrients, chemicals and microbes through a chemosensory system with many nerve and receptors (Furness et al., 2013). Recently, the gut chemosensory system has received a lot of attention due to the fact that the system can regulate digestion, absorption, and metabolism, with potential nutritional and pharmacological applications to improve gut growth, development and health (Mace and Marshall, 2013). Approximately 90% of cells in the gut epithelium are absorptive epithelial cells and the cells express various nutrient transporters (Henning et al., 1994). The gut epithelium has also a small amount of enteroendocrine cells that can secret gut hormone peptides, such as glucoinsulinotropic polypeptide, glucagon-like peptides 1 and 2 (GLP-1 and 2) and peptide YY (PYY) (Murphy et al., 2006). It has been reported that several taste receptors including the sweet taste receptor T1R1+T1R2, the umami taste receptor T1R1+T1R3, bitter taste-sensing type 2 receptors (T2Rs) and other taste receptors are not only expressed in taste

buds but also located in gut (Jeon et al., 2011; Daly et al., 2013; Shirazi-Beechey et al., 2014). Moreover, there are also other nutrient receptors including calcium-sensing receptor (CaSR) that is also called kokumi receptor and lipid receptors in the gut (Reimann et al., 2012; Huang et al., 2016). All these receptors are considered to belong to a group of G protein-coupled receptors (GPCR) (Reimann et al., 2012; Huang et al., 2016). The major function of nutrient transporters is to absorb luminal nutrients. However, it has been reported that nutrient transporters also contribute to the detection of nutrients in the lumen as transceptors (Hundal and Taylor, 2009). Therefore, amino acids, peptides, glucose, and lipids can be detected by these chemosensors. It can be assumed that other chemicals (e.g. essential oils) in feeds might also be detected by gut chemosensors that have not been identified. Studies indicate that fasting, refeeding and high protein diet can affect the expression of the taste signaling molecule α-transducin throughout the pig gastrointestinal tract, providing further support to the concept that taste receptors contribute to luminal chemosensing in the gut (Mazzoni et al., 2013; De Giorgio et al., 2016). The chemosensors transduce information regarding the nutrient profile and concentration of the lumen to regulate intestinal gene expression (e.g. transporters), digestive enzyme and gut peptide secretions, eventually to control feed intake, digestion, absorption, and metabolism. Several studies have been shown that phytogenic compounds can regulate the gene expression profile of ileal mucosa (Liu et al., 2013; Liu et al., 2014) and stimulate digestive secretions for improving nutrient digestibility (Janz et al., 2007; Maenner et al., 2011; Li et al., 2012b; Ahmed et al., 2013). However, the mechanisms of regulating gene expression relating to immune and digestive functions are still not fully clear. It is very important to identify specific receptors for essential oils, which will help us to understand the underlying mechanisms.

2.8 CONSIDERATIONS OF THE USE OF ESSENTIAL OILS IN SWINE NUTRITION

Viable alternatives to in-feed antibiotics should have several features: be safe to the public, cost-effective in swine production, and environmentally friendly (Gong et al., 2013). Due to these multiple requirements, so far there is no single alternative that has been identified to completely substitute antibiotics in feeds. It is also challenging to use comprehensive and systematic studies to evaluate the efficacy, cost-effectiveness, and safety of essential oils in swine production. Moreover, other challenges in using essential oils as antibiotic alternatives have some potential side effects (e.g. unpleasant odor/taste and toxic), regulatory concerns and possible interactions with other feed ingredients (e.g. fats) (Lambert et al., 2001; Friedman et al., 2002). The traceability of essential oils in feeds and pig tissues and feasible analytical methods are also important. A complete assessment (e.g. *in vitro* cell and *in vivo* animal models) on the toxicity and safety of essential oils is still needed before the compounds can be used extensively in pig feeds.

It is very important to fully understand the mechanism by which antibiotics can promote animal growth, which will help to develop effective alternatives to antibiotics in feeds. Several hypotheses on the potential mechanisms have been proposed: 1) reducing infection by the inhibition of pathogens; 2) making more energy and nutrients available for animals through reduction of total bacterial burden in the gut; 3) increasing nutrient absorption by thinning of the gut mucosal layer; and 4) reducing inflammation through modulation of the immune system (Niewold, 2007, Allen et al., 2013; Yang et al., 2015a). Although the above hypotheses are supported by some studies, the underlying mechanisms behind are still not fully understood. This has limited our effort to develop effective antibiotic alternatives including essential oils.

The gastrointestinal tract is a well-organized and complicated ecosystem and organ. It is mainly composed of epithelial cells, the mucosal immune system, and microbiome. The gut microbiota has commensal and beneficial bacteria as well as bacterial pathogens. The

ecosystem normally stays in homeostasis and the disruption of the homeostasis would affect gut functions and thus compromise gut health, animal growth, and well-being. Essential oils have multiple functions as a whole, including antimicrobial, anti-inflammatory, anti-oxidative and disruption of QS as well as enhancing digestion and immunity. It is very critical to define the specific effects and target sites (either animal host or its microbiota) of individual components in essential oils, which will facilitate the application of essential oils in feeds.

Essential oils have been investigated as alternatives to antibiotic in animal production. However, the results obtained from previous studies are highly inconsistent. There are two potential reasons associated with the inconsistency: (i) variable dosages that may not be efficacious (Cross et al., 2007) and (ii) different trial conditions (e.g. environment, animal age, genetics, feeds and health status). Moreover, most essential oils have the MIC values that are significantly higher than the levels that could be acceptable in swine production in terms of cost-effectiveness. The acceptance by the industry to use antibiotic alternatives to optimize the animal performance and health is also dependent on the cost of alternatives.

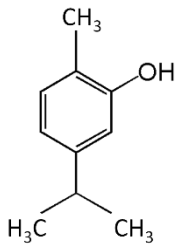
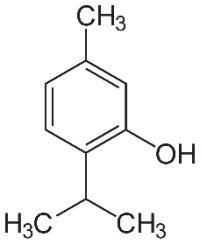
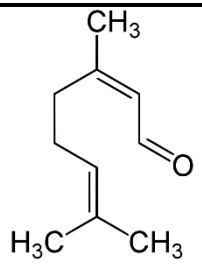
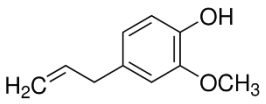
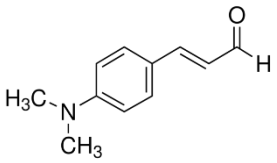
With their lipophilic characteristics, essential oil compounds may raise concerns about their potential toxicity and possible negative impact on animal health (Ambrosio et al., 2017). Most essential oils are also very volatile and can evaporate rapidly during feed processing and storage, resulting in the varied amount of essential oils that are in the feeds and delivered to animals (Lambert et al., 2001). It has been shown that most or all thymol, carvacrol, eugenol, and trans-cinnamaldehyde after oral ingestion were disappeared in the stomach and the upper small intestine in piglets (Michiels et al., 2008). Additionally, essential oils may interact with other components in feeds, leading to compromised antimicrobial activity (Si et al., 2006a) Therefore, if not properly protected, majority of essential oils will be lost during feed processing, storage and delivery to the animal gut and thus may fail to reach the lower intestine of animals where most pathogens are located. Also, it will affect the profitability of feed mills

and farmers eventually and become one of the major barriers for essential oil application in swine production.

A combination of different alternatives to antibiotic may hold the most promising method to substitute antibiotics in animal feeds. There are three major reasons: 1) one antibiotic alternative fail to cover all the performance-enhancing properties that antibiotics have; 2) there is a synergistic effect among different alternatives that will reduce effective dosages required to combat pathogens (e.g. organic acids and essential oils); and 3) an integrated approach should be taken to replace antibiotics, including nutrition (e.g. increasing feed intake after weaning by introducing a creep feed), biosecurity (e.g. prevention of farms against disease outbreak), and management (e.g. maintaining proper hygiene on farms) rather than supplementation of antibiotic alternatives alone (Yang et al., 2015a; Jayarawan and Nyachoti, 2017). Several recently published studies have shown that the combined use of different antibiotic alternatives had better effects on the performance and health of weaned pigs when compared with single compounds (Zeng et al., 2015; Walia et al., 2017). It is very important to understand the effects and mechanisms of action of various alternatives, which will help the design for more effective essential oils products to promote animal growth and improve feed efficiency in swine production. A method that can effectively and practically deliver essential oils is very important for the use of essential oils in swine production. Enteric protection (e.g. microencapsulation and coating) has become the most promising method to solve the problem (Gauthier, 2012). A widely used method is to microencapsulate essential oils in a lipid matrix that could release essential oils as it passes along the small intestine (Gauthier, 2012). Also, it has been reported that alginate-whey protein microparticles are a good carrier for enhancing the gut delivery of carvacrol in pigs (Zhang et al., 2016a). Therefore, obviously, proper protection technologies can reduce the required effective dosage of essential oils in feeds and reduce the cost of swine production. However, there are still some challenges to fully protect

and deliver essential oils into the lower gut, which lead to inconsistent results in the field. The potential reasons include: 1) A general drawback of microencapsulation due to the heating process involved in some methods of encapsulation that can lead to evaporation or dysfunction of sensitive compounds; 2) limited knowledge on the morphology and microstructure of lipid microparticles; and 3) no in-depth studies to elucidate the mechanisms underlying the phenomenon of stability or release of essential oils from the lipid microparticles. Thus, it is very important to investigate the physicochemical and molecular characterization of lipid microparticles, which will elucidate the mechanisms underlying the phenomenon of stability or release of essential oils and then optimize lipid microencapsulation to better protect and deliver essential oils. The advantages of biodegradable polymer-encapsulated essential oil nanoparticles have made nanotechnology an exciting essential oil delivery method in the animal gut (Aytac et al., 2017, Manukumar et al., 2017).

Table 2.1 Chemical properties of essential oils popularly used in pig feeds.

Compound	Carvacrol	Thymol	Citral	Eugenol	Cinnamaldehyde
Chemical structure					
Formula	$C_{10}H_{14}O$	$C_{10}H_{14}O$	$C_{10}H_{16}O$	$C_{10}H_{12}O_2$	C_9H_8O
Molecular mass (g/mol)	150.2	150.2	152.2	164.2	132.2
Density (kg/m^3)	976	969	893	1067	1050
Melting point (°C)	0-2	49-52	-10	-12 - -10	-7.5
Boiling point (°C)	234-238	232-233	229	253	246-251
Vapour pressure at 20°C (Pa)	35	250 (50°C) – 133 (64°C)	22	133 (78°C)	3.85

Solubility in water (g/L)	0.83-1.10	0.85-1.01 to 1.4 (40°C)	0.59	0.80-2.41	1.42-1.45
Solubility in ethanol (g/L)	Good	1000	Good	500 (in 70%), good	150 (in 60%), good
Octanol/water partition Coefficient (log K_{ow})	3.38-3.64	3.30	2.8-3.0	2.99	1.9
pKa value	10.4	10.4	-	-	-
Physical appearance at room temperature	Colorless to pale yellow liquid	White crystalline powder or large colorless crystals	Pale yellow liquid	Colorless or pale yellow, thin liquid	Clear yellowish liquid

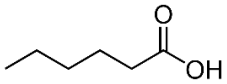
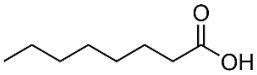
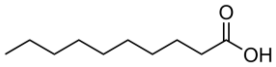
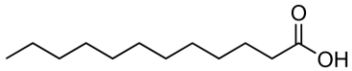
Source: Adapted from Michiels, 2009.

Table 2.2 Minimum inhibition concentration (MIC) of essential oils against various bacterial pathogens (Adapted from Yang et al., 2015a).

Product	Pathogenic microbe	Gram	MIC (unit)	MIC (#)	Reference
Thymol	*Lactococcus piscicum*	+	mg/L	320	Navarrete et al., 2010
	Streptococcus phocae	+	mg/L	640	Navarrete et al., 2010
	Flavobacteriaum psychrophilum	-	mg/L	320	Navarrete et al., 2010
	Vibrio anguillarum	-	mg/L	80	Navarrete et al., 2010
	V. parahaemolyticus	-	mg/L	320	Navarrete et al., 2010
	Pseudomonus sp.	-	mg/L	640	Navarrete et al., 2010
	Brachyspira hyodysenteriae	-	mM	1.25	Vande Maele et al., 2016
	Escherichia coli 0157:H7	-	ug/mL	166	Si et al., 2006a
	Salmonella Typhimurium DT104	-	ug/mL	233	Si et al., 2006a
	Escherichia coli k88	-	ug/mL	100	Si et al., 2006a
	Lactococcus lactis	+	mg/L	1280	Navarrete et al., 2010
Eugenol	*Vibrio sp.*	-	ug/mL	156	Seongwei et al., 2009
	Escherichia coli	-	ug/mL	625	Seongwei et al., 2009
	Salmonella	-	ug/mL	156	Seongwei et al., 2009
	Pseudomonas sp.	-	ug/mL	325	Seongwei et al., 2009
	Edwardsiella tarda	-	ug/mL	56–125	Seongwei et al., 2009
	Aeromonas hydrophilia	-	ug/mL	625	Seongwei et al., 2009
	Brachyspira hyodysenteriae	-	mM	2.5	Vande Maele et al., 2016
	Escherichia coli 0157:H7	-	ug/mL	466	Si et al., 2006a

	Salmonella Typhimurium DT104	-	ug/mL	400	Si et al., 2006a
	Escherichia coli k88	-	ug/mL	300	Si et al., 2006a
Carvacrol	*Listonella anguillarum*	-	ug/mL	25	Volpatti et al., 2013
	Brachyspira hyodysenteriae	-	mM	1.25	Vande Maele et al., 2016
	Escherichia coli 0157:H7	-	ug/mL	283	Si et al., 2006a
	Salmonella Typhimurium DT104	-	ug/mL	167	Si et al., 2006a
	Escherichia coli k88	-	ug/mL	100	Si et al., 2006a
Cinnamaldehyde	*Brachyspira hyodysenteriae*	-	mM	0.31	Vande Maele et al., 2016
	Escherichia coli 0157:H7	-	ug/mL	133	Si et al., 2006a
	Salmonella Typhimurium DT104	-	ug/mL	100	Si et al., 2006a
	Escherichia coli k88	-	ug/mL	133	Si et al., 2006a

Table 2.3 Molecular structure and physicochemical properties of medium chain fatty acids used in pig feed.

Name	Systemic name	Formula	Skeletal structure	Melting point (°C)	Boiling point (°C)	Density g/mL
Caproic acid	Hexamoic acid	$C_6H_{12}O_2$		-3.4	205.8	0.929
Caprylic acid	Octanoic acid	$C_8H_{16}O_2$		16.7	239	0.910
Capric acid	Decanoic acid	$C_{10}H_{20}O_2$		27-32	268-270	0.893
Lauric acid	Dodecanoic acid	$C_{12}H_{24}O_2$		43.8	225-297	1.007

CHAPTER 3 HYPOTHESES AND OBJECTIVES

3.1 HYPOTHESES

The following hypotheses were tested in this thesis:

1. Thymol can exert protective effects against lipopolysaccharide (LPS)-induced inflammation in porcine enterocytes;

2. With a proper microencapsulation, thymol and lauric acid can be effectively delivered to pig intestinal tract.

3.2 OBJECTIVES

The overall objective was to investigate the anti-inflammatory functions of thymol with *in vitro* porcine intestinal epithelial cells (IPEC-J2) cells and develop novel microparticles to deliver thymol and lauric acid to pig intestinal tract effectively. Specific objectives were to:

1. Determine the effects of thymol on barrier function, reactive oxygen species (ROS) and gene expression of nutrient transporters and cytokines in LPS-induced IPEC-J2 cells;

2. Develop novel microparticles to deliver thymol and lauric acid to pig intestinal tract effectively;

3. Validate and demonstrate slow release of thymol and lauric acid from novel microparticles with *in vitro* approaches;

4. Evaluate the stability of thymol and lauric acid in the developed microparticles during storage.

CHAPTER 4 MANUSCRIPT I THYMOL IMPROVES BARRIER FUNCTION AND ATTENUATES INFLAMMATORY RESPONSES IN PORCINE INTESTINAL EPITHELIAL CELLS DURING LIPOPOLYSACCHARIDE (LPS)-INDUCED INFLAMMATION

4.1 ABSTRACT

It is well known that thymol essential oil exhibits antibacterial activity. However, the protective effects of thymol on porcine intestine during inflammation is yet to be investigated. In the present study, an *in vitro* inflammation model using IPEC-J2 cells was established to evaluate the protective effects of thymol against lipopolysaccharide (LPS)-induced inflammatory responses in epithelial cells. Interleukin 8 (IL-8) secretion was measured using Enzyme-linked immunosorbent assay. The mRNA abundance of cytokines, nutrient transporters, and tight junction proteins was measured using real-time PCR. The results showed that LPS significantly increased IL-8 secretion, reactive oxygen species (ROS) production, tumor necrosis factor alpha (TNF-α) mRNA abundance in cells when compared with control cells without LPS treatment ($P < 0.05$). The ROS production induced by LPS was significantly blocked by 50 μM of thymol ($P < 0.05$). Thymol tended to decrease the production of LPS-induced IL-8 secretion ($P = 0.0766$). The mRNA abundance of IL-8 and TNF-α was significantly reduced in cells treated with thymol and LPS when compared with those treated with LPS only ($P < 0.05$). However, interleukin 6 (IL-6) mRNA abundance was not affected by LPS or thymol treatment ($P > 0.05$). The mRNA abundance of sodium-dependent glucose transporter 1 (SGLT1), excitatory amino acid transporter 1 (EAAC1) and system B^0 neutral amino acid transporter (B^0AT1) were significantly decreased by LPS treatment ($P < 0.05$), but the addition of thymol was unable to prevent these decreases ($P > 0.05$). The transepithelial electrical resistance (TEER) was reduced by LPS treatment for 1 h ($P < 0.05$), but this effect was prevented by thymol treatment ($P < 0.05$). Pre-treatment of IPEC-J2 cells with thymol

significantly decreased the leakage of fluorescein isothiocyanate (FITC)-dextran compared with the LPS group ($P < 0.05$). Moreover, thymol increased zonula occludens-1 (ZO-1) and actin staining in the cells. However, the mRNA abundance of ZO-1 and occludin-3 was not affected by either LPS or thymol treatment. These results suggest that thymol can enhance barrier function and reduce ROS production and pro-inflammatory cytokine gene expression in the epithelial cells during inflammation. The regulation of barrier function by thymol and LPS may be at post-transcriptional or post-translational levels.

Keywords: thymol, LPS-induced inflammation, barrier functions, nutrient transport, IPEC-J2 cells

4.2 INTRODUCTION

Intestinal epithelial cells (IECs) are continuously lined monolayer cells, which play important roles in the animal's physical defense. Normally, IECs function as the first line of defense against the invasion of pathogenic agents in the external environment of the gut lumen (Pitman et al., 2000). The maintenance of the barrier function of IECs contributes to the gastrointestinal (GI) homeostasis and GI tract (GIT) health of animals. Gut disorder and dysfunction might be harmful to the growth performance of livestock, and may induce gut diseases such as inflammatory bowel diseases (IBD) and diarrhea, possibly due to complex interactions among immunologic, genetic, microbial and environmental factors (Sartor et al., 2006). For instance, diarrhea is a common gut disease, which causes almost 5% mortality per year in weaned piglets (Sargeant et al., 2011). Therefore, it is necessary to prevent gut diseases by maintaining proper barrier function of IECs in animals.

In addition to the physical barrier, IECs also function as an extrinsic barrier. Under certain circumstances, IECs could secrete signaling molecules like mucins, cytokines, and chemokines to prevent the invasion of harmful microorganisms in the gut (Arce et al., 2010). Also, a series of immune responses within IECs and the interaction among IECs, leukocytes,

and adjacent cells is initiated by the invasion of pathogenic bacteria. When the toll-like receptors (TLRs) of IECs are activated by invading pathogen-associated molecular patterns (PAMs) like lipopolysaccharide (LPS), a signaling cascade in TLR-activated pathway leads to the activation of transcription factor nuclear factor kappa B (NF-κB) and the secretion of pro-inflammatory cytokines (Burkey et al., 2007), such as tumor necrosis factor α (TNF-α), interleukin 6 and 8 (IL-6 and IL-8). Therefore, it is necessary to improve gut health through suppressing unnecessary inflammatory responses.

Tight junctions (TJs) are one of the junctional multiprotein complexes which seal the paracellular space among adjacent epithelial cells. TJs lie at the apical side of the lateral membrane of epithelial cells and function as a barrier to separate the materials like solutes and water from the intercellular space (Tsukita et al., 2001). Tight junctions regulate ion transports, water, and solutes via the paracellular pathway and blockage of immunogenic macromolecules, and only controlled and selective movements are allowed during the passive permeability process (Pitman and Blumberg, 2000). Disruption of TJs would increase the paracellular permeability, which enables harmful substances like pathogens and endotoxins to translocate and resulting in tissue damage and inflammation eventually. TJs consist of over 30 structural or functional proteins (Tsukita et al., 2001). Occludin, zonula occluden-1 (ZO-1) and claudin-1 are three crucial proteins to maintain the physiological functions of tight junctions (Chiba et al., 2008). Previous studies demonstrated that the upregulation of ZO-1 and occludin could suppress the increase of intestinal permeability caused by disruption of tight junctions in weaned piglets (Zhang et al., 2009; Hu et al., 2013). Therefore, over-expression of occludin and ZO-1 is widely regarded as an effective target for the therapy of intestinal diseases.

Oxidative stress is also responsible for GI inflammation in animals. Generally, it results from the excessive accumulation of reactive oxygen species (ROS) and the imbalance between ROS and antioxidant agents. Oxidative stress could cause biomacromolecule damage, which

leads to inflammation and other diseases (Cai et al., 2013). Meanwhile, oxidative stress dysregulates the proliferation, differentiation, and apoptosis of IECs and is detrimental to the maintenance of physiological functions of intestinal epithelia in animals (Herring et al., 2007).

Many reports have elucidated that essential oils have anti-oxidative and anti-inflammatory properties (Baschieri et al., 2017) and they have been successfully applied to animal feeds (Alcicek et al., 2003; Brenes et al., 2010). Among them, thymol is a natural monoterpene phenolic compound, which exhibits antimicrobial (Liolios et al., 2009), anti-oxidative (Shabnum et al., 2011) and anti-inflammatory (Fachini-Queiroz et al., 2012) properties. It has been certified by the U.S. Food and Drug Administration (FDA), as having Generally Recognized as Safe (GRAS) status (Ye et al., 2016). Moreover, thymol has been identified as having therapeutic potential of fighting against pathogenic bacteria and boosting the immune system (Meeran, 2017). However, little is known about the protective effect of thymol on porcine intestinal epithelial cells during inflammation. Understanding the mechanisms underlying thymol effects is critical to its effective application in enhancing gut health and function in swine.

Thus, the objective of this study was to explore how thymol regulates the barrier function and inflammatory responses in porcine intestinal epithelial cells. We assumed that thymol may maintain barrier function by regulating inflammatory signaling pathway and the expression of crucial proteins of tight junctions, and eventually attenuating LPS-induced inflammatory responses in the IPEC-J2 cell line. The underlying molecular mechanism needed to be revealed further.

4.3 MATERIALS AND METHODS

4.3.1 Materials

Thymol (≥ 98.5%), Fluorescein Isothiocyanate-dextran (FITC-dextran, 4kDa) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

4.3.2 Cell Culture

The non-transformed neonatal jejunal epithelial cell line IPEC-J2 was grown in dulbecco modified eagles medium (DMEM–Ham's F-12 (1:1)) (Invitrogen, Fisher Scientific, Ottawa, ON, Canada) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Canadian Origin; Fisher Scientific, Ottawa, ON, Canada), penicillin (100 IU/mL), streptomycin (100 μg/mL) and 0.25 μg/mL of amphotericin B (Fisher Scientific, Ottawa, ON, Canada) and maintained in an atmosphere of 5% CO_2 at 37°C for cultures and assays. Culture medium was replaced every 2-3 days.

Inflammation was induced by LPS that was derived from *Salmonella Enterica* ser. *Typhimurium* (Sigma-Aldrich). IPEC-J2 cells cultured in a 12-well plate or Millicell membrane cell inserts (24 wells, Corning Costar) were first washed with plain medium and then were treated with 10 μg/mL of LPS (Arce et al., 2010) at different times. After treatments, cells cultured in a 12-well plate (Corning Costar) were used for RNA extraction and gene expression assay, and cells cultured in Millicell membrane cell inserts (Corning) were used for Transepithelial electrical resistant (TEER) and permeability assays.

For thymol treatment, 100 mM stock solution in dimethyl sulfoxide (DMSO) was freshly prepared and diluted in complete medium at an appropriate concentration (10 -100 μM). To eliminate the influence of DMSO, an equal concentration of DMSO was added to all the experimental groups. For viability assay, cells were treated with thymol at different concentration (10 -100 μM) for 24 h and for the rest of experiments, cells were pre-treated with thymol (50 μM) for 1 h and then continuously treated with the same concentration during LPS stimulation.

4.3.3 Cytokine Measurement by Enzyme-Linked Immunosorbent Assay (ELISA)

IL-8 and TNF-α concentration of culture supernatants were measured by ELISA kits (Invitrogen, Fisher Scientific), following the manufacturer's instruction. Briefly, 100 μL of

culture supernatant was used for both IL-8 and TNF-α assay. At the end of the reaction process, the plates were read at 450 nm using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek). Cytokine concentrations were calculated from a standard curve that had been created using seven 2-fold dilutions of porcine recombinant IL-8 and TNFα. The IL-8 level was expressed as pg/mL. TNF-α level was under the detection limit.

4.3.4 Reactive Oxygen Species (ROS) Assay

Cellular ROS was measured using a fluorescent dye, 2',7'dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich), which is a nonpolar compound that is readily diffusible into cells. It is hydrolyzed to the non-fluorescent polar derivative DCFH and thereby trapped within cells. In the presence of oxidants, DCFH is converted into the highly fluorescent 2',7'-dichlorofluorescein (DCF). Briefly, cells were cultured in the 96-well plate. After different treatments, cells were washed two times with PBS, and DCFDA at 10 μM in PBS was added to the wells. The cells were then incubated for 30 min and the fluorescence of DCF was detected by a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek) with a maximum excitation and emission spectra of 485 nm and 528 nm (Kobashigawa et al., 2014).

4.3.5 Cell Viability Assay

Cell viability was measured using the water-soluble tetrazolium salts (WST-1) Cell Proliferation Reagent (Sigma Aldrich) according to the manufacturer's instructions. Briefly, IPEC-J2 cells were seeded onto a 96-well plate (Corning Costar, New York City, NY) at density of 1×10^4 cells/mL and cultured in a medium for 2 weeks. After different treatments, the cells were washed one time with Phosphate-buffered saline (PBS), then 100 μL fresh culture medium containing 10% WTS-1 was added. The cells were then incubated for 1 h. The absorbance at 450 nm was measured using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). Cell viability was presented as a percentage of untreated control cells.

4.3.6 RNA Extraction and Real-time PCR

Total RNA was extracted from IPEC-J2 cells using Trizol reagents, (Invitrogen) following the manufacturer's protocol. RNA concentration, OD260/OD280, and OD260/OD230 were measured by Nanodrop-2000 spectrophotometer (Thermo Scientific, Ottawa, ON, Canada). The integrity of RNA was verified by visualization in an agarose gel. Two µg of total RNA was reverse transcribed into cDNA using the iScriptTM complementary deoxyribonucleic acid (cDNA) Synthesis kit (Bio-Rad, Mississauga, ON, Canada), following the manufacturer's instruction. Quantitative RT-PCR was performed using SYBR Green Supermix (Bio-Rad) on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). The Primers for real-time PCR analysis were designed with Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) based on the published mRNA sequence in the Genbank. All the primers spin at least two exons each. Sequences of primers are listed in Table1. The thermal profile for all reactions was 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. At the end of each cycle, the fluorescence was monitored for 10 s. Each reaction was completed with a melting curve analysis to ensure the specificity of the reaction. RT-PCR data were analyzed using the $2^{-\Delta\Delta}$CT method (Livak and Schmittgen, 2001) to calculate the relative fold change of target gene, using hypoxanthine phosphoribosyl transferase (HPRT) and Cyclophilin-A (CycA) as the reference genes (Farkas et al., 2015).

4.3.7 TEER Measurement

The TEER of cell monolayers was measured using a Millicell Electrical Resistance System (ESR-2) (Millipore-Sigma). IPEC-J2 cells were seeded onto Millicell membrane cell inserts (24 wells, Corning Costar) at a density of 7×10^4 cells/cm^2. The TEER was monitored every other day. When monolayer of cells was completely differentiated, cells were treated with LPS, and TEER was measured before and after treatments. The data was presented as a percentage of initial values before treatments.

4.3.8 Measurement of Cell Permeability

To quantify the paracellular permeability of cell monolayers, 1 mg/mL of 4 kDa FITC-dextran (Sigma-Aldrich) was added to the apical side of the inserts. The basolateral medium aliquots were taken after 6 h of incubation. The diffused fluorescent tracer was then measured by fluorometry (excitation, 485 nm; emission, 528 nm) using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek).

4.3.9 Immunofluorescent Staining

Cells were cultured onto coverslips (Fisher Scientific) and fixed with 4% paraformaldehyde (PFA) (Sigma). The cells were blocked with 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour and then incubated with an anti-rabbit ZO-1 polyclonal antibody (1:100 dilution, Thermal Scientific) at 4℃ overnight. The cells were then washed 3 times with PBS and incubated with an Alexa fluor 488 goat anti-rabbit antibody (Thermal Scientific, Cat# A-11034) for 1 h at the room temperature. Rinsed cells were mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc**.** Burlingame, CA, USA), For actin staining, fixed cells were washed 3 times with PBS, permeabilized with 0.5% Triton X-100 in PBS for 20 min and incubated with Phalloidin, CFTM488A (1:100 dilution in PBS, Biotium, Inc, Fremont, CA 94538, USA) at room temperature for 1 h. The cells were then washed 3 time with PBS and mounted with Vectashield Mounting Medium with DAPI (Vector Laboratories, Inc.). Images were taken by a Zesis Fluorescence Microscope (Car-Zesis Ltd, Toronto ON, Canada).

4.3.10 Statistical Analysis

Data were presented as means ± standard deviations. Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software, La Jolla, USA). Differences between the means were evaluated by one-way ANOVA. Multiple comparisons were done using Tukey's multiple comparisons test. Level of significance was set at $P < 0.05$.

Table 4.1 Primer sequences for gene expression of inflammatory cytokines, nutrient transporters, and tight junction proteins.

Gene symbol	Primer sequences	Product size (bp)	References
TNFα	5'-ATGGATGGGTGGATGAGAAA-3' 5'-TGGAAACTGTTGGGGAGAAG-3'	151	
IL-8	5'-CACCTGTCTGTCCACGTTGT-3' 5'-AGAGGTCTGCCTGGACCCCA-3'	126	
IL-6	5'-AAGGTGATGCCACCTCAGAC-3' 5'-TCTGCCAGTACCTCCTTGCT-3'	151	
SGLT1	5'-GGCTGGACGAAGTATGGTGT-3' 5'-GAGCTGGATGAGGTTCCAAA-3'	153	Yang et al., 2010
ASCT2	5'-GCCAGCAAGATTGTGGAGAT-3' 5'-GAGCTGGATGAGGTTCCAAA-3'	206	Yang et al., 2016b
EAAC1	5'-CCAAGGTCCAGGTTTTGGGT-3' 5'-GGGCAGCAACACCTGTAATC-3'	168	
B^0AT1	5'-AAGGCCCAGTACATGCTCAC-3' 5'-CATAAATGCCCCTCCACCGT-3'	102	Yang et al., 2016a
PepT1	5'-CATCGCCATACCCTTCTG-3' 5'-TTCCCATCCATCGTGACATT-3'	143	
ZO-1	5'-GATCCTGACCCGGTGTCTGA-3' 5'-TTGGTGGGTTTGGTGGGTT-3'	200	
CLDN3	5'-CTACGACCGCAAGGACTACG-3' 5'-TAGCATCTGGGTGGACTGGT-3'	123	
CycA	5'-GCGTCTCCTTCGAGCTGTT-3' 5'-CCATTATGGCGTGTGAAGTC-3'	160	Farkas et.al., 2015
HPRT	5'-GGACTTGAATCATGTTTGTG-3' 5'-CAGATGTTTCCAAACTCAAC-3'	91	Farkas et.al., 2015

Note: TNFα: Tumor necrosis factor α; IL-8: Interleukin 8; IL-6: Interleukin 6; SGLT1: Sodium/glucose cotransporter 1; ASCT2: glutamine transporter; EAAC1: excitatory amino acid transporter 1; B^0AT1: neutral amino acid transporter; PepT1: peptide transporter 1; ZO-1: Zonula occludens-1; CLDN3: Claudin 3; CycA: Cyclophilin-A; HPRT: hypoxanthine phosphoribosyl transferase.

4.4 RESULTS

4.4.1 Dose Effect of Thymol on the Viability of IPEC-J2 Cells

As shown in Figure 4.1, thymol concentrations less than 100 μM did not significantly affect cell viability ($P > 0.05$), but the viability of IPEC-J2 significantly decreased at the concentrations above 100 μM compared with control ($P < 0.05$). Therefore, 50 μM was set as the working concentration of thymol for further experiments.

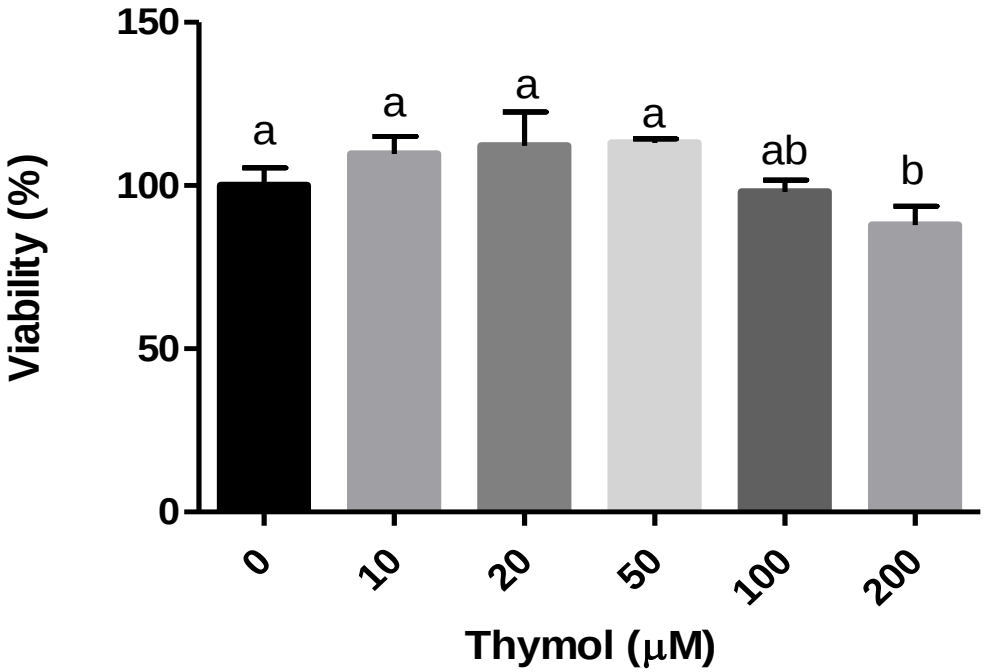


Figure 4.1 Dose-effect of thymol on the viability of IPEC-J2. IPEC-J2 cells were seeded onto 96-well plate and cultured for 10 d. Cells were then treated with thymol at the indicated concentration for 24 h. Cell viability was measured using WTS-1 as described in the Materials and Methods and expressed as a percentage of control. The data were presented as mean ± SD, n =5.

4.4.2 Effect of Thymol on IL-8 Secretion, ROS Production, and Cell Viability in IPEC-J2 Cells

As shown in Figure 4.2A and 4.2B, the levels of IL-8 and ROS in the LPS treatment were significantly higher than that in the control ($P < 0.05$). Although thymol pre-treatment significantly inhibited LPS-induced production of ROS ($P < 0.05$), thymol just tended to decrease IL-8 secretion ($P = 0.0766$). The effect of LPS and thymol treatment on cell viability was shown in Figure 2C. LPS and thymol treatment had no significant effect on the cell viability ($P > 0.05$).

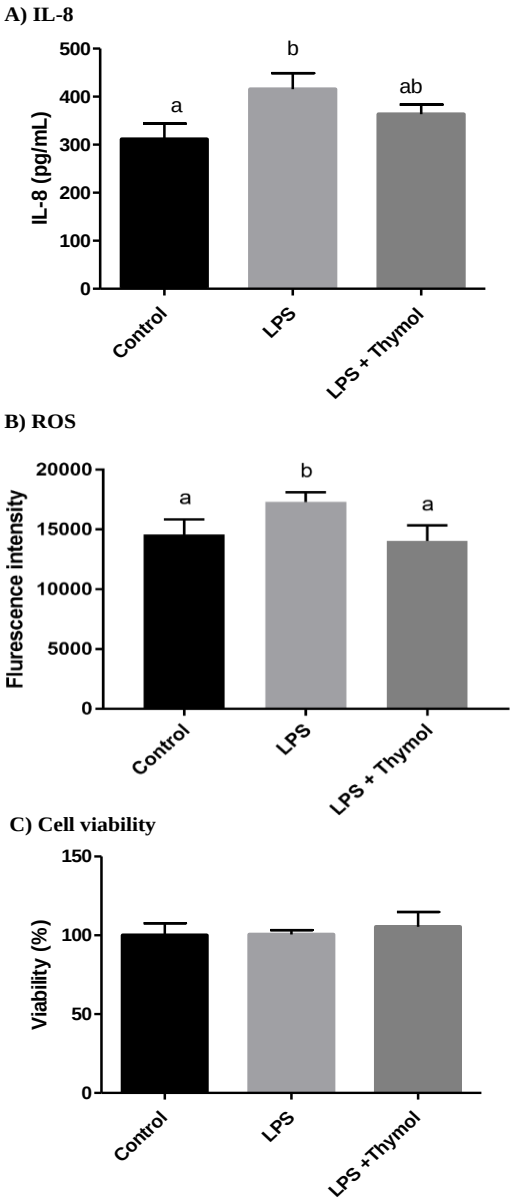


Figure 4.1 Effect of thymol on LPS-induced IL-8 secretion, ROS production, and cell viability. IPEC-J2 cells were seeded onto 12-well plates (for IL-8) or 96-well plate (for ROS production and viability) and cultured for 10 d. Cells were pre-treated with thymol (50 μM) for 1 h and then stimulated with LPS (10 μg/mL) for 1h (for ROS production and viability) and 4 h (for IL-8). IL-8 in medium (A), intracellular ROS production (B) and viability (C) were measured as described in the Materials and Methods The data were presented as mean ± SD, n = 4, the different letters represents a significant difference ($P < 0.05$).

4.4.3 Effect of Thymol on Cytokine Gene Expression in IPEC-J2 Cells

As shown in Figure 4.3A, there was no significant effect on the relative gene expression of IL-8 in the LPS treatment compared with the control ($P > 0.05$), whereas thymol treatment before LPS stimulation significantly down-regulated the gene expression of IL-8 compared with LPS treatment group ($P < 0.05$). The relative gene expression of TNF-α was significantly up-regulated in cells stimulated with LPS compared with the control ($P < 0.05$), but thymol showed a significant suppressive effect on gene expression of TNF-α in cells treated with thymol + LPS ($P < 0.05$) (Figure 4.3B). The relative gene expression of IL-6 did not respond to LPS stimulation in IPEC-J2 cells compared with the control ($P > 0.05$), and also LPS + Thymol treatment had no effect ($P > 0.05$) (Figure 4.3C).

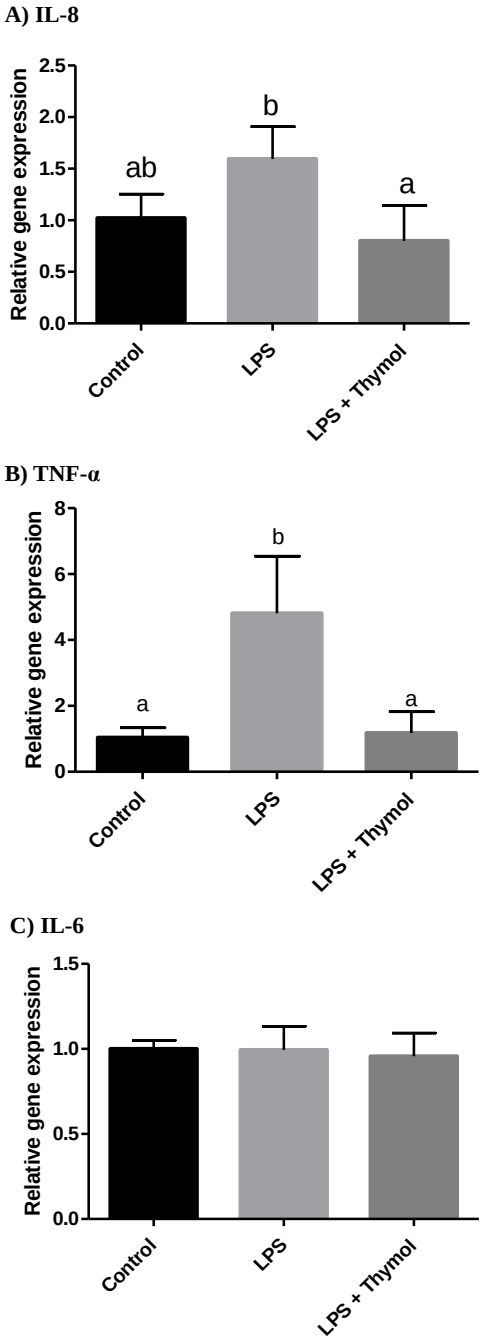


Figure 4.3 Effect of thymol on LPS-induced cytokine gene expression. IPEC-J2 cells were seeded onto 12-well plate and cultured for 10 d. Cells were pre-treated with thymol (50 μM) for 1 h and then stimulated with LPS (10 μg/mL) for 4 h. Total RNA was extracted from cells and the mRNA abundance of IL-8 (A), TNF-α (B) and IL-6 (C) were detected by RT-PCR. The data were presented as mean ± SD, n = 4, the different letters represents a significant difference ($P < 0.05$).

4.4.4 Effect of Thymol on Transporter Gene Expression in IPEC-J2 Cells

Figure 4.4A and 4.4C showed that LPS significantly downregulated the expression of intestinal Na^+/glucose cotransporter 1 (SGLT-1) ($P < 0.05$) and excitatory amino-acid carrier 1 (EAAC-1) ($P < 0.05$) compared with the control. However, thymol pre-treatment of cells did not significantly ($P > 0.05$) upregulate the expression of these two transporter genes compared with only LPS-stimulated cells. LPS did not show a significant effect on ASC amino-acid transporter 2 (ASCT2) transporter gene expression ($P > 0.05$) (Figure 4.4B). Figure 4.4D showed that LPS downregulated the expression of H^+/peptide cotransporter 1 (PepT1) significantly compared with the control ($P < 0.05$), but pre-treatment of cells with thymol did not significantly upregulate the expression of this gene ($P > 0.05$). As shown in Figure 4.4E, LPS significantly upregulated the gene expression of B^0-type amino acid transporter 1 (B^0AT1) in IPEC-J2 cells compared with the control ($P < 0.05$), but the pre-treatment of cells with thymol did not show a significantly positive effect on LPS-induced B^0AT1 transporter gene expression ($P > 0.05$).

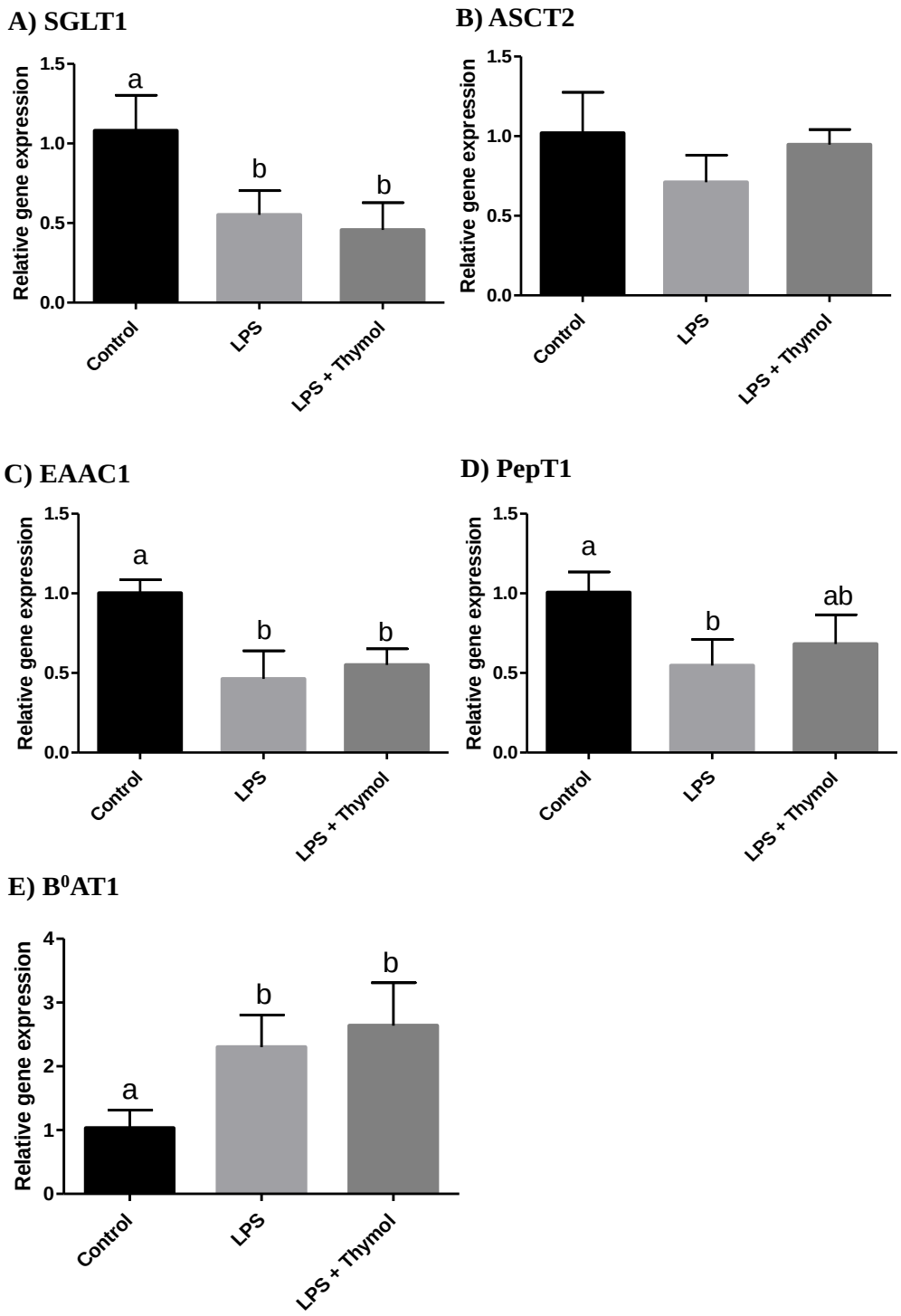


Figure 4.4 Effect of thymol on LPS-induced transporter gene expression. IPEC-J2 cells were cultured and treated using the same condition as Fig. 4.3. Total RNA was extracted from cells and the mRNA abundance of SGLT1 (A), ASCT2 (B), EAAC1 (C), PepT1 (D) and B^0AT1 (E) was detected by RT-PCR. Data were presented as mean ± SD, n = 4, the different letters represents a significant difference ($P < 0.05$).

4.4.5 Effect of Thymol on Relative Gene Expression of Tight Junction Proteins in IPEC-J2 Cells

There were no significant differences in the gene expression of ZO-1 and CLDN3 in both LPS and LPS + thymol treatment groups compared with the control (P > 0.05) (Figure 5).

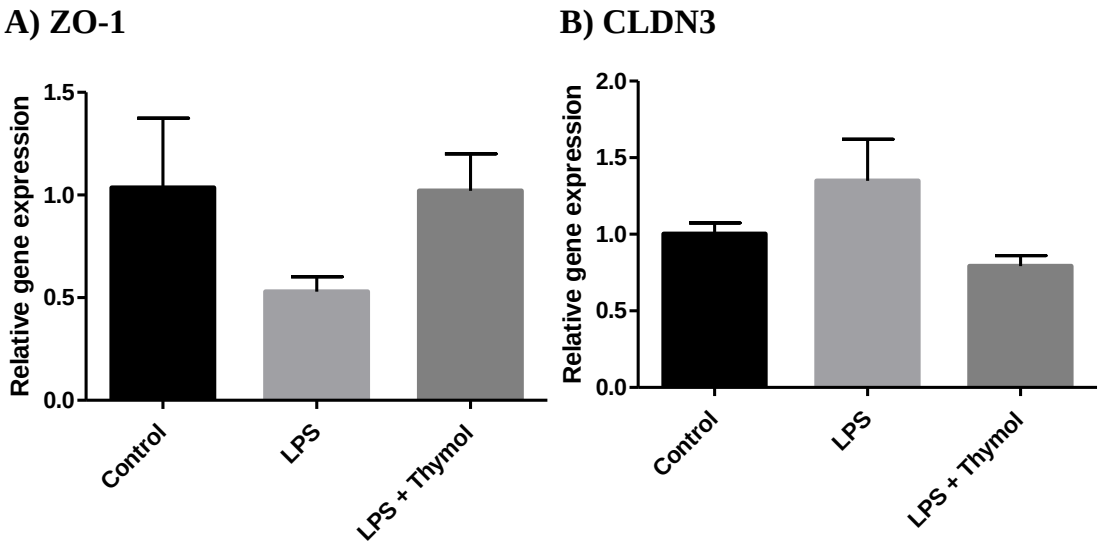


Figure 4.5 Effect of thymol on LPS-induced tight junction protein gene expression. IPEC-J2 cells were cultured and treated using the same condition as Fig. 4.3. Total RNA was extracted from cells and mRNA abundance of ZO-1 (A) and claudin3 (CLDN3) was detected by RT-PCR. Data were presented as mean ± SD, n = 4, the different letters represents a significant difference ($P < 0.05$).

4.4.6 Effect of Thymol on TEER and Permeability of IPEC-J2 Cells

As shown in Figure 4.6A, the percentage of relative TEER value was the lowest for 1 h LPS stimulation at 0 h, 2h and 3h ($P < 0.05$), whereas after 1 h LPS stimulation, there was no significant difference ($P > 0.05$) at 2h and 3 h. This indicated that TEER adapted to LPS stimulation after 1 h, and thus 1 h LPS stimulation was chose to test the effect of thymol on TEER values in LPS-induced IPEC-J2 cells. As shown in Figure 4.6B, the percentage of relative TEER value of cells stimulated with LPS for 1 h was significantly lower compared with the control ($P < 0.05$), whereas pre-treatment of cells with thymol restored the value to the same level as the control ($P > 0.05$). The protective effect of thymol on LPS-induced damage in IPEC-J2 was also apparent in the FITC-dextran flux experiment (Fig. 4.6C). Pre-treatment with thymol significantly decreased the leakage of FITC-dextran caused by LPS stimulation compared with the LPS treatment group ($P < 0.05$), and the measurements were similar to the control ($P > 0.05$).

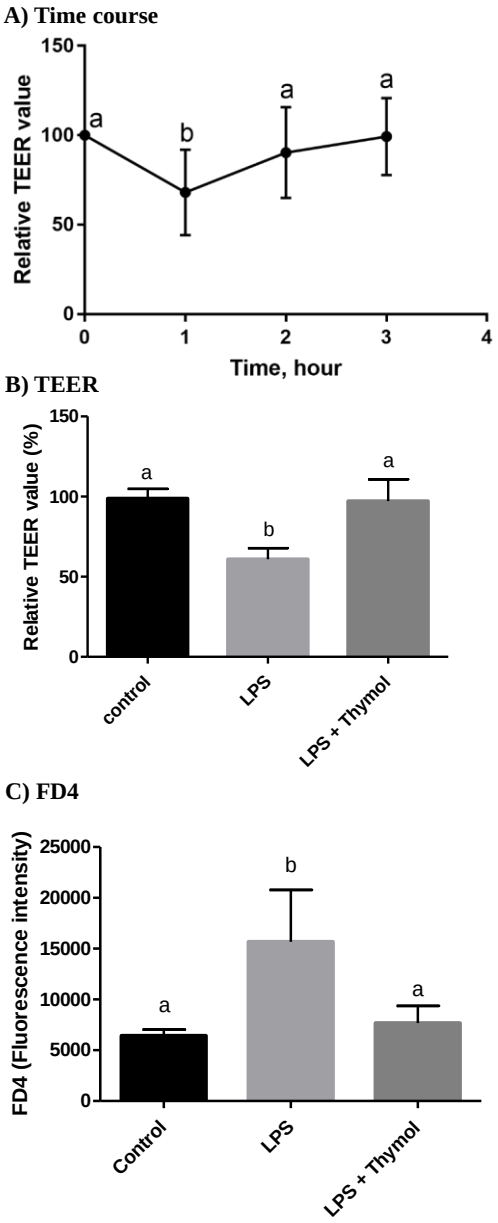


Figure 4.6 Effect of thymol on LPS-induced trans-epithelial electrical resistance (TEER) and permeability. IPEC-J2 cells were seeded onto millicell membrane cell inserts (24-well) with a density of 1×10^5/well and cultured for 2 weeks. TEER was monitored every other day. LPS (10 μg/mL) induced time course change of TEER was measured (A). To test the effect of thymol (50 μM) on LPS-induced TEER, cells were pre-treated with thymol (50 μM) for 2h and then stimulated with LPS for 1h. TEER was measured before and after LPS-stimulation (B). Permeability was tested by FD4 (C). Data were presented as a percentage of initial TEER value and given as means ± SD, n = 4. Bars with different letters are significantly different ($P < 0.05$).

4.4.7 Effect of Thymol on LPS-Induced Morphological Changes in Tight Junction and Actin Fiber in IPEC-J2 Cells

As shown in Figure 7, the distribution of ZO-1 protein within the tight junction was broken after LPS stimulation compared with the control, whereas pre-treatment of cells with thymol can promote the even redistribution of ZO-1 protein within the tight junction. The cytoskeletal structure of the actin fiber in IPEC-J2 cells was disorganized due to LPS stimulation compared with the control, whereas the pre-treatment of cells with thymol can alleviate the severity.

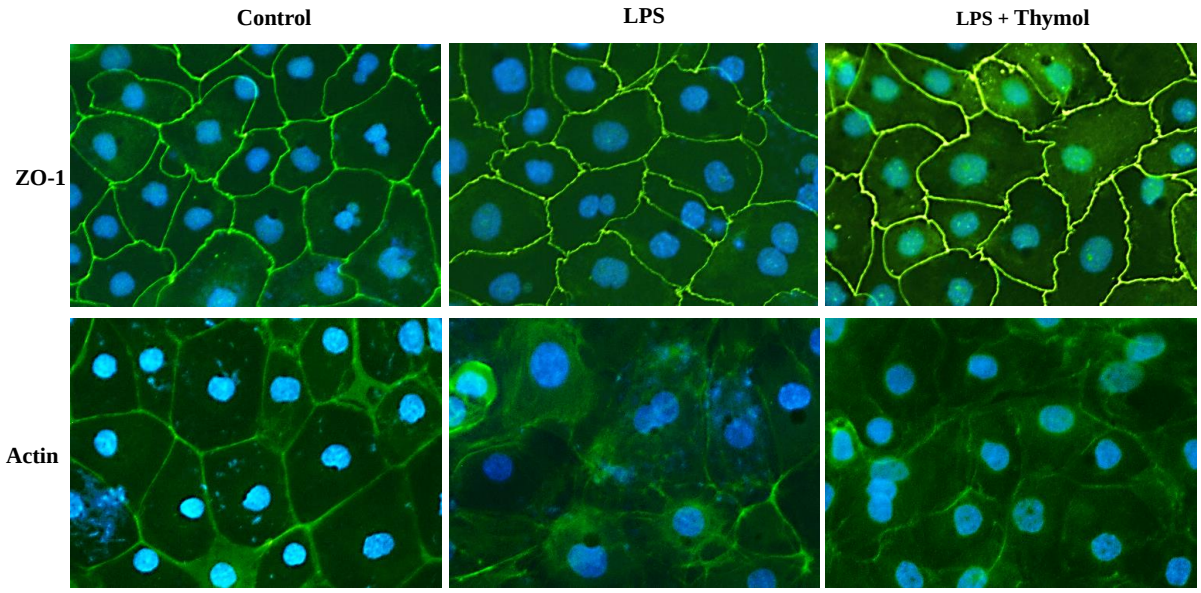


Figure 4.7 Effect of thymol on LPS-induced morphological changes of tight junction and actin fiber. IPEC-J2 cells were seeded onto coverslips at a density of 1×10^5/well and cultured for 2 weeks. Cells were pre-treated with thymol (50 μM) for 2h and then stimulated with LPS for 1h. Cells were then fixed for ZO-1 and actin staining as described in the Materials and Methods.

4.5 DISCUSSION

Gut inflammation can be caused by infection, diet allergy and weaning in weaned piglets (Yang et al., 2015a; Omonijo et al., 2018). Although gut inflammation may not cause the full-blown clinical symptoms in weaned piglets, it could lead to increased mucosal permeability and reduced growth performance. Wondrak et al. (Wondrak et al., 2010) demonstrated that cinnamaldehyde reduced or inhibited the activation of NF-κB through increasing the expression or translocation of nuclear factor E2-related factor 2 (Nrf2). Wang et al.[25] also indicated that the supplementation of cinnamon oil in the diet alleviated LPS-induced injury by suppressing inflammatory responses. A previous study reported that cinnamaldehyde and eugenol could suppress nitric oxide (NO) release and inducible NO synthase expression in LPS-treated murine macrophages (Wang et al., 2015). Recently, a study indicated that cinnamaldehyde improves intestinal mucosal barrier function and amino acid transporter gene expression in intestinal porcine epithelial cells (IPEC-1) isolated from neonatal pigs (Sun et al., 2017). The dietary supplementation of thymol can prevent the intestinal injury by improving the intestinal integrity and modulating immune responses in the clostridium perfringens–challenged broiler chickens (Du et al., 2016). However, the potential anti-inflammatory effects of thymol on porcine intestinal cells remain to be elucidated. Therefore, an *in vitro* inflammation model using IPEC-J2 cells was established in the present study to evaluate the protective effects of thymol on LPS-induced inflammatory responses in epithelial cells.

Our results showed that LPS significantly increased IL-8 secretion (Figure 4.2A) and TNF-α mRNA abundance (Figure 4.3B) in cells when compared with control cells without LPS treatment, indicating inflammation was successfully induced by LPS. The thymol treatment was able to attenuate the inflammatory responses induced by LPS evidenced by reduced IL-8 and TNF-α mRNA abundance (Figure 4.3A and 4.3B) as well as the tendency to decrease the production of LPS-induced IL-8 secretion (Figure 4.2A). Interestingly, IL-6 mRNA abundance

(Figure 3C) was not affected by LPS or thymol. The possible explanation was that different LPS sources and different treatment time might cause different inflammatory responses in the IPEC-J2 cells. It has been reported that IPEC-J2 cells treated with 1 μg/mL LPS (*E. coli* 055:B5) with different treatment time showed a varied effect on IL-6 secretion (Yang et al., 2015). Previous studies indicated that oxidative stress affected IL-8 and TNF-α expression, but IL-6 expression changed insignificantly in IPEC-J2 cells (Paszti-Gere et al., 2012). Farkas et al. (Farkas et al., 2015) found that modified apigenin did not influence IL-6 expression as apigenin did in IPEC-J2 cells during LPS-induced inflammation. Moreover, TNF-α protein was not detectable in the present study. The reason might be that IPEC-J2 cells as a porcine intestinal epithelial cell line, would not respond to LPS as sensitively as macrophages to produce a number of pro-inflammatory cytokines like TNF-α and IL-6. Many reports have indicated that the signaling pathways like NF-κB and mitogen-activated protein kinase (MAPK) are involved in the transcriptional regulation of cytokine genes. Thymol could inhibit LPS-induced inflammation by suppressing NF-κB and MAPK signaling pathways (Wang et al., 2018; Liang et al., 2014), and activating Nrf2 pathways (Yao et al., 2018). The underlying mechanismS might be that the anti-inflammatory effects of thymol are related to the regulation of these signaling pathways. Therefore, supplementation with thymol might be a potential approach to reduce inflammatory responses in weaned piglets.

Generally, during LPS-induced inflammation, phagocytes might be stimulated to generate excessive ROS and thereby leading to the imbalance of ROS and antioxidants (Li et al., 2007). The imbalanced redox state could induce the oxidative stress, which causes the dysfunction of porcine IECs by damaging the structure of biomacromolecules like DNA, proteins, and lipids (Cai et al., 2013). However, the ROS production induced by LPS was significantly blocked by thymol (Figure 4.2B). The results were consistent with the recent study indicating that thymol attenuated the ROS production and showed myeloperoxidase inhibitory activity in isolated

human neutrophils (Perez-Roses et al., 2007). Thymol as a phenolic compound is an effective antioxidant, which could scavenge the ROS and free radicals to maintain the normal cellular functions and signaling pathways. The decrease of ROS intensity might be a characteristic that the barrier function of IPEC-J2 cells was enhanced. Meanwhile, the dose of thymol addition did not show any harmful effects on cell viability (Figure 4.2C).

According to the physiological function, the small intestine is the major organ for the digestion and absorption of dietary carbohydrates and proteins. Hence, nutrient transporters SGLT1, ASCT2, EAAC1, PepT1, and B^0AT1 play important roles in the absorption of carbohydrates, amino acids, and proteins (in the form of glucose, amino acids, di- and tripeptides) (Pan et al., 2004). Previous studies demonstrated that the dietary supplementation of essential oils could enhance carbohydrate and protein absorption by improving the gene expression of nutrient transporters in animals (Colombo et al., 2014; Fernandez-Alarcon et al., 2017; Reyer et al., 2017). In the present study, LPS reduced the mRNA abundance of these nutrient transporters except ASCT2 and B^0AT1 (Figure 4.4), which demonstrated that LPS-induced inflammation damaged the structure and barrier functions of porcine intestinal epithelia and the major nutrient transport through a transcellular pathway. Meanwhile, oxidative stress might cause mitochondria injury and energy metabolisms disorder, but because these transporters are energy-dependent, it is possible that this might poses a threat to the process of energy-dependent active transport. However, they were not totally restored by the addition of thymol on the level of transporter mRNA abundance. It does not mean that thymol did not affect the transport activities. Many post-translational modifications (PTMs) could regulate the activity of transporters, such as glycosylation, phosphorylation, trafficking and ubiquitination (Pochini et al., 2014). We assumed that thymol might mainly regulate the activities of these transporters at the PTMs level, but further research is needed to confirm this assumption. It is reasonable that the mRNA abundance might not thoroughly reflect the activity

of these transporters. Furthermore, many reports have indicated that nutrient transporters function depends on ions like Na^+ and H^+ (Anderle et al., 2004). We inferred that thymol might not be able to completely rescue the disruption of ions homeostasis caused by LPS stimulation, which explains the insignificant changes in the expression of transporters at the transcription level under the thymol treatment.

TEER reflects variations in the ion permeability both through transcellular and paracellular pathways, but it is mainly thought to depend on the status of tight junctions (Madara et al., 1988). Thus, TEER is considered as a good indicator of the integrity and tightness of intestinal epithelial barrier model (Tsukita et al., 2001). Similarly, the FITC-dextran flux reflects the permeability of cells. The TEER was reduced by LPS stimulation for 1 h. After LPS stimulation, the structure of IECs, as well as tight junctions were disrupted (Figure 4.7). Thus, the relative TEER value significantly decreased and FD-4 significantly increased (Figure 4.6A and 4.6B). However, these effects were prevented by thymol, and the TEER value was normalized by using thymol as a pre-treatment. Also, pre-treatment of the monolayers with thymol significantly decreased the leakage of FITC-dextran and increased ZO-1 and actin staining in the cells (Figure 7). These results indicated that thymol can enhance barrier function in the IPEC-J2 cells, which were consistent with recent studies with cinnamaldehyde in porcine epithelial cells (Sun et al., 2017) and with oregano essential oil in pigs (Zou et al., 2016). Therefore, supplementation with thymol might be a potential nutritional approach to improve intestinal barrier functions in weaned piglets.

Although thymol improved the protein level of ZO-1 in the IPEC-J2 cells, the mRNA abundance of ZO-1 was not affected by either LPS or thymol (Figure 4.5A). Similarly, as a crucial protein of tight junctions, the mRNA abundance of CLND-3 was not significantly upregulated (Figure 4.5B). The results were consistent with the findings that a carvacrol-thymol blend decreased intestinal oxidative stress and influenced selected microbes without

changing the mRNA levels of tight junction proteins in the jejunal mucosa of weaning piglets (Wei et al., 2017). This discrepancy between ZO-1 mRNA and ZO-1 protein levels suggested that thymol may regulate ZO-1 expression by the post-transcriptional or post-translational mechanisms in the present study, although tight junction proteins are regulated at both the transcriptional and post-transcriptional levels (Robinson et al., 2015). However, the molecular basis for thymol actions on the barrier function requires further investigations, especially thymol's molecular target (e.g. receptor or protein kinase). For instance, thymol might be a transient receptor potential cation channel subfamily M member 8 channel (TRPM8) agonist to inhibit downstream inflammatory responses, and it has been reported for eucalyptol, a plant essential oil (Caceres et al., 2017).

4.6 CONCLUSION

Thymol is derived from cymene and recognized as a bioactive compound existing essential oils, which can effectively enhance gut barrier structure and function under inflammatory status by reducing ROS production and pro-inflammatory cytokine gene expression in the IPEC-J2 cells. Supplementation with thymol might be a potential nutritional or therapeutic strategy to improve the intestinal mucosal barrier function and reduce inflammatory responses in weaned piglets. *In vivo* studies with experimentally infected pigs are still needed to validate this beneficial effect of thymol on intestinal barrier functions in the future.

CHAPTER 5 MANUSCRIPT II DEVELOPMENT OF NOVEL MICROPARTICLES FOR EFFECTIVE DELIVERY OF THYMOL AND LAURIC ACID TO PIG INTESTINAL TRACT [2]

5.1 ABSTRACT

Antibiotics have been widely supplemented in feeds at sub-therapeutic concentrations to prevent post-weaning diarrhea and increase the overall productivity of pigs. However, the emergence of antimicrobial-resistant bacteria worldwide has made it urgent to minimize the use of in-feed antibiotics. The development of promising alternatives to in-feed antibiotics is crucial for maintaining the suitability of swine production. Both medium-chain fatty acids (MCFA) and essential oils exhibit great potential to post-weaning diarrhea; however; their direct inclusion has compromised efficacy because of several factors including low stability, poor palatability and low availability in the lower gut. Therefore, the objective of this study was to develop a formulation of microparticles to deliver a model of essential oil (thymol) and MCFA (lauric acid). The composite microparticles were produced by the incorporation of starch and alginate through a melt-granulation process. The release of thymol and lauric acid from the microparticles was *in vitro* determined using simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), consecutively. The microparticles prepared with 2% alginate solution displayed a slow release of thymol and lauric acid in the SSF (21.2 ± 2.3%; 36 ± 1.1%), SGF (73.7 ± 6.9%; 54.8 ± 1.7%) and SIF (99.1 ± 1.2%; 99.1 ± 0.6%), respectively, whereas, the microparticles without alginate showed a rapid release of thymol and lauric acid from the SSF (79.9 ± 11.8%; 84.9 ± 9.4%), SGF (92.5 ± 3.5%; 75.8 ±

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5.9%) and SIF (93.3 ± 9.4%; 93.3 ± 4.6%), respectively. The thymol and lauric acid in the developed microparticles with or without alginate both exhibited excellent stabilities (> 90%) during storage at 4˚C for 12 weeks and 23˚C for 2 weeks. These results evidenced that the approach developed in the present study could be potentially employed to deliver thymol and lauric acid to the lower gut of pigs, although, further *in vivo* investigations are necessary to validate the efficacy of the microparticles.

Keywords: Encapsulation; Gut, Lauric acid, Microparticles, Slow release, Thymol

5.2 INTRODUCTION

Young animals are very vulnerable to diseases, and using antimicrobials is the most cost-effective method to improve the health and productivity of food production animals raised with conventional agricultural techniques (Looft et al., 2012; Yang et al., 2015a). Although this practice has been banned in Europe and other countries have also started to minimize the use of antibiotics in the animal production, it still exists in major parts of the world (Hassan et al., 2018). Therefore, replacing antibiotics with cost-effective alternatives remains crucial to ensure a sustainable food animal production.

Essential oils are considered as valid candidates to replace antibiotics in the feed industry (Li et al., 2012a; Gong et al., 2014; Nikolic et al., 2014; Omonijo et al., 2018). Essential oils (e.g., thymol) are extracted from plants and can promote growth performance and health in animals because of their biological activities and antimicrobial activities (Si et al., 2006b; Edris, 2007; Del Nobile et al., 2008; Brenes et al., 2010; Puvaca et al., 2013; Rassu et al, 2014). With the identification and characterization of bioactive components in plant extracts and significant progress in mechanistic research with these components in food production animals, many research efforts have been made to use essential oils substituting antibiotics within the animal production chain (Omonijo et al., 2018). The rationales for using essential oils in animal feeds have relied on their abilities to inhibit bacterial growth, reduce

virulence through quorum-sensing disruption, and regulate innate immunity of animals (Hassan et al., 2018). Therefore, it is vital to ensure the delivery of essential oils to the target site for increasing their efficacy.

Essential oils have very high volatility, and their bioactive compounds are readily degradable when exposed to heat, oxygen, light, or during their interactions with other compounds, thus, negatively affecting their biological activities and antimicrobial activities (Si et al., 2006a; Zhang et al., 2016a; Gonçalves et al., 2017). Additionally, several studies have demonstrated that several essential oils including thymol and carvacrol were almost completely vanished in the upper digestive tract of pigs (Michiels et al., 2008; Zhang et al., 2016a). Therefore, unprotected essential oils can be significantly vanished at the manufacture, transportation, and storage of feeds and as well as during delivery to the pig gut, thus hindering access to the distal part of pig intestine (Omonijo et al., 2018). This serves as a major challenge to the use of essential oil in pig feeds. Thus, it is crucial to establish a useful and practical delivery approach for using essential oils in feeds.

Medium-chain fatty acids (MCFA) including lauric acid (C_{12}) and its ester derivatives also have potential to substitute antibiotics in weaning piglets (Han et al., 2011; Zentek et al., 2012; 2013; Hanczakowska et al., 2013; De Smet et al., 2016; Wang and Johnson, 1992). Several studies indicated that MCFA could inhibit Salmonella growth (Van Immerseel et al., 2004; Messens et al., 2010). Synergistic antimicrobial activities between oregano oil and caprylic acid were observed with several strains including Salmonella (Hulánková and Bořilová, 2011). Similarly, Vande Maele et al. (2016) demonstrated in an *in vitro* study that a combination of lauric acid and cinnamaldehyde had synergistic effects in inhibiting the growth of *Brachyspira hyodysenteriae* that causes swine dysentery. The use of MCFA is popular both in the food and feed industries. However, some MCFA and their ester derivatives can compromise feed palatability and acceptance and reduce feed intake in pigs due to their

unpleasant odors (Omonijo et al., 2018). Thus, it is also essential to develop a useful and practical delivery approach for using MCFA in feeds.

Microencapsulation has been becoming one of the most popular and practical approaches to mask the unpleasant taste/odor, and deliver bioactive compounds in food production animals (Piva et al., 2007; Chitprasert et al., 2014). Ideal microencapsulation should not only stabilize essential oils but also release them specifically in the targeted regions of the intestine (Chen et al., 2017; Omonijo et al., 2018). Therefore, the objective of the present study was to develop a formulation of microparticles containing both thymol and MCFA for effective delivery to pig intestinal tract.

5.3 MATERIALS AND METHODS

5.3.1 Materials

Thymol (≥ 98.5%), lauric acid (LA**,** C_{12}**)**, palmitic acid (PA, C_{16}), stearic acid (SA, C_{18}), amylase, sodium alginate (low viscosity), pepsin originated from porcine and pancreatin originated from porcine were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Cornstarch was purchased from Cargill (Cargill Inc., Minneapolis, MN, USA) and pre-gelatinized starch (1500) from Coloran (West Point, PA, USA).

5.3.2 Selection of a Suitable Fatty Acid

Three fatty acids including lauric acid, palmitic acid, and stearic acid were used in this experiment because those have melting point above a melting point (42˚C) of thymol and have been used to deliver bioactive compounds (Ma et al., 2016; Pitigraisorn et al., 2017). The melting points of lauric acid, palmitic acid, and stearic acid are 43˚C, 63˚C, and 69˚C, respectively. Ten grams of each fatty acid was mixed with 10 g of thymol, respectively. The mixtures were then melted in a water bath at 70˚C. After melting, the mixtures were stirred for 30 min. The molten mixture of each fatty acid with thymol was left to stay at 55˚C without stirring for 2 h before placing at room temperature (23˚C) up to 6 h to allow for solidification.

To observe the crystal morphology of thymol, lauric acid, and their mixture, an emulsion of thymol, lauric acid and the mixture of thymol and lauric acid (ratio 1:1) were prepared. Lauric acid and thymol were melted at 70˚C individually or mixed at a ratio of 1:1, and then added into the water at 10% with 1% tween 80 as a surfactant. The mixture was mixed using a Polytron (PT10-35GT, Kinematica AG, Switzerland) for 2 min at 13,000 rpm to make an emulsion. Then, three emulsions were stored at 4˚C overnight allowing the emulsions to crystallize. The crystal morphology was examined under a microscope (Eclipse Ci, Nikon, Japan).

5.3.3 The Melting Point of Thymol, Lauric acid, and Their Mixture

Among the three fatty acids tested, lauric acid was selected for further study because its mixture with thymol remained a homogeneous liquid at room temperature for 6 h. Before use, 1 g of thymol and lauric acid each were kept at -80˚C for 30 min and then mixed by vortexing for 30 sec at 3,000 rpm. The mixture was kept in -80˚C for 3 h and then ground to a fine powder using a grinder. The grinder was kept in -20˚C for 3 h before use to avoid increasing temperature to higher than the melting temperature of thymol and lauric acid. The melting temperature of thymol, lauric acid, and their mixture (50: 50 wt%) was measured by differential scanning calorimetry (DSC). For the measurement, 12.1 mg thymol, 13.1 mg lauric acid, and 10.7 mg mixture were weighed into individual Tzero Aluminum hermetic pans. The pan was placed in the chamber of DSC (Q Series DSC, TA Instrument). The DSC was programmed as follow: 1) Equilibrate at 25˚C; 2) Jump to -10˚C; 3) Ramp 10˚C/min to 80˚C (1st run); 4) Cooling; 5) Equilibrate at -10˚C; 6) Isothermal for 5 min; and 7) Ramp 10˚C/min to 80˚C (2nd run).

5.3.4 Preparation of Microparticles

As shown in Figure 5.1, for preparing microparticles without adding 2% alginate solution, 5 g of lauric acid and 5 g of thymol were weighed into a closed vial separately and

melted at 70˚C in a water bath, mixed together and stirred for 30 min. Thirty grams of cornstarch and 5 g of pre-gelatinized starch (a ratio of 6:1) were weighed separately and then mixed in a container by handshaking. The molten thymol and lauric acid mixture were added into the starch mixture and then mixed by hand stirring. Fifteen milliliters of distilled water (3 times of pre-gelatinized starch) was added to the mixture. The containers were immediately placed into an ice-water bath for 1.5 h and kept in a refrigerator (4˚C) overnight for solidification. The solid particles were then granulated into micro-particles with a granulating machine (UAM Pharmag, Germany) at 90 rpm using a pore size of 0.1 mm and dried at room temperature (23˚C) for 1 h before being stored in a refrigerator (4˚C).

For preparing microparticles with alginate, a total of 0.3 g of alginate was weighed and dissolved in 15 mL of distilled water to make a 2% (w/v) alginate solution. The same protocol described above was used to make the microparticles except for replacing the 15 mL of water with the 2% alginate solution.

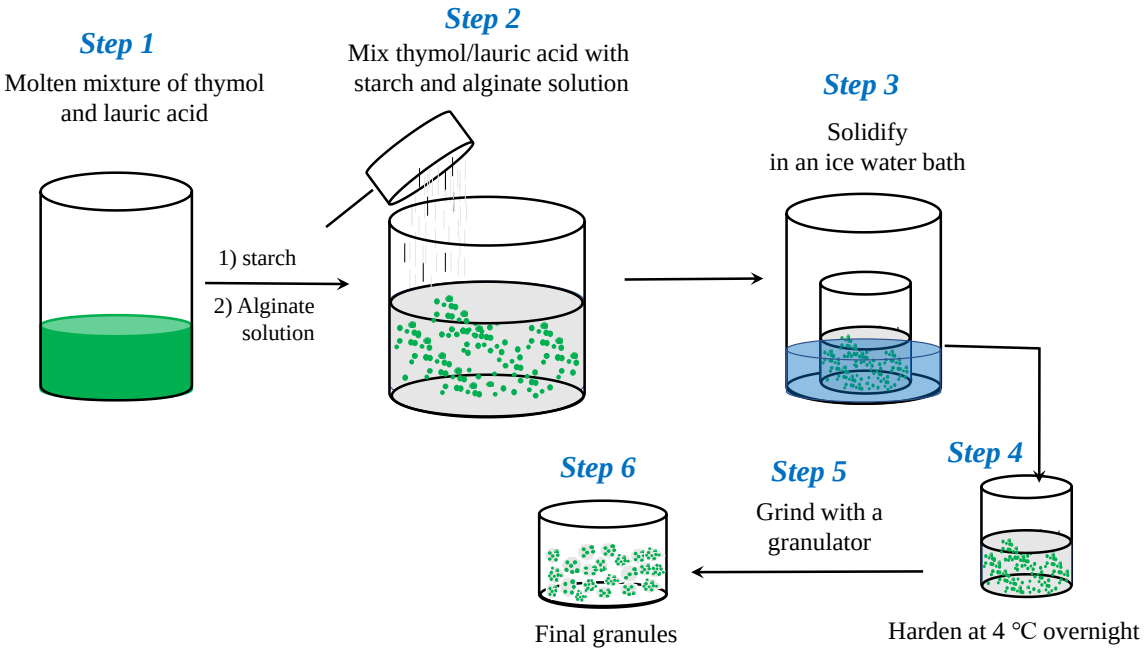

Figure 5.1 Production of microparticles with 2% alginate solution.

5.3.5 Morphology of Microparticles

The morphology of the microparticles produced with or without adding a 2% alginate solution was determined with a light microscope (Axio Cam 105, Carl-Zeiss, Switzerland; Nikon eclipse, Japan) at 10 × magnification and the Zein Image Software (2012) was used to determine the surface diagram of the microparticles.

5.3.6 *In vitro* Release of Thymol and Lauric acid from the Microparticles

In vitro release of thymol and lauric acid from the microparticles was determined with simulated digestive fluid using previously published procedures with some modifications (Minekus et al. 2014). The simulated salivary fluid (SSF) contained 15.1 mmol/L KCl, 13.6 mmol/L $NaHCO_3$, 3.7 mmol/L KH_2PO_4, 0.15 mmol/L $MgCl_2(H_2O)_6$ and 0.06 mmol/L $(NH_4)_2CO_3$. The simulated gastric fluid (SGF) contained 47.2 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 6.9 mmol/L KCl, 0.9 mmol/L KH_2PO_4, 0.5 mmol/L $(NH_4)_2CO_3$ and 0.1 mmol/L $MgCl_2(H_2O)_6$. The simulated intestinal fluid (SIF) contained 85 mmol/L $NaHCO_3$, 38.4 mmol/L NaCl, 6.8 mmol/L KCl, 0.8 mmol/L KH_2PO_4 and 0.33 mmol/L $MgCl_2(H_2O)_6$. The pH of SSF, SGF, and SIF was adjusted using HCl or NaOH to 7.0, 3.0 and 7.0, respectively. The final digestion mixtures of the electrolyte solution for SSF, SGF and SIF contained 1.5, 0.15 and 0.6 mmol/L of $CaCl_2(H_2O)_2$, respectively. Respective enzymes were also added to simulate digestion in pig digesta. Alpha-amylase originated from human saliva was included in the SSF final digestion mixture at a concentration of 75 U/mL. Pepsin originated from porcine gastric mucosa was added to the SGF final digestion mixture at a concentration of 2000 U/mL and pancreatin originated from porcine pancreas was added to the SIF final digestion mixture at a concentration of 100 U/mL.

Forty microparticle samples (each 0.5 g) were employed to mimic digestion within the mouth, stomach and small intestine in pigs. Four samples were taken from each sampling point (0, 2, 30, 60, 90, 120, 150, 180, 210 and 240 min) with points between 0 to 2 min representing

the digestion in the mouth, 2 to 120 in the stomach and 120 to 240 min in the small intestine. All simulated solutions were maintained at 37˚C. The SSF was added to each of the samples at a ratio of 1:1 and placed in the incubator with shaking (Innova TM. 4200, New Brunswick Scientific, Edison/ NJ. USA) for 2 min. The pH was adjusted to 3 with 1M HCl before SGF was added. At the end of the SGF stage, the pH was adjusted to 7 with 1 M NaOH followed by addition of the SIF. To measure the concentration of thymol and lauric acid, 5 mL of oil extraction solvent (hexane) was added to each of the supernatants, shaken (IKA Vibrax VXR Basic, U.S.A) for 20 min and allowed to stay for 30 min. Each of the supernatant from each point was diluted 10 times and the diluent was filtrated using a syringe-driven filter unit (polytetrafluoroethylene, 0.22 nm) and further analyzed by gas chromatography (GC) following the method explained below. Two replicates for each sample were used.

The column installed was SUPELCO WAX^{TM} 10 (fused silica capillary column; 60 m × 0.25 mm × 0.50 nm film thickness and the temperature limits from 35-280˚C). Thymol and lauric acid were identified by comparing the retention time with the standard thymol and lauric acid and their concentrations were calculated by comparing the total peak area of thymol and lauric acid with the standard curve. Released thymol or lauric acid content = thymol or lauric acid concentration in GC vial × 5 (volume of added hexane) × dilution times/thymol or lauric acid in the dry samples × 100%.

5.3.7 Determining the Stability of Thymol and Lauric acid in the Microparticles

The stability of thymol and lauric acid in the microparticles with or without alginate was measured after being stored at room temperature (23˚C) for 2 weeks and during the storage at 4˚C for 12 weeks. The recovery rate of thymol and lauric acid were determined with the procedure described as below. Samples were taken at different time points (1 week, 3 weeks, 6 weeks and 12 weeks) for analysis. Each sample (0.5g) was suspended in 15 mL of distilled

water containing Pancreatin (100 U/mL). The mixture was incubated and analyzed as described above. Each of the samples was measured in triplicate.

5.4 RESULTS AND DISCUSSION

5.4.1 Selection of a Fatty Acid

There was no visible phase separation for all three mixtures at the molten state (Figure 5.2a). After being placed at room temperature (23˚C) for 6 h, the molten mixture of thymol and lauric acid was still in a clear liquid state without having phase separation, however, the other two molten mixtures (thymol / palmitic acid and thymol / stearic acid) solidified and formed a gel-like mixture (Figure 5.2B). These results are consistent with the DSC measurements. As shown in Figure 5.3, the mixture of lauric acid and thymol exhibited a single melting peak with a value of 30.6˚C, which is lower than that of thymol (52.8˚C) and lauric acid (47.4˚C). This suggested that the mixture of lauric acid and thymol was in a eutectic solution, that is, a mixture of two or more pure chemicals at certain ratios, in which the chemicals inhibit the crystallization process of one another, resulting in a system having induced melting point depression (Washburn, 1924). Once cooling the emulsions, thymol crystalized in irregular shapes (Figure 5.4A), whereas lauric acid crystalized in round shapes (Figure 5.4B). The resulted mixture of the two crystalized into somewhat ovular shaped particles without visible distinctions between the two individual components (Figure 5.4C). This observation indicates that thymol and lauric acid co-crystalized together. Both results from DSC and microscopy observation showed that thymol and lauric acid form a pair of a good candidate for a formulation of antimicrobial microparticles for the following reasons. Firstly, since lauric acid significantly reduced the melting point of thymol, it served as a liquid carrier for thymol at room temperature for a period up to 6 h. This property provides an excellent convenience for processing of thymol products such as in the present study. This is because when at a liquid state, thymol and fatty acids can be easily mixed and better absorbed by the starch granules

which helps to ensure even distribution and better protection of the core ingredients within the encapsulation matrix. Secondly, a combination of thymol with lauric acid in one product may provide additional protective benefits to the animals. An *in vitro* study demonstrated that lauric acid could effectively inhibit the growth of *Brachyspira hyodysenteriae* with a MIC value less than 1.5 mM (Vande Maele et al., 2016). Dietary fats with a considerable level of lauric acid and myristic acid increased broiler growth performance that may be related to lauric acid's antimicrobial properties (Zeitz et al., 2015). Most recently there was a study showing that lauric acid can reduce *Campylobacter spp.* in broiler meat (Zeiger et al., 2017). Lauric acid's ester derivatives (e.g., monolaurin) are also known for their protective biological activities as antimicrobial agents (Seleem et al., 2016). The exact mechanism of lauric acid anti-microbial effect is still unclear. However, it is believed that some MCFA can damage the cell membrane, therefore, causing bacterial death (Desbois et al., 2010). It has been believed that the amphipathic structure of MCFA allows them to cause pores with a different size in the cell membrane. MCFA also could cause bacteria death by reducing enzyme function, blocking nutrient absorption and producing toxic compounds for bacteria (Desbois et al., 2010). Therefore, in this study lauric acid is not only a suitable carrier for thymol but also a bioactive compound with antimicrobial properties.

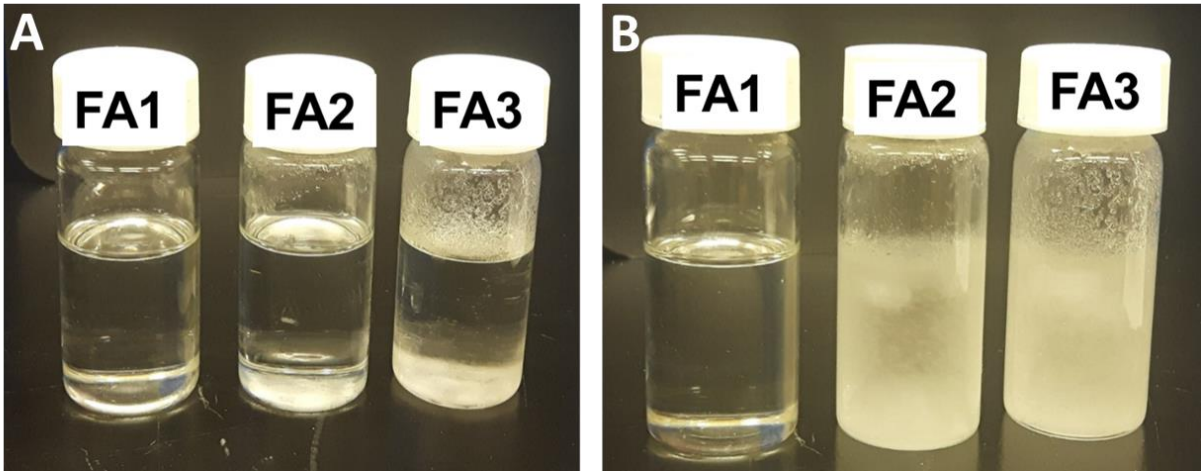

FIGURE 5. 2 PICTURES SHOWING THE MOLTEN MIXTURE OF THYMOL AND FATTY ACIDS AT 0 MIN AT ROOM TEMPERATURE (23°C) (A). PICTURE SHOWING THE MOLTEN MIXTURE OF THYMOL AND FATTY ACIDS AT 6 H AT ROOM TEMPERATURE (23°C) (B). FA1- MIXTURE OF THYMOL AND LAURIC ACID, FA2 – MIXTURE OF THYMOL AND PALMITIC ACID; FA3 – MIXTURE OF THYMOL AND STEARIC ACID.

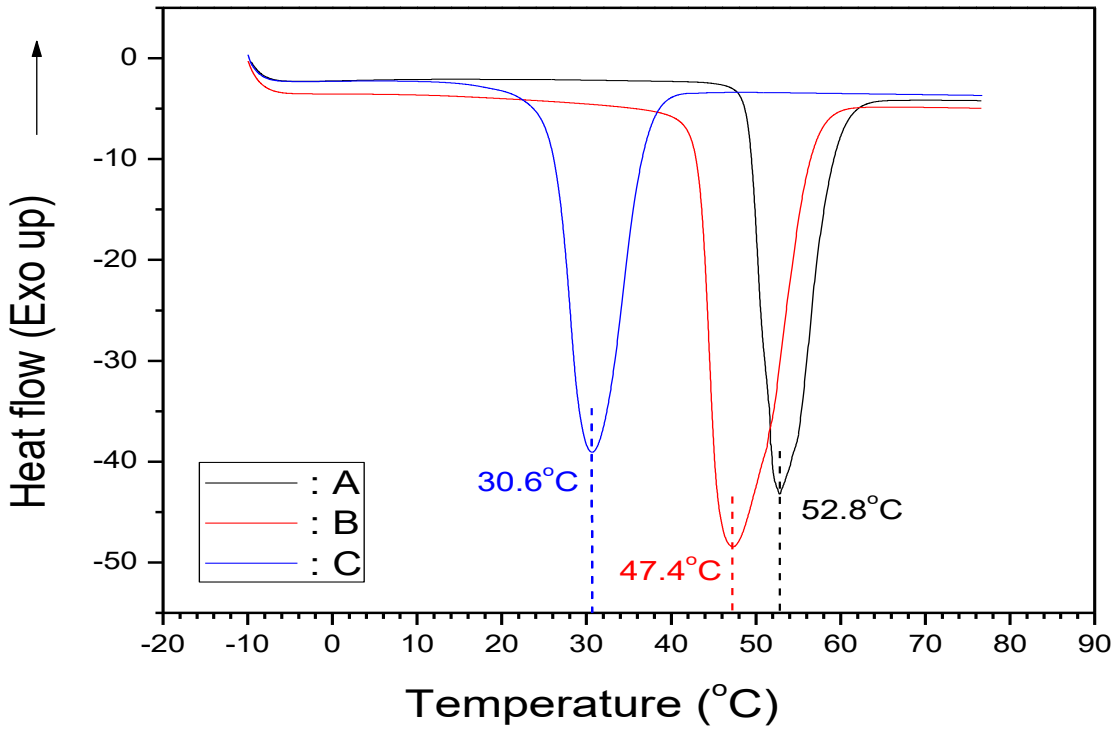

Figure 5.3 Differential scanning calorimetry (DSC) of (A)Thymol, (B) Lauric acid, and (C) Mixture of thymol and lauric acid (50: 50wt%). The second run with a heating rate of 10 °C/min from -10°C to 80°C.

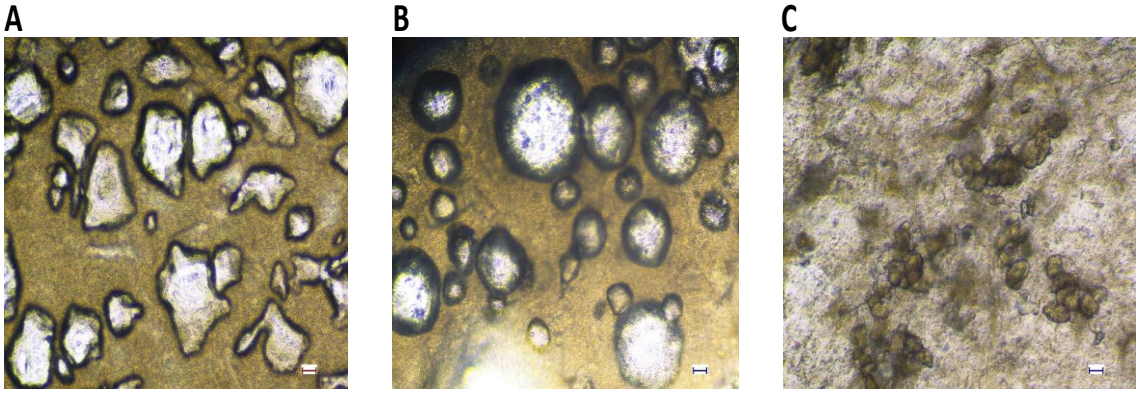

Figure 5.4 Morphology of crystals of thymol (A) and lauric acid (B) and a mixture of thymol and lauric acid (C) after crystallization. The scale bars in the pictures are 1µm.

5.4.2 Morphology of Microparticles

The compositions of microparticles with/without alginate include 66.22%/66.67% cornstarch, 11.03%/11.11% pre-gelatinized starch, 11.03%/11.11% thymol, 11.03%/11.11% lauric acid and 0.7%/0% alginate. The average particle sizes of the microparticles were 800 μm in diameter, and this was similar to the average size of 890 μm for microparticles produced by Benavides et al. (2016) through the method of ionic gelation of alginate. There is no difference in the average particle size between the microparticles produced with or without alginate; however, the shapes and surfaces of the two types of microparticles were different (Figure 5. 5). The microparticles with alginate were mostly spherical with a relatively smooth surface, whereas those without alginate had irregular shapes with rough edges and coarse surfaces. Many kinds of polymers have been employed to encapsulate and deliver bioactive compounds in both food and feed applications (Almeida et al., 2013; Zhang et al., 2016b; Chen et al., 2017). For applications in animal feeds, it is better to use natural polymers that have been approved for use in feeds. Starch is popularly used for microencapsulation because it is biodegradable, edible, commonly available at low cost, nonallergic, easy to use and thermo-processable (Zhu, 2017). Starch consists of both amylose and amylopectin (Tester et al., 2004; Udachan et al., 2012). Pre-gelatinized starch has undergone processing under intense heat conditions by cooking, drying and making into fine powder thus, leading to better solubility in water and being readily solubilized at room temperature (Romano et al., 2018; Fiorda et al., 2015). The combined use of cornstarch and pre-gelatinized starch in this study increases the water retentivity (Romano et al., 2018), thus promotes hydrogen bonding and the formation of the network in the encapsulation matrix. As a natural polymer derived from brown seaweed, alginate is a linear and anionic polysaccharide (Dragan, 2014). At room temperature, alginate is soluble in water allowing the formation of gel without heating and cooling cycles, which make alginate as an attractive microencapsulation material for feed applications (Benavides et

al., 2016; Agüero et al., 2017). The inclusion of alginate to the starch matrix improved the shape and surface properties. This could be attributed to its remarkable crosslinking capability and excellent film-forming properties (Costa et al., 2018).

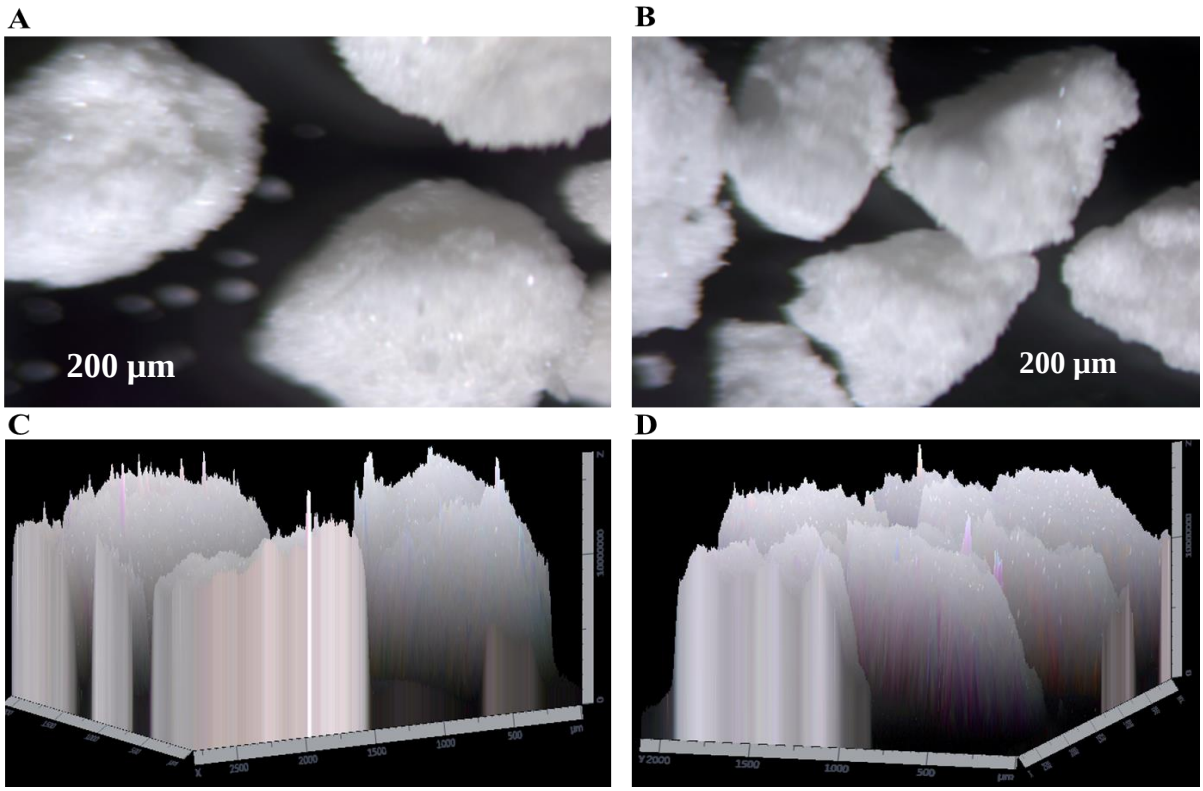

Figure 5.5 Morphology and surface diagram of the microparticles of lauric acid and thymol with and without 2% alginate observed with a light microscope. (A) Morphology of microparticles with alginate; (B) Morphology of microparticles without alginate; (C) Surface diagram of microparticles with alginate and (D) Surface diagram of microparticles without 2% alginate.

5.4.3 *In vitro* Release Profiles of Thymol and Lauric acid from the Microparticles

As shown in Figure 5.6A, both thymol and lauric acid encapsulated in the microparticles with alginate exhibited slow release profiles in the simulated gastrointestinal fluids. The cumulative release (%) of thymol and lauric acid increased gradually to 21.2 ± 2.3 and 36.0 ± 1.1 in the SSF, 73.7 ± 6.9 and 36.8 ± 0.6 in SGF. Both thymol and lauric acid were completely released in the SIF within 240 min. However, as shown in Figure 5.6, the microparticles produced without alginate had a rapid release of thymol (79.9 ± 11.8%) and lauric acid (80.8 ± 5.9%) after incubation in the SSF for 2 min. When the microparticles were placed in the SGF for 120 min, the cumulative release rates reached 92.5 ± 3.5% and 75.8 ± 5.9% respectively for thymol and lauric acid. The rest of thymol and lauric acid were released from both types of microparticles in less than 40 mins after they were placed in the SIF. The goal of a current delivery method is to release thymol and lauric acid at a low percentage in the mouth and stomach but have a sustained release as it passes through the intestine (Piva et al., 2007). The fast release of thymol and lauric acid in SSF from the microparticles without alginate is primarily due to the presence of alpha-amylase in the SSF, an enzyme that is known to digest starch quickly. The excellent solubility of pre-gelatinized starch could also have contributed to the fast release of the active components. The inclusion of alginate to the starch matrix markedly reduced the release rate in the SSF. This is mainly due to the existences of carboxylic groups in alginate molecules and calcium ions in the simulated digestive fluids. Calcium ions may form crosslinks between carboxylic groups in addition to hydrogen bondings, leading to enhanced networks of encapsulation matrix, therefore, retard the dissolution of starch molecules and slow the release of thymol and lauric acid. The globular shaped and smooth surface of microparticles with alginate would have a smaller specific surface area compared to the irregular shape and rough surface of microparticles without alginate. This may be another factor contributing to the better release property of alginate

containing microparticles. Notably, alginate also effectively reduced the release of active components in the SGF which can be explained by the pH sensitivity of alginate molecules. When it is under very acidic conditions (e.g., pH at stomach) that are lower than its pKa, the carboxylic groups are not ionized and stay as COOH resulting in an insoluble structure (Agüero et al., 2017). When pH is close to 7 which is similar to the intestinal pH, the carboxylic groups became ionized (COO-) resulting in that the polymer chain significantly expands and the hydrophilic alginate matrix enlarges (Agüero et al., 2017). In this study, the results indeed demonstrated that alginate significantly decreased the release of thymol and lauric acid in SGF and increased their release in the SIF. Many studies have shown that alginate matrix prevented a quick release of active components in the acidic environment of the stomach and allowed a prolonged release under the intestinal conditions (Zastre, 1997, Zhang et al., 2016b). However, compounds that are highly soluble and have a low molecular weight cannot be prevented from releasing in the mouth and stomach even though the granules matrix does not erode or swell. The alginate-containing microparticles developed in this study need to be further optimized to reduce the release rates in the SSF and SGF.

Although the release behavior of thymol and lauric acid from the microparticles provides very valuable information, it is challenging to precisely demonstrate release behavior in pig gut because of the complexity of gut physiological environments. This was supported by the study indicating that the rate of release of encapsulated carvacrol in the pig stomach via *in vivo* studies was 25% higher than the rate obtained from *in vitro* studies (Zhang et al., 2016b), which may be due to the phenolic binding to other components such as fats and hydrophobic compounds present in the diet (Lallès et al., 2009). Therefore, *in vivo* release behavior of the microparticles has to be determined eventually in the gastrointestinal tract of pigs.

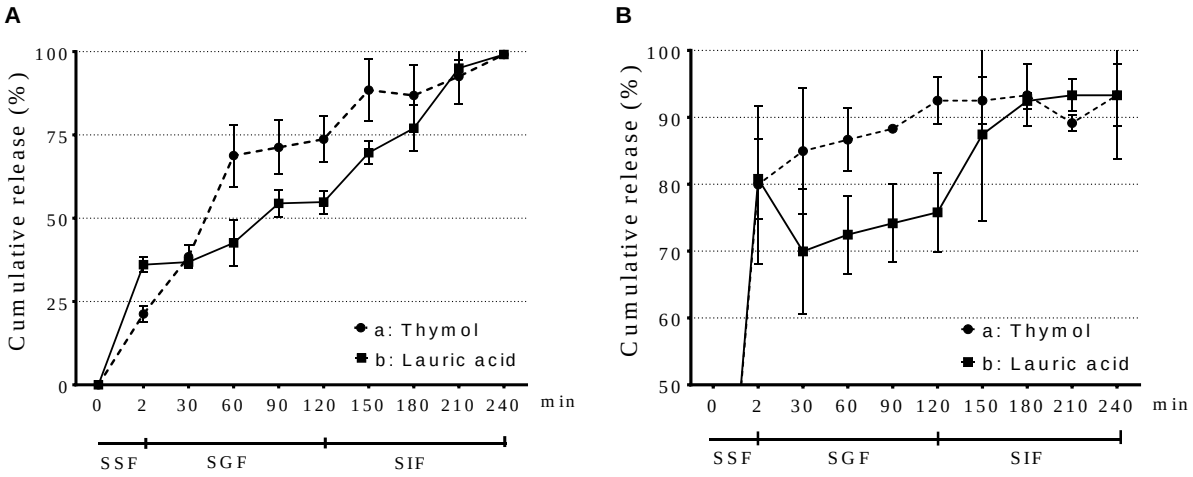

Figure 5.6 *In vitro* release profile of thymol and lauric acid from the microparticles with (A) and without (B) alginate using simulated fluids (SSF - simulated salivary fluid, SGF - simulated gastric fluid and SIF - simulated intestinal fluid). (Mean ± SD, n = 4).

5.4.4 The Stability of Microparticles with/without Alginate During Storage

As shown in Figure 5.7, thymol and lauric acid had good stabilities (> 95%) in both types of microparticles with or without alginate after being stored at room temperature (23˚C) for 2 weeks. As shown in Figure 5.8, thymol and lauric acid had good stabilities (> 90%) in both types of microparticles with or without alginate after being stored at 4˚C for 12 weeks. Durante et al. (2012) showed that the encapsulation of wheat bran oil into 2% (w/v) sodium alginate beads significantly increased the stability of wheat bran oil at 4˚C. This was also found in the research conducted by Otálora et al. (2016), that the encapsulation of betalain with calcium-alginate had good stability when stored at low relative humidity. Stability during storage is an essential factor that should be considered for a feed additive. Feed additives have a 1-2-year shelf life under current industry practice. Our preliminary data demonstrated that the current microparticles are stable during short-term storage. However, the stability of long-term storage (e.g.1-2 year) must be further investigated. The inclusion of antioxidants in the formula may be considered to enhance the stability of encapsulated thymol and lauric acid.

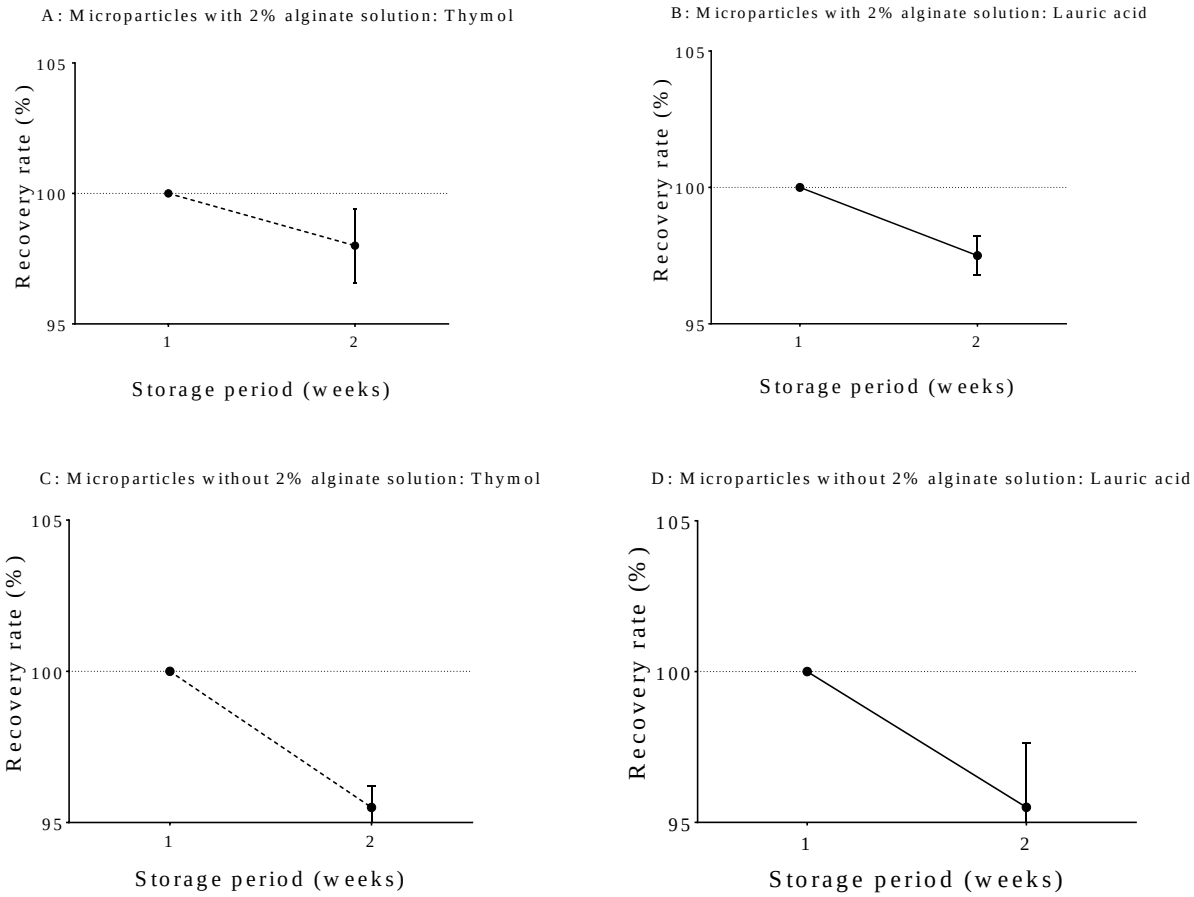

Figure 5.7 Stability of the microparticles of (A) thymol in the microparticles with alginate, (B) lauric acid in the microparticles with alginate, (C) thymol in the microparticles without alginate and (D) lauric acid in the microparticles without alginate. Samples were stored at room temperature (23˚C) for 2 weeks. (Mean ± SD, n = 4).

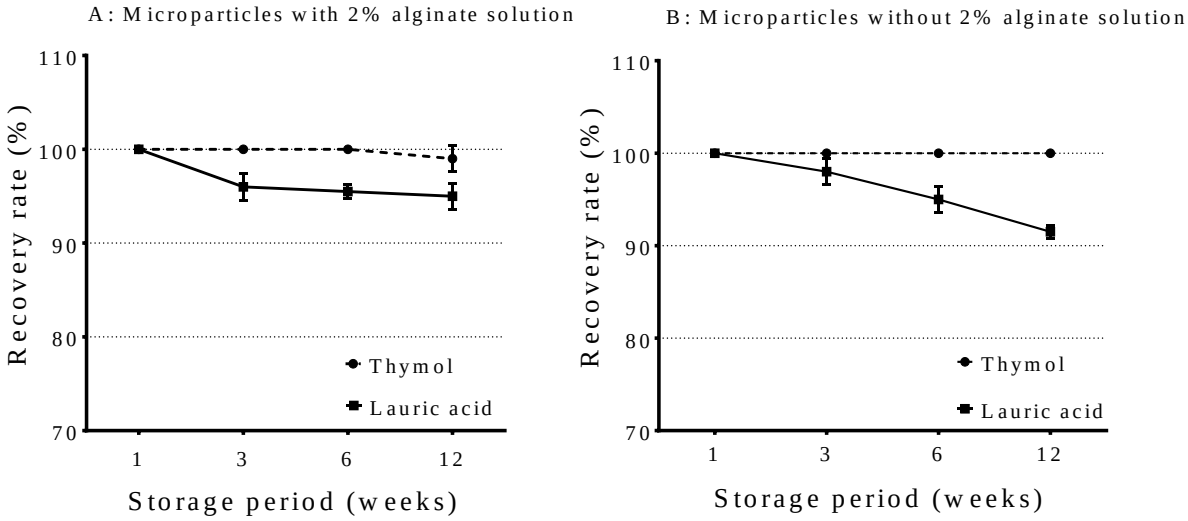

Figure 5.8 Stability of the microparticles of thymol and lauric acid with (A) and without (B) alginate stored at 4˚C for 12 weeks. (Mean ± SD, n = 4).

5.5 CONCLUSION

The formulation and method established in this study for the encapsulation of thymol and lauric acid in microparticles are relatively simple and can be used as a potential method to effectively deliver essential oils and MCFA to the pig intestinal tract. This unique essential oil formula will be further optimized for better-controlled release though investigating the physicochemical and molecular property of the microparticles. Retention of encapsulated thymol and lauric acid during feed processing will be mimicked by the treatments of steam for different time periods and validated in a real pelleting process. Further investigations are needed to validate the efficacy of the microparticles with *in vivo* studies.

CHAPTER 6 GENERAL DISCUSSION AND CONCLUSION

6.1 GENERAL DISCUSSION

The gastrointestinal tract is the major site where feed digestion takes place. Because, the gastrointestinal tract consists of enzymes that can break down feeds into smaller molecules for better absorption, digestion and metabolism (Yang et al., 2015a). However, the gut is susceptible to pathogenic bacteria (mostly populated at the distal part of the small intestine) as a result of different stressors e.g. weaning and disease, etc. thus affecting animal growth and performance (Zhao et al., 2007). It is popular to use AGP to improve the growth and prevent incidences of postweaning diarrhea in animals e.g. pig, but its application as in-feed antibiotics has been banned in some countries (e.g. Europe) due to its negative effect on public health. However, antibiotics lack the ability to distinguish pathogenic bacteria from beneficial bacteria and thus kills both (Yang et al., 2015a). Researchers have proved that essential oils e.g. thymol can serve as a potential alternative to antibiotics due to their anti-inflammatory, antimicrobial and anti-oxidative properties (Hanczakowska et al., 2013; De Smet et al., 2016). In this study, we developed a novel microparticle that can deliver thymol and lauric acid to pig gut.

The composite microparticles were produced by the incorporation of starch and alginate through a melt-granulation process. The novelty of this technique lies in the 1) Innovative adoption of lauric acid to serve 2 purposes: a. acting as a carrier for thymol; b. presenting an additive, if not synergistic, antibacterial effect with thymol; 2) Controlled and target release of thymol, achieved by using an economical and scalable encapsulation process; and that 3) Lauric acid has significantly reduced the melting point of thymol which provides the convenience of processing thymol at room temperature in liquid form. The optimized encapsulation techniques will be a gateway to more research on how to protect and deliver other bioactive compounds (e.g., enzymes, amino acids and probiotics) in the pig gut.

It is believed that the retention time of feed in the mouth is very short (Minekus et al., 2014) and therefore, the digestion of the microparticle in the mouth should be at the minimal level. Also, there should be less digestion in the stomach and the bulky part should be released at the proximal part of the small intestine. Further optimization is required to investigate the physicochemical and molecular properties of the developed microparticles, which will provide insight for the mechanisms underlying the phenomenon of stability or release of bioactive ingredients and will help to further optimize microencapsulation to better protect and deliver bioactive ingredients.

Stability during storage is an important factor that is needed to be considered as a feed additive. Feed additives have a 1-2-year shelf life under current industry practice. Our preliminary data demonstrated that the lipid matrix granules are stable during short-term storage. However, the stability of long-term storage (e.g.1-2 year) must be further investigated. The inclusion of antioxidants in the formula could be considered to improve the stability of the granules. Feed manufacturing often involves a heating process (e.g. pelleting). Inclusion of antioxidants (e.g. butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), eugenol, ethoxyquin, propyl gallate, tertiary butyl hydroquinone, (TBHQ), tocopherols) in the formula could be considered to improve the stability and shelf life of microparticles because these antioxidants can reduce the rate of rancidity conversion in the microparticles. In addition to the future studies, the retention of thymol and lauric acid in the microparticles during simulating feed process by steaming for different time periods and a real pelleting process needs to be determined.

Likewise, the *in vivo* release profile of thymol and lauric acid from the microparticles in the pig intestinal tract should be verified. Moreover, the anti-inflammatory effect of thymol against LPS-induced inflammation in IPEC-J2 cells needs to be investigated in experimental animals. Therefore, further *in vivo* studies are required to elucidate the molecular mechanisms

of the function of novel microparticles containing thymol and lauric acid in experimentally infected pigs by measuring nutrient absorption, immune responses, microbiota, and gut barrier function.

6.2 GENERAL CONCLUSION

The novel approach developed in this study can be likely used to deliver thymol and lauric acid effectively to pig intestinal tract and the microparticles containing thymol may improve gut health by enhancing barrier function and reducing inflammatory cytokine secretion in pigs under physiological challenges.

CHAPTER 7 FUTURE DIRECTIONS

Future directions include:

1. To further optimize the microparticles for better-controlled release through investigating their physicochemical and molecular properties;
2. To determine retention of thymol and lauric acid in the microparticles during simulating feed process by steaming for different time periods and a real pelleting process;
3. To evaluate the *in vivo* release profile of thymol and lauric acid from the microparticles in the pig intestinal tract;
4. To elucidate the molecular mechanisms of the function of novel microparticles containing thymol and lauric acid in experimentally infected pigs by measuring nutrient absorption, immune responses, microbiota, and gut barrier function; and
5. To evaluate the efficacy of novel microparticles containing thymol and lauric acid as an alternative to infeed antibiotics in pigs.

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