EFFECTS OF PLANT EXTRACTS ON IMMUNE FUNCTION AND DISEASE RESISTANCE IN PIGS

BY

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DISSERTATION

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ABSTRACT

Plant extracts (PE) are bioactive substances, extracted from some foods or traditional herbs. It has been known that PE possess antioxidant, antibacterial, anti-inflammatory, and perhaps immunoregulatory effects. The 3 studies below demonstrate the anti-inflammatory effects of PE in vitro and the effect of PE on immune function and disease resistance of pigs in vivo. The first study evaluated the effects of 7 PE (anethol, capsicum oleoresin, carvacrol, cinnamaldehyde, eugenol, garlic, and turmeric oleoresin) on cell viability and cytokine secretion of porcine alveolar macrophages (PAM) with or without lipopolysaccharide (LPS) stimulation. Without LPS stimulation, anethol (35 to 52%) and capsicum oleoresin (39 to 59%) increased cell viability of PAM, whereas other PE reduced ($P < 0.05$) it. Anethol (12 to 34%), capsicum oleoresin (53 to 92%), or carvacrol (46 to 61%) enhanced ($P < 0.05$) the cell viability of LPS-treated PAM. Without LPS stimulation, anethol, capsicum oleoresin, cinnamaldehyde, or turmeric oleoresin stimulated TNF-α secretion from PAM, whereas all PE except eugenol enhanced IL-1β secretion from PAM. However, all PE suppressed ($P < 0.05$, 15 to 100%) TNF-α, and carvacrol, cinnamaldehyde, eugenol, or garlic decreased ($P < 0.05$, 31 to 95%) IL-1β secretion from LPS-induced PAM. This study indicates all PE may have potent anti-inflammatory effects to varying degrees. Based on the in vitro study, three PE (capsicum oleoresin (CAP), garlic (GAR), and turmeric oleoresin (TUR)) showing diverse effects in vitro were selected to investigate their effects in vivo with two different disease models, *Escherichia coli* (*E. coli*) and porcine reproductive and respiratory syndrome (PRRS). The second study evaluated the effects of 3 PE on diarrhea, immune response, intestina morphology, and growth

performance of weaned pigs experimentally infected with a pathogenic F-18 *E. coli*. The *E. coli* infection increased ($P < 0.05$) white blood cells (WBC), tumor necrosis factor (TNF)-α, and hapotoglobin (Hp), and reduced overall ADG, G:F, and villi height (VH) of the small intestine as expected. In the challenged group, the supplementation of 10 mg of CAP, GAR, or TUR/kg diet reduced average diarrhea score from d 0 to 2 and d 6 to 11 and overall frequency of diarrhea, decreased ($P < 0.05$) TNF-α and Hp on d 5 and WBC and NEU on d 11, and increased ($P < 0.05$) ileal VH on d 5, and tended ($P = 0.10$) to increase jejunum VH and villi height:crypt depth compared with the control diet (CON). In the sham group, the PE treatments reduced ($P < 0.05$) average DS from d 3 to 5, overall frequency of diarrhea, and Hp on d 5, compared with the CON. In addition, the 3 PE tested here showed different influences on the inflammatory mediators. In conclusion, the 3 PE tested reduced diarrhea, increased the VH of the small intestine, and affected total WBC, the populations of immune cells, and inflammatory mediators in *E. coli*-infected piglets, which may be beneficial to pig health. The third study was conducted to determine the effects of these 3 PE on growth efficiency and immune responses of pigs experimentally infected with PRRS virus (PRRSV). Infection of PRRSV reduced pig performance ($P < 0.01$), but increased rectal temperature (RT), viral load (VL), and PRRSV specific antibody titer (AT), and serum inflammatory mediators ($P < 0.05$). In addition, the PRRSV infection reduced ($P < 0.01$) leukocytes on d 7, but increased ($P < 0.01$) leukocytes on d 14. In the PRRSV challenged group, the PE treatments increased ($P < 0.05$) growth efficiency, IL-10, and Hp, but reduced ($P < 0.05$) viral load, TNF-α, C-reactive protein, and RT on d 4 as compared to the CON. In the unchallenged group, all piglets were PRRSV negative during the overall period PI. The CAP increased ($P < 0.05$) ADFI from d 0 to 7 and overall period PI, and final weight of piglets compared with the CON. Similar to the second study with *E. coli* infection,

the 3 PE tested showed diverse effects on growth efficiency and inflammatory mediators of pigs infected with PRRSV, and TUR appeared to strengthen the immune responses and efficiency of pigs infected with PRRSV. In summary, PE are potent s in both in vitro and in vivo systems. Dietary supplementation of different PE for pigs may bring different influences to pigs infected with a bacterial or viral model. In the *E. coli* infection model, PE may bring the benefits by preventing over-stimulation of the immune system, while in the PRRSV infection model, PE may exhibit the benefits by boosting the host's disease resistance in the early stage of disease and maintaining it in the later stage.

Key words: cytokines, *Escherichia. coli*, immune responses, plant extracts, porcine alveolar macrophages, porcine reproductive and respiratory syndrome

DEDICATION

This dissertation is dedicated with my deepest gratitude to

my parents, Mr. Zhixiu Liu & Mrs. Xiuyun Mao,

my mother-in-law, Mrs. Xia Wu,

my husband, Peng Ji, my future baby in my belly.

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× g	relative centrifugal force
ADFI	average daily feed intake
ADG	average daily gain
ANOVA	analysis of variation
APC	antigen presenting cells
APP	acute phase proteins
AT	antibody titer
B	B lymphocytes
C	Carbon
Ca	calcium
cAMP	cyclic adenosine monophosphate
CAP	dietary treatment with 10 mg capsicum oleoresin per kg diet
CD	cluster of differentiation
CDH	crypt depth
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	cyclic guanosine monophosphate
cm	centimeter
CNS	central nervous system
CO_2	carbon dioxide
CoA	coenzyme A
CON	control
COX-2	cyclooxygenase-2
CRP	C-reactive protein
Ct	cycle threshold
d	day
DM	dry matter
DMAPP	dimethyylallyl-pyrophosphate

DMI	dry matter intake
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DS	diarrhea score
dsRNA	double-stranded ribonucleic acid
E. coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EO	essential oil
ERK 1/2	mitogen-activated protein kinase
ETEC	enterotoxigenic *Escherichia coli*
F	fimbriae
FD	frequency of diarrhea
g	gram
G:F	gain:feed
GAR	dietary treatment with 10 mg garlic per kg diet
Gb	globotriaosylceramine
GC-C	guanylate cyclase
GM1	monosialotetrahexosylganglioside
GPP	geranyl purophosphate
h	hour
HCl	hydrogen chloride
HCO_3	bicarbonate
HCT	hematocrit
HGB	hemoglobin
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
Hp	haptoglobin
IFN	interferon

Ig	immunoglobin
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	interferon gamma-induce protein 10 kDa
IPP	isopentenyl-5-pyrophosphate
IU	international unit
IκB	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
K	potassium
kDa	kilodalton
kg	kilogram
L	liter
LBP	lipopolysaccharide binding protein
LPS	lipopolysaccharide
LSMEANS	least squares means
LT	heat-labile toxin
LYM	lymphocytes
MAPKs	mitogen-activated protein kinase
mCD14	membrane cluster of differentiation 14
MD2	lymphocyte antigen 96
mg	miligram
MHC	major histocompatibility complex
MIG	monokine induced by gamma interferon
min	minutes
mL	milliliter
mM	millimolar
MONO	monocytes
mRNA	messenger ribonucleic acid

MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
N	nitrogen
Na	sodium
NDP	NanoZoomer Digital Pathology System
NEU	neutrophils
NF- κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLRs	NOD-like receptors
nm	nanometer
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
NOS	nitric oxide synthase
NAHMS	National Animal Health Monitoring System
NRC	National Research Council
^{o}C	degrees celsius
OD	optical density
OH	hydroxyl
OM	organic matter
PAM	porcine alveolar macrophage
PAMPs	pathogen-associated molecular patterns
PBS	phosphate-buffered saline
PE	plant extract
PI	post inoculation
ppm	g/ton
PRR	pattern recognition receptors
PRRS	porcine reproductive and respiratory syndrome
PRRSV	procine reproductive and respiratory syndrome virus
PWD	*Escherichia coli* postweaning diarrhea

qPCR	quantitative real time polymerase chain reaction
RBC	red blood cells
RelA	v-rel reticuloendotheliosis viral oncogene homolog A
RHT	ration of β-hemolytic coliforms to total coliforms
RPMI	Roswell Park Memorial Institute medium
RT	rectal temperature
S/P	sample to positive ratio
SAS	Statistical Analysis System
sCD14	soluble cluster of differentiation 14
SDS	sodium dodecyl sulfate
SLT-2	shiga-like toxin
ssRNA	single-stranded ribonucleic acid
ST	heat-stable toxin
STaH	heat-stable toxin a, human
STaP	heat-stable toxin a, porcine
T	T lymphocytes
TGF-β	transforming growth factor-beta
Th	T helper cells
TLR	toll-like receptor
TNF-α	tumor necrosis factor
TUR	dietary treatment with 10 mg turmeric oleoresin per kg diet
USDA	United States Department of Agriculture
VFA	volatile fatty acids
VH	villi height
VIP	vasoactive intestinal peptide
VL	viral load
WBC	white blood cells
wk	week

wt	weight
XT	a mixture of plant extract standardized to 5% (wt/wt) carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin
μg	microgram
μL	microliter
μm	micrometer

CHAPTER 1

LITERATURE REVIEW

1.1. The Changes of Swine Nutrition Research

Swine products occupy an important position in the structure of human food consumption. The need to continue to increase food production with the earth's limited sources places the onus squarely on the swine industry to increase both efficiency and production. In swine production, nutrition, genetics, and management have been largely applied to improve production. But, on the other side, protecting the health of animals in the future livestock production systems has been put in an important position to successfully meeting this goal.

Post-weaning is one of the most challenging and critical stages in swine production. Its effects are many, affecting behavior, environment, disease, immunity and nutrition. In this period, piglets are immediately subjected to a combination of stressors that predispose them to diarrhea, which can adversely impact survival at a very early and most vulnerable stage (Moeser et al., 2007). Weaning is usually associated with low and variable feed intake, resulting in decreased pig performance and alterations in gut environment and function, making piglets highly sensitive to digestive disease (Pluske et al., 1997). As the Swine 2006 survey (NAHMS, USDA, 2008) reported, the mortality of post-weaning pigs (Figure 1.1) increased recently from 2.6% to 2.9% because of diarrhea, respiratory problem, or CNS/meningitis caused by diarrheic *Escherichia coli (E. coli)*, porcine reproductive and respiratory syndrome (PRRS), *Streptococcus*,

Haemophilis, or others (Figure 1.2 & 1.3). Thus, it becomes particular important to improve post-weaning piglet's health.

Many production technologies that include age segregation, all-in/all-out pig flow, biosecurity measures, sanitation, vaccination and depopulation/repopulation have been used in the swine industry to improve disease resistance and keep pigs from disease (Hardy, 2002; Adjiri-Awere and van Lunen, 2005; NAHMS, USDA, 2008). All of these technologies are powerful, but they cannot guarantee freedom from disease for pigs.

In addition, scientists focus on several methods to improve the health of weaning pigs. One of the most important is modulating the microbial ecology in the digestive tract, which plays an important role in regulating pig performance and health. A second is ensuring the proper function of the immune system. One good example is in-feed antibiotics that have been widely used in the pig industry to enhance production efficiency for several decades (Cromwell, 2002). Antibiotics have been used not only to treat sick animals, but also prevent disease among animals susceptible to infections. Dierick er al. (2002) stated that growth promotion by in-feed antibiotics is related and proportional to the inhibition of the total microbial load and microbial metabolism in the stomach and the jejunum. A reduction in the general bacterial growth in the small intestine and pathogen proliferation should be major targets to improve animal performance and/or health (Apajalahti and Kettunen, 2006). However, the potential threat to human health from the use of antibiotics has led to their ban as growth promoters throughout the European Union since 1 January 2006 (Regulation (EC) No. 1831/2003) and outside the European Union. Risks to human health include the possibility of antibiotics residues in meat, unapparent carriage of anti-microbial drug-resistant bacteria, and exchange of plasmids from antibiotic-resistant bacteria of swine to human pathogens making them resistant to antibiotics (Dewey et al., 1997; Anadon and

Martinez-Larrranaga, 1999; Pugh, 2002). But, the initial experience of an in-feed antibiotics ban in Sweden and Denmark indicated that there was a reduced performance and increased morbidity in nursery pigs (Stein, 2002). This increases the importance of other reliable alternative strategies of maintaining pig health.

In the pig industry, many of the feed ingredients and additives now available for use as "alternatives to antibiotics" either alter microbial populations in the gastrointestinal tract or influence the immune system. An incomplete list of dietary ingredients and other technologies that may improve pig health is offered in Table 1.1 (Adapted from Pettigrew, 2006).

1.2. Plant Extracts

Plant extracts (PE) have been largely employed for human nutrition and improvement of human health. At present, thousands of PE are known, hundreds of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. Plant extracts are of potential interest due to their antiviral (Sökmen et al., 2004), antimicrobial (Baydar et al., 2004; Sökmen et al., 2004), antioxidant (Dundar et al., 2008), anti-inflammatory (Sosa et al., 2005), and other biological effects (Lee et al., 2004). This may lead to the ability to use these PE, instead of antibiotics, in diets to improve performance and health of animals (Pettigrew, 2006; Stein and Kil, 2006). Based on the literature, many in vitro and in vivo studies have shown that PE can improve animal health through several mechanisms such as direct suppression of the proliferation of pathogens, alteration of gut microbial populations, and enhancement of immune functions. Lee et al. (2004), Calsamiglia et al. (2007), and Bakkali et al. (2008) have well reviewed essential oils and their biological effects.

1.2.1. Description of Plant Extracts

Plant extracts are responsible for the odor and color of plants, and are composed of more than a hundred individual components. Plant extracts are secondary plant metabolites and can be obtained naturally from parts of plant materials, such as, flowers, buds, seeds, leaves, twigs, bark, wood, fruits, and roots. There are 4 commonly used methods to extract PE from plants, steam distillation, maceration, cold pressing, and solvent extraction (Kerrola, 1995). Otherwise, PE can be synthesized directly. They are in two different forms, liquid oil and solid powder. Most of oil formed PE are commonly called essential oils (EO), which are mixed oil compounds with variable chemical compositions and concentrations of individual compounds depending on the plants and extraction methods (Lee et al., 2004). Most EO are water-insoluble.

Most PE extracted from plants, vegetables, or flowers are not pure. The chemical compositions of lots of EO are summarized by Surburg and Panten (2006) and Burt (2004). Plant extracts can contain about 20-60 components at quite different concentrations. The major components can constitute up to 85% of the EO, whereas other components are present only as a trace (Surburg and Panten, 2006). For example, the concentration of thymol from same species of plant, *Origanum vaulgare* can vary from trace to 64%, *Thymus vulgaris* from 10 to 64%. Another predominant component, carvcarol has been reported to range from trace to 80% in *Origanum vaulgare* and 2-11% in *Thymus vulgaris* (Burt, 2004; Lawrence and Reynolds, 1984). Cinnamaldehyde, a main component of cinnamon EO, amounts to approximately 60 to 75% of the total oil (Duke, 1986; Lens-Lisbonne et al., 1987). The chemical properties and biological activities of several selected EO components are summarized in Table 1.2. Because of the large variation in composition, the biological effects of different batches of the same EO may differ.

The diversity among sources of EO prompted us to select pure principles for evaluating their possible role as alternatives to antibiotics in livestock production.

The plant extracts basically consist of two classes of compounds, the terpenes and phenylpropenes (Bakkali et al., 2008). Terpenes are made from combinations of several isoprenes, 5-carbon-base units. The biosynthesis of the terpenes is mainly through the mevalonic acid pathway (Miziorko, 2011; Figure 1.4). Briefly, three acetate units form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is converted to mevalonic acid by HMG-CoA reductase. The mevalonic acid can be further converted to isopentenyl-5-pyrophosphate (IPP) and dimethyylallyl-PP (DMAPP), which then combined in a 1:1 molar ratio to genereate the precursor of monoterpenes, geranyl pyrophosphate (GPP). The monoterpenes (C10) are the most representative molecules consistituting 90% of the EO and allow a great variety of strutures. Thymol and carvacrol are synthesized from GPP and classified as monoterpene products. The repetitive addition of IPPs to DMAPP can form the precursors of the various classes of terpenes, such as, C15, C20, C30. Phenylpropenes are synthesized by the shikimic acid pathway (Herrmann and Weaver, 1999; Figure 1.5), which produces the aromatic amino acid phenylalanine. Then phenylalanine can be transconfigured to cinnamic acid and p-coumaric acid (Seigler, 1998; Wilson et al., 1998). Eugenol, trans-cinnamaldehyde, and capsaicin are the most important phenylpropene compounds.

1.2.2. Biological Effects of Plant Extracts

Antimicrobial effects. The antimicrobial activity of plant extracts has been recognized for a long time. Various PE have been shown to exhibit a wide spectrum of antibacterial activity against Gram-negative and Gram-positive bacteria including *Escherichia, Salmonella,*

Staphylococcus, Klebsiella, Proteus, Bacillus, Clostridium, and *Mycobacterium* (Hammer et al., 1999; Dorman and Deans, 2000; Wong et al., 2008). Besides antibacterial properties, PE or their components have been shown to exhibit antifungal (Pinto et al., 2006; Abed, 2007), antiparasitic (Pandey et al., 2000; Pessoa et al., 2002; Moon et al., 2006), antiviral (Bishop, 1995; Garozzo et al., 2009), and antitoxigenic (Ultee and Smid, 2001) properties. Several commonly used PE and their main components displaying antimicrobial activities are shown in Table 1.3.

Considering the large number of different groups of chemical compounds present in PEs, it is not surprising that several modes of action are involved in the antimicrobial activity of PE. First, the hydrophobicity of PE enables them to partition into the lipids of the bacterial cell membrane and mitochondria, disturbing the structures and rendering them more permeable (Knobloch et al., 1989; Burt, 2004; Xu et al., 2008). The greater permeability of the membrane results in leakage of critical intracellular materials and finally leads to cell death (Juven et al., 1994; Helander et al., 1998; Carson et al., 2002). Second, the structural properties, such as the presence of the functional groups (Farag et al., 1989) and aromaticity (Bowles and Miller, 1993) are also responsible for the antibacterial activity of PE. The PE possessing the strongest antibacterial properties, such as carvacol, eugenol and thymol, often contain a high percentage of phenolic compounds (Farag et al., 1989; Dorman and Deans, 2000; Lambert et al., 2001). Phenolics are generally considered to disturb the cytoplasmic membrane, disrupting the proton motive force, electron flow, active transport and coagulation of cell contents (Helander et al., 1998; Dorman and Deans, 2000; Burt, 2004). Third, plant extracts exert antibacterial activity through modifying the enzyme systems of bacteria. Ankri and Mireman (1999) indicated that allicin, the main active component in garlic, can rapidly react with the thiol groups of certain enzymes in microorganisms and subsequently inhibit their enzymatic activity. The inhibition of

thiol-dependent enzymatic systems may block the microbe's virulence, even be lethal for the microorganism. Finally, carvacrol can prevent the development of flagella in *E. coli* O157:H7, which is critical for bacterial adhesion to the intestinal cell membranes (Burt et al., 2007).

Anti-inflammatory effects. Many in vitro experiments show anti-inflammatory activity of PE (Aggarwal and Shishodia, 2004; Lang et al., 2004; Tung et al., 2008). Table 1.4 shows several commonly used PE and their main components displaying anti-inflammatory activities. A variety of inflammatory mediators, including tumor necrosis factor (TNF)-α and IL-1β, are involved in the development of inflammatory diseases (Dinarello, 2000). Dung et al. (2009) concluded that the EO of the *C. operculatus* buds had potential anti-inflammatory effects due to inhibition of TNF-α and IL-1β expression and secretion from lipopolysaccharide (LPS)-induced RAW 264.7 cells. Hart et al. (2000), Lang et al. (2004), and Lee et al. (2007) demonstrated that eugenol, tea tree oil and garlic extract can inhibit the secretion of both TNF-α and IL-1β. Another important molecule involved in the immune defense is nitric oxide (NO), which is produced mainly by macrophages through the activity of nitric oxide synthase (NOS) (MacMicking et al., 1997). A high concentration of NO is associated with inflammatory diseases. Previous studies from Lee et al. (2005), Li et al. (2006), and Tung et al. (2008) reported that cinnamaldehyde and eugenol were able to suppress the NO release and suppress the inducible NOS expression in LPS-treated murine macrophages. Moreover, Kim et al. (2003), Li et al. (2006), and Landa et al. (2009) observed carvacrol, eugenol, and cinnamaldehyde suppressed cyclooxygenase-2 (COX-2) gene expression in LPS-stimulated mouse macrophage cells. Cyclooxygenase -2 is mainly responsible for the production of prostaglandins, which are involved in various pathophysiological process including inflammation and carcinogenesis. Otherwise, Lang et al. (2004) found allicin derived from garlic also can inhibit intestinal

epithelial cell secretion of several chemokines, IL-8, interferon gamma-induced protein 10 kDa (IP-10), and monokine induced by gamma interferon (MIG), which mediate the inflammatory response by recruitment of various circulating leukocytes into the flamed tissue.

The modes of action for the anti-inflammatory activity of PE are still not clear, but evidence suggests that these effects are mediated, at least in part, by blocking the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway. This factor is a key regulator of various genes involved in immune and inflammatory responses (Xie et al., 1994). In resting cells, NF-κB exists in an inactive state in the cytoplasm, complexed with an inhibitory protein, called IκB. Upon activation, IκB undergoes phosphorylation and degradation, and NF-κB is translocated into the nucleus, where it binds to DNA and activates transcription of various genes, including TNF-α, IL-1β and iNOS (Rice and Ernst, 1993; Hiscott et al., 1993; Ghosh et al., 1998). Jobin et al. (1999) found that curcumin can block cytokine-induced NF-κB DNA binding activity, RelA nuclear translocation, IκBα degradation, IκB serine 32 phosphorylation, and IκB kinase (IKK) activity, all of which are involved in the upstream NF- κB signaling pathway. Lee et al. (2005) and Choi et al. (2007) also demonstrated blockade of p50 and p65 translocation, phosphorylation of ERK 1/2 and p38 kinase, and degradation of I-κBα by cinnamaldehyde and eugenol. A minireview from Aggarwal and Shishodia (2004) also demonstrated that a large number of spice-derived phytochemicals can mediate therapeutic effects, possibly through suppression of the NF-κB activation pathway.

Anti-oxidant effects. Animals in intensive farming systems are frequently exposed to oxidative stress which can result in damage to proteins, lipids and DNA (McCall and Frei, 1999). Antioxidants act as radical scavengers, inhibiting lipid peroxidation and other free-radical-mediated processes, and protecting the animal from oxidative damage caused by free radicals.

The antioxidative properties of extracts of oregano, thyme, clove, pepper, lavender, and basil have been evaluated by many studies in vitro (Economou et al., 1991; Gülçin et al., 2004; Oboh et al., 2007). Several commonly used PE showing antioxidant activities are summarized in Table 1.5. Otherwise, some in vivo studies also reported the antioxidant properties of some PE. Slamenova et al. (2008) indicated that carvacrol given in drinking water reduced the level of DNA lesions induced in freshly isolated hepatocytes and testicular cells by H_2O_2, which could be associated with an increase of antioxidant activity of liver and testicular cells in these animals. Frankič et al. (2010) showed the supplementation of PE to pigs reduced the DNA damage in lymphocytes, which indicated their potentially beneficial effects on the immune system under dietary-induced oxidative stress. Botsoglou et al. (2002) found the dietary administration of oregano oil increased the antioxidative status of broiler meat.

The high correlation between the total phenol content of PE and low-density-lipoprotein oxidation indicated that the high antioxidant activity of PE is related to their chemical compositions (Teissedre and Waterhouse, 2000). The presence of phenolic OH groups in thymol, carvacrol, and other PE act as hydrogen donors to the peroxy radicals produced during the first step in lipid oxidation, thus retarding the hydroxyl peroxide formation (Farag et al., 1989; Djeridane et al., 2006).

1.2.3. Plant Extracts on Growth Performance and Animal Health

Swine. Plant extracts have been proposed and reviewed as alternatives for in-feed antibiotics in the pig industry. Use of phytogenic products as feed additives for swine has been reviewed by Windisch et al. (2008). The inclusion of PE, especially EO during different periods of pig production falls within the scope of this discussion.

Sows are the physical engines of the swine industry and keeping them healthy and performing efficiently is a major challenge. Many studies support a beneficial effect of EO on sow's performance. Allan and Bilkei (2005) showed that sows fed diets containing 1000 ppm oregano (dried leaf and flower of *Origanum vulgare,* consisted of 50% cold-pressed essential oil of *O. vulgare*) had lower annual sow mortality rate, lower sow culling rate during lactation, increased farrowing rate, increased number of liveborn piglets per litter, and decreased stillbirth rate. Ilsley et al. (2002) reported that sows fed 100 ppm of the combination of several PE (1% spices capsicum, 1.25% cinnamaldehyde, and 0.85% oregano oil) had greater litter performance, such as heavier piglets and higher piglet average daily gain. However, no beneficial effect was observed from feeding sows *Yucca shidigera* and *Quillaja saponaria* extract (Ilsley et al., 2002), and oregano EO (Ariza-Nieto et al., 2011).

The supplementation of PE to the diet has resulted in a large variation in growth performance of newly-weaned pigs. Simonson (2004) used nursery pigs to identify potential benefits of several PE (horseradish, mustard, oregano, and cassia) in diets for newly weaned pigs. They found only mustard and cassia increased ADG, ADFI and G:F. Another study from Sads and Bilkei (2003) found that weaner piglets fed 1000 ppm of an oregano supplement had higher weight gain and lower disease incidence compared to the unsupplemented control animals. But, some other researchers found no beneficial effect of PE on weaned pig performance (Manzanilla et al., 2004; Neill et al., 2006; Nofrarías et al., 2006).

Although failing to find beneficial effect on productive performance, Manzanilla et al. (2004) and Nofrarías et al. (2006) suggested PE might improve gut health. They reported that a mixture of PE (XT) standardized to 5% (wt/wt) carvacrol, 3% cinnamaldehyde, and 2% capsicum oleoresin (oregano, cinnamon and Mexican pepper), increased stomach contents and

percentage of DM, suggesting an increased gastric retention time. In addition, the XT decreased ileal total microbial mass and increased the lactobacilli:enterobacteria ratio. Otherwise, Michiels et al. (2010) also indicated that supplementing with 500 ppm carvacrol and thymol reduced the number of intra-epithelial lymphocytes and increased villus height/crypt depth in the distal small intestine.

In the grower-finisher period, the application of different levels and different sources of PE shows some benefits on growth performance. Cullen et al. (2005) and Janz et al. (2007) reported pigs fed a garlic-treated diet had higher ADG, ADFI, and feed conversion ratio compared to the control diets. Grela et al. (1998) observed a significant improvement in ADG and feed conversion ratio with the use of an herb mixture (great nettle, garlic, wheat grass) in the diet of pigs from 25 to 105 kg. Dunshea et al. (2003) demonstrated an improvement in growth performance with the inclusion of vanillylnonamide, a capsaicin analogue, in the diets of finisher pigs. Otherwise, an experiment with growth-retarded low-weight growing-finishing pigs found that a diet with 3000 ppm commercial oregano containing 60 g carvacrol and 55 g thymol/ kg improved daily gain and feed conversion rate and reduced mortality of pigs (Walter and Bilkei 2004).

Poultry. Use of plant extracts in poultry has also been reviewed in the papers of Lee et al. (2004) and Windisch et al. (2008). The observed effects of PE on growth performance in chickens are either positive (Langhout, 2000; Ertas et al., 2005; Al-Kassie, 2009) or non-significant (Botsoglou et al., 2002; Hernández et al., 2004). The inclusion levels varied from 20 to 400 ppm. Al-Kassie et al. (2009) showed that chicks fed 200 ppm EO derived from thyme and cinnamon had significantly higher feed intake, body weight and feed conversion ratio than did controls. In addition, Ertas et al. (2005) showed that compared with the control group and

antibiotic group, the usage of 200 ppm essential oil mix derived from oregano, clove and anise in the broiler diets increased daily live weight (16% and 8%, respectively) and feed conversion ratio (12% and 6%, respectively). The authors indicated the improvement of growth performance might be due to enhanced enzyme activities and digestibility (Langhout, 2000; Hernández et al., 2004) caused by the active materials (i.e., thymol, carvacrol, cinnamaldehyde, and eugenol) in these plants (Kamel, 2001). On the other hand, Botsoglou et al. (2002) reported that 50 or 100 ppm of dietary oregano EO fed to rapidly growing broiler chickens for a period of 38 days did not affect body weight and feed conversion ratio. Hernández et al. (2004) also failed to find beneficial effects on growth performance when broilers were fed diets containing 200 ppm of an EO blend from oregano, cinnamon, and pepper.

Some relevant studies were conducted under commercial poultry production conditions. Alçiçek et al. (2003) observed the effects of supplementation of 24, 48, and 72 ppm essential oil combinations (EOC) on growth performance of broiler chickens. The results showed that the inclusion of 48 ppm EOC significantly improved the body weight, feed conversion ratio and carcass yield of broilers after a growing period of 42 days, but the higher level had no additional beneficial effect on these production traits. Moreover, Dalkiliç and Güler (2009) reported the effects of different levels (100, 200, and 400 ppm) of clove extract on performance and nutrient digestibility in broilers. The 400 ppm clove-supplemented group had the best feed conversion ratio and the best digestibility.

In addition, supplementation of PE has shown beneficial effects on the disease resistance of birds. Allen et al. (1997) reported that diets containing camphor or 1,8-cineole (5% *Artemisia anna* dried leaf) led to significantly increased weight gains when the birds were infected with coccidia, but no beneficial effect was found in uninfected birds. Mitsch et al. (2004)

demonstrated that PE reduced *Clostridium perfringens* infection, which causes necrotic enteritis in broilers. McElroy et al. (1994) and Vicente et al. (2007) stated that the dietary capsaicin administration (5, 20, or 36 ppm) increased resistance to *S. enteritidis* colonization and organ invasion throughout the normal growth period without detrimental effects on growth in broiler chickens and laying hens. The reduced bacterial infection or colonization might be explained by increased release of mucin and the creation of a thick layer of mucus on the small intestine of broilers fed PE (Jamroz et al., 2006) Otherwise, the enhanced local innate immunity may be also contribute to the greater protective immunity against bacterial infection when feeding dietary PE to broiler chickens (Lee et al., 2010). However, there is still a need for a systematic approach to explain the modes of action of these extracts. Kim et al. (2010) investigated the gene expression changes of intestinal intraepithelial lymphocytes after oral feeding of carvacrol, cinnamaldehyde or capsicum oleoresin by microarray analysis. They indicated that these phytonutrients exert significant effects on host immunity, protein and nuclear metabolism, and physiology by changing the expression of important genes related to these pathways.

Ruminants. Plant extracts have potential benefits on rumen microbial fermentation. Calsamiglia et al. (2007) extensively reviewed the essential oils as modifers of rumen microbial fermentation, especially in vitro or in situ. They concluded that some EO reduced ammonia nitrogen (N), methane, and acetate concentrations and increased propionate and butyrate concentrations by inhibiting deamination and methanogenesis. But these effects may vary depending on the different types and doses of EO or combination of EO supplemented, and different conditions under which these EO are used to modify rumen microbial fermentation. For example, Castillejos et al. (2006) reported that the addition of 50 mg/L of thymol had no effect on in vitro rumen microbial fermentation, but 500 mg of thymol/L reduced total VFA and

ammonia N and increased acetate-to-propionate ratio. The rumen fluid used in Castillejos et al. (2006) was from lactating dairy cows fed a 60:40 forage:concentrate diet with pH 6.4. However, Cardozo et al. (2005) reported reduction of acetate-to-propionate ratio when thymol was incubated in rumen fluid from cattle fed a 10:90 straw:concentrate diet at pH 5.5. In addition, many other in vitro studies also indicated that garlic oil, cinnamaldehyde, eugenol, capsicum, and anethol improved the fermentation profile of rumen microorganisms in continuous culture (Cardozo et al., 2004; Basquet et al., 2005; 2006).

Shaver and Tassoul (2008) reviewed the effects of plant extracts as dietary supplements for dairy cows. They summarized that feeding a mixture of natural and synthesized EO, including thymol, eugenol, vanillin, guaiacol, and limonene, may increase DMI and feed efficiency, milk yield and composition (fat and protein percentages), ruminal OM and N digestibility, ruminal pH and total tract ADF digestibility and reduce total VFA based on several reports (Benchaar et al., 2006; 2007; Yang et al., 2007). Otherwise, Bravo and Doane (2008) indicated that the supplementation of EO comprised of cinnamaldehyde and eugenol increased DMI and milk yield based on a meta-analysis of lactating dairy cow trials. However, another review from Benchaar et al. (2008) summarized the effects of EO in ruminant nutrition and production, and indicated that more in vitro and in vivo research is needed to clarify the mechanisms of these effects, and to find more active products.

In summary, several benefits from the use of PE in animals and the beneficial biological effects of PE have been discussed, but whether immunity of pigs is improved by PE remains unclear. Understanding of the mechanisms of PE in modulation of immune responses of pigs is necessary to gain maximum benefit from PE under practical applications. The data from in vitro studies suggest that PE may directly interact with immune cells and induce changes in

expression of molecules involved in immune regulation such as cytokines, chemokines, molecules in the signaling pathway etc. Thus, the special effects of PE on immune response need to be evaluated under various conditions.

1.3. The Immune System

Immunity is divided into two parts, the innate immunity and the adaptive immunity, based on the speed and specificity of the immune reaction, although in practice there is much interaction between them.

1.3.1. The Innate Immune System

The innate immune system encompasses the elements of the immune system, such as, neutrophils, monocytes, complement, cytokines, and acute phase proteins, which provide immediate host defense. Otherwise, this system also includes physical, chemical, and microbiological barriers (Parkin and Cohen, 2001).

The innate immune system gives protection against a broad variety of pathogens using a variety of pattern recognition receptors (PRR), which can be expressed on the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids (Janeway and Medzhitov, 2002). The PRRs can recognize pathogen-associated molecular patterns (PAMPs), such as microbial membranes, cell walls, proteins and DNA, and further, stimulate opsonization and phagocytosis, and activation of the complement system and proinflammatory signaling pathways. The PRRs include the members of toll-like receptors (TLRs) family and the nucleotide-binding oligomerization domain proteins (NOD-like receptors, NLRs). The TLRs are type I transmembrane proteins characterized by an extracellular leucine-rich domain and an

intracellular domain. So far, 13 mammalian TLRs have been identified (Albiger et al., 2007). Toll-like receptor 4 is the most extensively studied PRR and it recognizes a variety of ligands, such as mannan from yeast, host heat-shock proteins, envelope proteins from viruses, and LPS, the main component of the outer membrane of gram-negative bacteria. Toll-like receptor 5 recognizes flagellin, the major structural component of bacterial flagella. Toll-like receptor 3 recognizes the double-stranded RNA (dsRNA) that appears in cells after infection by RNA viruses, whereaa the TLR7 and TLR8 recognized the viral single-stranded RNA (ssRNA). Numerous studies have successfully mapped out the signaling pathways that are activated within the cell upon TLR-ligand recognition (O'Neill and Greene, 1998; Medzhitov, 2001). A simplified model of the current view of TLR signaling is depicted in Figure 1.6. The TLR-recognition leads finally to activation of the nuclear NF-κB and members of the mitogen-activated protein kinase (MAPKs) family, which directly regulate gene expression profiles following TLR stimulation. Jobin et al. (1999), Aggarwal and Shishodia (2004), Lee et al. (2005), and Choi et al. (2007) demonstrated that several PE can block the activation of NF-κB, and further suppress the signaling pathway of NF-κB. But the mechanism of action is still unclear.

Innate immune responses typically involve the participation of many different cell types, including neutrophils, macrophages, monocytes, natural killers (NK) cells, and dendritic cells (Dempsey et al., 2003). These cells migrate towards the source of the infection. Neutrophils are the first cells to migrate from the blood to sites of infection. The main functions of neutrophils include phagocytosis. But, the display of several TLRs and other pattern receptors on their surface allows neutrophils to directly recognize pathogens, engulf and digest them. In addition, neutrophils secrete antimicrobial peptides, include defensins and cathelicidins, cationic peptides with a broad range of antimicrobial activity (Parkin and Cohen, 2001). Macrophages are also

phagocytic cells that reside in many tissues and produce high levels of cytokines and chemokines that function as the "red alert" of infection. Besides killing and clearing pathogens, macrophages also play a role in the coordination of other cells and tissues of the immune and other supporting systems by the secretion of cytokines (Bilitewski, 2008). Numerous studies have shown the anti-inflammatory properties of PE, mainly through suppressing the cytokine production of macrophages, such as TNF-α, IL-1β, and nitric oxide (Hart et al., 2000; Hodge et al., 2002; Lang et al., 2004; Lee et al., 2007). In addition, Abe et al. (2003) demonstrated that EO suppressed the neutrophil adhesion through signal transduction.

1.3.2. The Adaptive Immune System

The innate response is rapid but lacks specificity, so sometimes it can damage normal tissues. The adaptive response is more precise and has memory, so subsequent exposure leads to a more vigorous and rapid response, but the development of the adaptive response takes several days or weeks (Dempsey et al., 2003). The adaptive response includes two stages. First, the antigen is recognized by antigen-presenting cells (APC) and presented to the antigen specific T or B cells leading to cell priming, activation, and differentiation, which usually occurs within the specialized environment of lymphoid tissue. Second, the activated T cells leave the lymphoid tissue to the disease site and the antibodies are released from activated B cells into blood and tissue fluids, and then to the infective place. In this immune system, three important components, APC, T lymphocytes, and B lymphocytes, drive the targeted effector responses (Parkin and Cohen, 2001).

An APC can be defined as any cell that expresses the major histocompatibility complex (MHC) that binds antigenic components and can be recognized by T cells (Knight and Stagg,

1993; Austyn, 2000). Macrophages, dendritic cells, and B cells are three main APC involved in the antigen presenting process. There are 2 ways in which APC recognize antigens and load onto their surface MHC. The antigen may have been produced endogenously within the cell (such as viral or tumor proteins) and is assembled with MHCI through the intracellular processing pathway. All nucleated cells can express MHCI, so any such cell can work as an APC. Alternatively, the exogenous antigen is combined with MHC II and presented to T cells or B cells (Knight and Stagg, 1993; Austyn, 2000). Antigen-presenting cells can internalize antigens by phagocytosis, endocytosis, or both. Macrophage and dendritic cells internalize antigens by both processes, whereas most other APC, like B cells, are poorly phagocytic or not phagocytic and therefore internalize antigen by receptor-mediated endocytosis.

The T cells can be divided into 2 populations according to their expression of CD4 and CD8 membrane molecules (Jenkins et al., 2001; Parkin and Cohen, 2001). $CD4^+$ T cells are T helper (Th) cells, which recognize antigen that is combined with class II MHC, whereas $CD8^+$ T cells recognize antigen that is combined with MHC I and function largely as cytotoxic cells. The MHC molecule associated with different T cells determines the type of effector response generated (Lippolis, 2008). For example, any nucleated cell that is infected with a virus or other intracellular pathogen, or is producing abnormal tumor antigens can assemble these antigens with MHCI and active CD8+ T cells, which remove this cell by cytotoxic attack (Williams and Bevan, 2007). Therefore, the CD8+ T cells responses are highly targeted to the cells they recognize. However, CD4+ T cells only can be activated by small parts of cells that express MHCII and the activation can lead to production of cytokines which in turn activate a wide range of cells around them. On stimulation, precursor Th0 cells become Th1 or Th2 cells, which can stimulate different immune responses by releasing different cytokines. Th1 cells secrete IFN-γ,

IL-2, TNF-α, and TNF-β, which induce mainly a cell-mediated inflammatory response. For example, IFN-γ activates macrophages to kill intracellular pathogens such as mycobacteria, fungi, and protozoa and induces natural killer cells to cytotoxicity. Tumor necrosis factor-α is one of most important pro-inflammatory cytokines that stimulates systemic inflammation and acute phase reaction. Th2 cells produce the cytokines IL-4, IL-5, IL-6, IL-10, and IL-13, which are essential for optimal antibody production and for the elimination of extracellular organisms. For instance, IL-4 induces class-switching in B cells to IgE production and provides positive feedback to induce further Th2 responses and suppress Th1 differentiation. Thus the Th2 response is associated with allergic disease (Jenkins et al., 2001; Szabo et al., 2003). Otherwise, IL-6 is an important cytokine that induces the synthesis of acute phase proteins by hepatocytes (Bode and Heinrich, 2001). The limited data from previous studies indicated that the supplementation of PE might enhance the immune response through increasing the percentage of CD4+, CD8+, CD4+CD8+, and MHCII+ in peripheral blood (Walter and Bilkei, 2004).

The B cells can be activated by 2 types of antigens: T cell-independent antigens and T cell-dependent antigens. The former antigens can activate B cells without the involvement of T cells. These antigens, such as, polysaccharides, can effectively cross-link surface receptors and initiate response even though they are not recognized by Th cells. However, the latter antigens need to bind to the B cells and are internalized into endosomal vesicles, assembled with MHCII, and then presented to Th cells. The interaction between Th and B cells stimulates B cell clonal expansion, isotype switching, affinity maturation, and differentiation into memory cells (Parkin and Cohen, 2001). The activated B cells can produce antibodies, which serve to neutralize toxins, prevent organism adhering to mucosa surfaces, activate complement, opsonise bacteria for phagocytosis, and sensitize tumor and infected cells for antibody-dependent cytotoxic attachment

by killer cells (Parkin and Cohen, 2001; Carroll, 2008). Thus antibodies also act to enhance elements of the innate system.

In summary, the interaction between PAMPs and PRRs initiates the innate immune response, leading to upregulation of both MHC I and MHC II and co-stimulatory molecules, as well as secretion of inflammatory cytokines. All of these factors drive the activation of T cells and B cells. The activated adaptive immune cells feed-forward stimulate innate cells to amplify antigen responses. Th1 cells activate macrophages through IFN-γ secretion and cell-cell contact, whereas Th2 cells activate B cells secrete antibodies to stimulate the cascade of complement proteins, phagocytes, NK cells, and mast cells. Therefore, the combination of activation of two arms of the immune system amplifies the immune responses effectively and efficiently against abnormal antigens in the host or foreign pathogens (Zhao et al., 2008).

1.4 *Escherichia coli* Infection

1.4.1. Definition of *Escherichia coli*

Escherichia coli (E. coli) was first discovered in 1885 by Theodor Escherich, who noted that *E. coli* are highly prevalent in the intestinal microflora of healthy individuals and have potential to cause disease when directly inoculated into extra-intestinal sites (Robins-Browne and Hartland, 2002). *Escherichia coli* is a Gram-negative rod-shaped bacterium that is commonly found in the intestine of animals. Most *E. coli* strains are harmless, but some others are the important cause of diarrhea and intestinal infection in both humans and animals. According to the different adhesion characteristics, virulence genes and mechanisms of pathogenicity, diarrheagenic *E. coli* can be divided into six groups: enterotoxigenic *E. coli* (ETEC),

enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enteroinvasive *E. coli*, diffusely adhering *E. coli* and enteroaggregative *E. coli* (Torres et al., 2005).

Escherichia coli postweaning diarrhea (PWD) is an important cause of death in weaned pigs. This diarrhea is responsible for economic losses due to mortality, morbidity, decreased growth performance, and cost of medication (Fairbrother et al., 2005; Nagy and Fekete, 2005). Enterotoxigenic *E. coli* are the most predominant types of pathogenic *E.coli* that cause diarrhea in both pre-weaning and post-weaning piglets (Hampson, 1994; Nagy and Fekete, 1999).

1.4.2. Clinical Signs

Clinical signs of ETEC infection in pigs include reduced appetite, depression, weakness, rapid dehydration, watery diarrhea (light orange-colored feces), anorexia, and shock due to hypovolemia and electrolyte imbalance (Bohl and Cross, 1971; Sarmiento et al., 1988; Nollet et al., 1999). The rectal temperature is normal. Many pigs show cyanotic discoloration of the tip of the nose, the ears, and the abdomen. Mild cases may recover spontaneously but several cases may result in death in about 12 hours, sometimes without external evidence of diarrhea. Dehydration of the carcass with distension of the small intestine by colorless, slightly mucoid fluid is characteristic of the infection. Enterotoxigenic *Eshcherichia coli* infection does not normally cause gross and histological lesions because the bacteria do not invade the mucosa; however, with optimal fixation, bacteria may be seen lining the epithelial cells of the intestine or adhered diffusely to the luminal surface of enterocytes. However, the toxins released by ETEC may induce physiological changes (Faubert and Drolet, 1992; Berberov et al., 2004).

1.4.3. The Pathogenesis of *E. coli* Infection

The pathogenesis of ETEC infection depends on two main virulence factors. First is the expression of fimbriae that enable bacteria to adhere to the small intestinal epithelial cells. Second, the colonized *E. coli* produce one or more endotoxins, such as heat-labile (LT), heat-stable (STa and STb) toxins, etc. They induce functional changes in the small intestinal epithelial cells, resulting in increased secretion of H_2O, Na^+ and Cl^- and decreased fluid absorption, leading to dehydration and acidosis (Weikel and Guerrant, 1985; Forte et al., 1992; O'Brien and Holmes, 1996). In this step, other components, including capsular polysaccharides, cell wall LPS and iron binding proteins may also be involved in the pathogenicity of these bacteria in the host (Gyles, 1993). Lipopolysaccharide and Shiga toxin induce disease through stimulating cytokine release or directly killing cells and organisms (Sandvig, 2001; Bannerman and Goldblum, 2003).

1.4.4. Fimbriae and Attachment of *E. coli*

Fimbriae are straight or kinky proteinaceous appendages from the outer membrane of the bacterial cells, which facilitate the adhesion of ETEC to small intestinal mucosa and facilitate the colonization of ETEC in the small intestine (Nagy et al., 1976; Isaacson et al, 1978; Morris et al., 1982). Epidemiological studies have shown that many kinds of fimbria may be expressed in ETEC isolated from piglets with diarrhea, including F4 (K88), F5 (K99), F6 (987p), F18, and etc. Among these, F4 (K88) and F18 are the 2 best studied fimbria of animal ETEC.

Fimbriae F4 are typically associated with diarrhea in nursing pigs as well as in weaned pigs. In neonatal pigs, ETEC carrying F4 fimbriae tend to colonize throughout the whole length of the small intestine (Nagy et al., 1976). The F4 fimbrial adhesin is a filamentous surface appendage composed of repeating protein subunits, FaeG, that enable $F4^+$ ETEC to bind to

specific receptors on intestinal brush border cells (Van den Broeck et al., 2000; Fairbrother et al., 2005; Nagy and Fekete, 2005). Three antigenic variants of F4 fimbria have been identified: F4ab, F4ac, and F4ad. Among these, F4ac variant is the most common one that was found in pathogenic *E.coli* isolates from a major pork-producing region of the United States (Fairbrother et al., 2005; Nagy and Fekete, 2005). The adhesion receptors of F4 fimbriae appear to be glycoconjugates, including glycoproteins and glycolipids, which have been identified from the brush borders of epithelial cells, intestinal membranes, and mucosa (Blomberg et al., 1993; Grange et al., 1999). It is interesting to note that F4ad adhesin appears to preferentially bind to glycolipids, whereas F4ab and F4ac adhesins preferentially bind to glycoproteins (Erickson et al., 1994; Grange et al., 1999; Jin and Zhao, 2000).

Fimbriae F18 is associated with *E.coli* strains isolated from post-weaning diarrhea and oedema disease in pigs. These fimbriae are long flexible appendages that show a characteristic zigzag pattern (Nagy et al., 1997). Based on morphological, serological, functional and genetic characteristics, two antigenic variants of the F18 fimbriae were determined and designated: F18ab and F18ac (Rippinger et al., 1995). The F18ab-positive strains are usually isolated from cases of oedema disease, whereas F18ac-positive strains are associated with the cases of post-weaning diarrhea (Wittig et al., 1995; Nagy et al., 1997). The F18 fimbriae are composed of protein subunits (FedA) with molecular weights of approximately 15.1 kDa (Imberechts et al., 1992). Five structural genes (*fed*A, *fed*B, *fed*C, *fed*E, and *fed*F) encoded on a plasmid have been identified (Smeds et al., 2001). Among these genes, the *fed*E and *fed*F genes are essential for F18 adhesion and fimbrial length (Imberechts et al., 1996). However, receptors for F18 fimbriae actually increase with age and have not been detected in newborn pigs (Nagy et al., 1992). This

may in part explain the reason why ETEC strains carrying F18 are more prevalent in weaned pigs.

To effectively colonize a host animal and cause disease, it is necessary for bacterial pathogens to bind to host cells and tissues. Adhesion is required for several reasons: keeping the bacteria from being swept away by the natural cleansing mechanism of the host; providing the pathogens with better access to sources of nutrition; facilitating the delivery of toxins into the host tissues; and helping the bacteria penetrate into the tissues. Thus, the binding of pathogenic bacteria to the small intestinal wall is absolutely necessary for the process of pathogenesis. But we still do not know the detailed mechanism.

1.4.5. Toxin Effects

After adhering to the small intestinal surface, ETEC induce diarrheal disease through release of enterotoxins which stimulate copious secretion by the mucosa of the small intestine. The enterotoxins include LT, ST, LPS, and Shiga toxin.

Heat-labile toxins. Heat-labile toxin mainly accumulates in the periplasmic space, only a little amount of this toxin can be found on the surface of the bacteria. Heat-labile toxin consists of a single A subunit and five B subunits. The B subunits bind predominantly to the monosialotetrahexosylganglioside (GM1) ganglioside on the cell surfaces and fix the toxin (O'Brien and Holmes, 1996). After that, a fragment of A domain will translocate into the cell and activate the adenylate cyclase system and increase cyclic adenosine monophosphate (cAMP) levels, which mediate several different changes. First, cAMP stimulates the phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR), which cause chloride secretion from the apical region of enterocytes (Thiagarajah and Verkman, 2003). Second, cAMP

stimulates the activation of an apical chloride channel and a basolateral Na/K/2Cl cotransporter, which result in the release of prostaglandin E2, release of vasoactive intestinal peptide (VIP), and loosening of tight junctions (Nataro and Kaper, 1998; de Haan and Hirst, 2004). These activities all contribute to increased chloride secretion, reduced sodium absorption, and a concomitant massive loss of water into the intestinal lumen. The effect of LT is irreversible (O'Brien and Holmes, 1996).

Heat-stable toxin. Heat-stable toxins are further classified as STaH found in human isolates, STaP found in porcine and bovine isolates, and STb found in porcine isolates (Gyles, 1994; Nataro and Kaper, 1998). Heat-stable toxin a is a small, nonimmunogenic protein with a molecular weight of approximately 2 kDa (Lallier et al., 1982; Lazure et al., 1983). The major receptor for STa is a particular transmembrane form of guanylate cyclase (GC-C) (Schulz et al., 1990; de Sauvage et al., 1991). The biological activity of STa is exerted through stimulation of the GC-C system, leading to excessive levels of cyclic guanosine monophosphate (cGMP) in enterocytes. Signals resulting from cGMP accumulation lead to activation of CFTR and elevated secretion of Cl^- and H_2O in crypt cells and inhibition of Na^+ and Cl^- absorption from cells at the tips of villi (Forte et al., 1992). Heat-stable toxin a can induce small intestinal fluid secretion in newborn but not in weaned pigs, all in contrast to STb which can induce fluid secretion in both age groups of pigs (Nagy and Fekete, 1999).

Heat-stable toxin b is a 48-amino acid protein with a molecular weight of approximately 5.1kDa, which has no homology to STa (Arriaga et al., 1995). Heat-stable toxin b is antigenically and genetically unrelated to STa and poorly immunogenic (Dubreuil, 1997). Production of STb is restricted to porcine ETEC (Gyles, 1994). The mechanism of action and molecular characteristics of STb are still less known than LT and STa. Heat-stable toxin b does not

stimulate an increase in intracellular levels of either cAMP or cGMP, either Na^+ or Cl^- (Weikel et al., 1986), but does stimulate the secretion of HCO_3^- from intestinal epithelial cells (Argenzio et al., 1984; Weikel and Guerrant, 1985). Heat-stable toxin b toxin causes mild histological damage in the intestinal epithelium, including loss of villous epithelial cells and partial villous atrophy. This damage may be responsible for impaired absorption of fluids (Whipp et al., 1987). Another proposed mechanism of action is to increase the level of prostaglandin E_2 (Harville and Dreyfus, 1995). Otherwise, it has been found that STb opened a G-protein-linked receptor-operated calcium channel in the plasma membrane, thus elevating intracellular Ca^{++}, which activates the prostaglandin endoperoxidase synthetase system, leading to the formation of prostaglandins (Dreyfus et al., 1993).

LPS. Lipopolysaccharide is the major surface component of outer membrane of most Gram-negative bacteria, including ETEC (Alexander and Rietschel, 2001). Lipopolysaccharide consists of three distinct regions: Lipid A, with low structural variability; a core oligosaccharide with little variability; and the O-antigen polysaccharide, with high variability. The lipid A anchors the molecule in the outer membrane and is the primary immunostimulatory center of LPS due to the highly sensitive recognition of lipid A by numerous cellular signaling pathways of innate immunity. The receptors that respond to LPS are mainly located on the cells in the innate immune system, such as macrophages and endothelial cells (Raetz and Whitfield, 2002). Therefore, LPS not only participates in the physiological membrane functions but also plays an important role in the pathogenesis of gram-negative bacterial infection (Raetz and Whitfield, 2002). LPS induces pathological effects mainly through stimulating the immunological cells of the host, resulting in the release of large amounts of cytokines, which play critical role in regulating the immune system.

A prerequisite for the activation of cells by LPS is the binding of LPS to the surface of LPS-responsive target cells. The CD14 and LPS binding protein (LBP) are involved in this process. The CD14 are membrane glycoproteins (mCD14) or soluble molecules (sCD14), expressed on monocytes or macrophages. In the case of LPS binding to the cell surface, LBP and sCD14 play important roles in facilitating the interaction of LPS and CD14. After binding to mCD14, LPS is delivered to the other LPS receptors on the cell surface, such as TLR and MD2, which is a protein physically associated with TLR4. Both TLR4 and MD2 are involved in LPS signaling. The complex of TLR4 and MD2 transduce intracellular LPS signals via several signal pathways (Wiese et al., 1999), ultimately resulting in the target cells responding to LPS by activation of NFκB and subsequently the stimulation of the release of TNF-α, IL-1, and IL-6 (Bannerman and Goldblum, 2003).

Shiga toxin. Some strains of ETEC that cause PWD possess additional genes that encode Shiga toxin, allowing them to cause edema disease as well (Nagy and Fekete, 1999). Shiga toxin is a large group of protein toxins, which can kill cells after binding to the cell surface. Similar to LT, Shiga toxin consists of one A subunit and five B subunits.

Completely different mechanisms are involved in their infection of cells. Shiga toxin binds to only a few types of cells, which possess the glycolipid receptors for Shiga toxin, globotriaosylceramine (Gb)3 or Gb4 (Paton and Paton, 1998). In Gb3 and Gb4, both carbohydrate and lipid components play important roles in regulating the binding of Shiga toxin to the cell surface (Sandvig and Van Deurs, 1996). After binding to the cell surface, Shiga toxin is transported to the Golgi apparatus through endocytosis. Then, the Golgi is responsible for transporting Shiga toxin to the endoplasmic reticulum, in which the subunit A is cleaved into A1 and A2 subunits by trypsin. The A1 subunit is the enzymatically active fragment that is released

into the cytosol and exerts its effects on ribosomes (Sandvig, 2001). Shiga toxin has the ability to inhibit protein synthesis and induce synthesis of cytokines, including IL-1, IL-6, IL-8, and TNF-α (Hughes et al., 2001; Paton and Paton, 1998). Otherwise, Shiga toxin also induces DNA degradation and release of the cellular contents, which facilitates proteolytic attack on neighboring cells and contributes to cell apoptosis and a toxic effect in the whole organism (Sandvig, 2001).

In summary, postweaning E. coli diarrhea in pigs, characterized as anorexia, depression, rapid dehydration, decreased growth performance, and increased mortality, remains a major cause of economic losses for the pig industry. The attachment of E. coli in the small intestine induces the secretion of one or several toxins, such as LT and ST that cause the imbalance of the small intestine secretion and diarrhea. Another important molecule on the surface of Gram-negative bacteria, LPS, stimulates the activation of the innate immune system, and subsequently the adaptive system.

1.5. Porcine Reproductive and Respiratory Syndrome

Porcine reproductive and respiratory syndrome. Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important diseases. The characteristics of this syndrome are reproductive failure in late-term gestation in sows, respiratory disease in pigs of all ages, decreased performance, and increased mortality (Hall, 2005). The syndrome was first recognized in the mid-1980's as "Mystery disease" or "Blue-ear Disease" in North America and Europe. However, the causative agent was not clear until 1991 Dutch researchers identified the PRRS virus (Wensvoort et al., 1991).

The PRRS virus (PRRSV) is the causative agent of this disease. Porcine reproductive and respiratory syndrome virus is an enveloped virus, with a 15 kb single-stranded positive-sense RNA genome (Meulenberg et al., 1997; Van Breedam et al., 2010). Two genotypes are recognized (American and European), and both are thought to derive from a common ancestor, but genetic similarity between these two genotypes is about 55-56% (Meng et al., 1995; Dea et al., 2000). In addition, genetic diversity of strains within a given genotype is high (Forsberg et al., 2002; Mateu et al., 2003). Porcine reproductive and respiratory syndrome virus is approximately 50-65 nm in diameter, belongs to the family *Arteriviridae,* and is grouped along with the *Voronaviridae* and the *Roniviridae* in the order of the *Nidovirales* (Cho and Lee, 2006). In vivo, the virus shows a very narrow cell tropism and targets specific subsets of porcine macrophages, porcine alveolar macrophages (PAM) (Mardassi et al., 1994; Van Breedam et al., 2010).

The attachment and internalization of PRRSV is the first step of PRRSV infection. Recently, three PRRSV receptors have been identified on PAM: heparin sulphate, sialoadhesin (Sn; CD169) and CD163 (Delputte et al., 2002; Vanderheijden et al., 2003; Calvert et al., 2007). The binding of virus will lead to uncoating and release of viral RNA into the cytoplasm, RNA replication, and infection (Calvert et al., 2007).

Clinical signs. Depending on the class of animal infected, prior exposure history and the virulence of the strain of PRRSV, the clinical signs vary widely. General signs include reproductive failure, fever, lethargy, decreased appetite and respiratory distress. However, many different factors also can affect the clinical signs of PRRS. First, the intensity of the disease appears to vary among isolates and variation in the pathogenicity of PRRSV virulence. Halbur et al. (1995 and 1996) found that pigs experimentally infected with 9 different isolates of PRRSV (from USA) had many differences in clinical disease, rectal temperatures, and lung lesions. In

these studies, animals infected with mildly virulent isolates had transient pyrexia, dyspnea, and tachypnea, whereas infection with highly virulent isolates resulted in pyrexia, lethargy, and anorexia. Secondly, animal age can influence virus replication and clinical signs. Younger pigs (4-8 weeks) infected with PRRSV had a longer viremia, and higher excretion rates and replication rates in macrophages compared to the older pigs (16-24 weeks) (Thanawongnuwech et al., 1998; van der Linden et al., 2003). In addition, Bacterial co-infection also can influence PRRSV infection. Certain bacterial agents, e.g. *Bordetella bronchiseptica* and *Mycoplasma hypneumoniae* appeared to enhance the duration and severity of PRRSV-induced pneumonia and lung lesions (Thacker et al., 1999; Brockmeier et al., 2001). On the other hand, PRRSV infection also can increase the susceptibility of pigs to *Streptococcus suis* type 2 infection and enhance the severity of *Salmonella choleraesuis* infection (Wills et al., 2000; Feng et al., 2001).

Immune responses. The course of PRRSV infection can be divided into three stages: 1) acute infection characterized by systemic infection centered in the lung and lymphoid tissues, viremia, and seroconversion; 2) chronic persistent infection characterized by declining antibody titers and declining levels of virus replication in lymphoid tissues; and 3) clearance of infectious virus by immune mechanisms (Molina et al., 2008). Porcine reproductive and respiratory syndrome virus has a complex interaction with the immune system because the primary targets of PRRSV are immune cells, such as, macrophages, monocytes, and dendritic cells.

The innate immune responses of the host against PRRSV involve alveolar macrophages, pulmonary epithelial cells, natural killer cells, dendritic cells and IFN-α/β responses in the lung. These responses affect the initial virus infection and also regulate adaptive responses. It has been reported that PRRSV can attenuate innate immune responses, evade the antiviral cytokine (IFN-α) response, and block IFN-α production in the cytoplasm of infected alveolar macrophages

(Borghetti et al., 2011). Many studies reported that macrophages infected with PRRSV demonstrate reduced phagocytic activity, microbial killing, production of reactive oxygen species, (e.g., superoxide anion and hydrogen peroxide) and production of antiviral cytokines (e.g., TNF-α and IFN-α) compared to uninfected macrophages (López-Fuertes et al., 2000; Borghetti et al., 2011).

Apoptosis is often considered as an innate defense mechanism that limits virus infection by elimination of infected cells (Everett and McFadden, 1999). Therefore, many viruses inhibit apoptosis to prevent premature cell death and thus increase viral replication. However, it has been reported that PRRSV infection induced the apoptosis of infected macrophages as well as uninfected bystander lymphocytes (Miller and Fox, 2004; Charerntantanakul et al., 2006; Colsters et al., 2008). In these studies, a high percentage of monocytes, macrophages, and immature dendritic cells were dead after 48 h of inoculation with PRRSV, and the percentage of APC death was much higher than the percentage of APCs infected with virus, maybe due to bystander cell death (Charerntantanakul et al., 2006). The APC apoptosis might be related with the delayed adaptive immune response in PRRSV-infected pigs.

The poor innate immune responses induced by PRRSV compromise the following onset and development of the antigen-specific adaptive immune response. The weak adaptive immune responses are characterized as weak cell-mediated immune responses, delayed appearance of neutralizing antibody, often prolonged viremia and persistent infection of pigs (Murtaugh et al., 2002; Miller and Fox, 2004; Mateu and Diaz, 2008). Pertaining to the development of adaptive immunity, circulating antibodies can be detected as early as 5 to 14 days post infection, but most of them are specific to non-neutralizing epitopes of the virus (Yoon et al., 1995; Mulupuri et al., 2007). Neutralizing antibodies to PRRSV appear much later, approximately 4 wk after infection,

and have relatively low titers throughout the course of infection (Yoon et al., 1995; Mulupuri et al., 2007). Anti-PRRSV IgM antibodies appear in serum by 5-7 days post inoculation (PI) and then decline rapidly to undetectable levels after 2-3 weeks PI (Loemba et al., 1996; Joo et al., 1997). Anti-PRRSV IgG antibodies are first detected 7-10 PI, peak at 2-4 weeks PI, remain constant for a period of months, and decrease to low levels by 300 days PI (Yoon et al., 1995; Loemba et al., 1996; Joo et al., 1997). Anti-PRRSV IgA can be detected in serum at 14 days PI, reaches a maximum at 25 days PI, and remains detectable until 35 days PI (Labarque et al., 2000).

The cell-mediated immune response to PRRSV is extremely late compared to other swine viral agents, which appear within 3 d to 1 week PI (López Fuertes et al., 1999). Porcine reproductive and respiratory syndrome virus causes a transient decline of $CD4^+$ and $CD8^+$ T cells in peripheral blood 3 days PI (Shimizu et al., 1996; Nielsen and Bøtner, 1997). The decrease of $CD4^+$ T cells lasts at least 2 weeks PI, whereas the decrease of $CD8^+$ T cells lasts 4 weeks after infection (Shimizu et al., 1996; Lamontagne et al., 2003). Following a decrease, the population of $CD8^+$ T cells, primarily $CD4^-CD8^+$, increases significantly in peripheral blood and bronchoalveolar lavage fluid (Shimizu et al., 1996; Samsom et al., 2000; Lamontagne et al., 2003). In addition, Charerntantanakul et al., 2006 determined that PRRSV had the ability to suppress T cell responses, by reducing IFN-γ and TNF-α production. It has been reported that IFN-γ plays a critical role in cell-mediated immune responses. Interferon-γ not only mediates protection against viral infections in vivo, but also blocks PRRSV replication in cultured cells in vitro (Bautista et al., 1999; Schroder et al., 2004).

The poor innate and adaptive immune responses to PRRSV have been suggested to be attributable at least in part to virus-induced IL-10 production (Suradhat et al., 2003;

Charerntantanakul and Kasinrerk, 2010). Porcine reproductive and respiratory syndrome virus has been reported to upregulate IL-10 production correlated with reduced IFN-γ production in virus-infected cells (Suradhat et al., 2003; Flores-Mendoza et al., 2008). In pigs, IL-10 has been demonstrated to suppress IL-2, IL-4 and IFN expression by peripheral blood mononuclear cell (PBMC), Th1, Th2, and NK cells (Charerntantanakul et al., 2006). Charerntantanakul and Kasinrerk (2010) also proved the suppression of IL-10 expression in PBMC can increase the percentage of the IFN-γ+ population in lymphocytes and CD8β+ T cells.

In summary, PRRS is an infectious disease caused by PRRSV and characterized by failure of reproduction in pregnant sows, respiratory problems, decreased pig performance, and increased mortality of growing pigs. The primary targets of PRRSV are macrophages, monocytes, and dendritic cells in the lung, later spread to other lymph organs. It causes leucopenia and lymphopenia after 14 days PI and a weak initial innate response due to the apoptosis of APC and decreased pro-inflammatory cytokine production. Porcine reproductive and respiratory syndrome virus infection also induces delayed and reduced humoral and cell-mediated immune responses due to the increased expression of the anti-inflammatory cytokine, IL-10 and decreased pro-inflammatory cytokines, such as TNF-α and IFN-γ. The modified immune responses might induce prolonged viremia and persistent infection of pigs.

SUMMARY

Weaning is one of the most challenging and critical stages in swine production due to the abrupt exposure of piglets to a combination of stressors. Weaning is generally related to low and variable feed intake, decreased performance, and high sensitive to digestive disease, such as *E. coli* infection, and respiratory disease, such as PRRS. In recent decades, many strategies, such as,

nutrition, genetics, and management have been applied to improve production. In addition, in-feed antibiotics have been used as growth promotants and for therapeutic treatment of gastrointestinal diseases in newly weaned piglets. Because of concerns about residues in animal products and bacterial resistance to antibiotics, alternatives to in-feed antibiotics are needed. Various feed additives, such as plant extracts have been studied as alternatives to antibiotics in newly weaned piglets. Plant extracts are secondary plant metabolites and can be obtained naturally from parts of plant materials. Based on the diversity of sources and extraction methods, PE may exert different influences in vivo and in vitro. This diversity of PE prompted us to select pure principles for evaluating their possible role as alternatives to antibiotics in swine production. Plant extracts have shown potential anti-inflammatory effects on human or rat cells. However, the potential anti-inflammatory effects of PE on porcine cells remain to be elucidated. Otherwise, the changes in intestinal microorganisms by PE have been known; however, their effects on humoral and cell-mediated immunity have not been clearly understood yet. Particularly, there is little information about the effects of PE on immune responses of weaned pigs reared under disease challenge conditions. More efforts, therefore, need to be made so as to fully understand the role of PE involved in regulation of the immune system and in prevention of bacterial and viral infection. This will help at least to substantially reduce the amount of antibiotics in animal feeds, and at the same time maintain animal health and performance at the optimal level.

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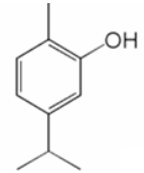
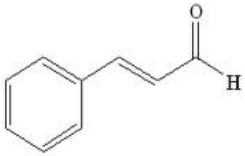
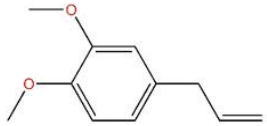
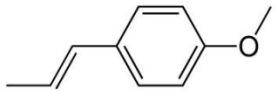
Tables and Figures

Table 1.1. An incomplete list of potential dietary technologies to improve pig health and productive performance (Adapted from Pettigrew, 2006)

Energy & protein sources	Additives	Feeding management
Spray-dried plasma	Immune egg products	Low-protein diets
Milk protein products	Mannan oligosaccharides	Restricted feeding
Egg products, conventional	Fructo-oligosaccharides	Fermented liquid feed
Fibrous ingredients	Other oligosaccharides	
Rice	Probiotics	
Lactose	Essential oils & plant extracts	
N-3 fats	Acids	
Medium-chain fats	Zinc oxide	
	Copper sources	
	Yeasts/yeast products	
	Bacteriocins	
	Bacteriophage	
	Enzymes	
	Glutamine/glutamate	
	Conjugated linoleic acid	
	Antioxidants	

Table 1.2. Chemical properties of several essential oil constituents carvacrol, cinnamaldehyde, eugenol, and anethol[1]

	Carvacrol	Cinnamaldehyde	Eugenol	Anethol
Molecular weight	150 $C_{10}H_{14}O$	132 C_9H_8O	164 $C_{10}H_{12}O_2$	148 $C_{10}H_{12}O$
Synonym	2-methyl-5-(1-methylethyl) phenol	3-phenyl-2-propenal	4-Allyl-2-methoxyphenol	1-methoxy-4-(1-propenyl)benzene
Found in	Oregano (*Lamiaceae*)	Cinnamon (*Lauraceae*)	Clove (*Eugenia aromaticum*)	Anise *(Pimpinella anisum)*
Appearance	Colorless to pale yellow liquid	Slightly yellow liquid	clear to pale yellow liquid	Solid
Odor	Thymol-odor like	Cinnamon	Clove	Anisic odor
Boiling point	237	246	256	234
Density, g/mL	0.976	1.048	1.060	0.998
LD_{50}	810 mg/kg, orally rat	2,220 mg/kg, orally rat	1,930 mg/kg, orally rat	2,090 mg/kg, orally rat
Stability	Good	Fair to poor	Good	Good
Structure				
Biological activity	Antimicrobial Antiinflammatory Antimelanomic Antioxidant Carminative Flavor	Antimicrobial Antiinflammatory Antipasmodic Antiurease Cancer-preventive Flavor Hyperglycemic	Antimicrobial Antiinflammatory Antioxidant Antifungal Flavor	Antimicrobial Antiinflammatory Antifungal Antihelmintic Flavor

[1]adapted from Lee et al., 2004a

Table 1.3. Plant extracts and their main components exhibiting antimicrobial activities

Scientific name	Common name	Main components	References
Allium saticum	Garlic	Allicin	Ankri and Mirelman, 1999
Origanum vulgare spp. *hirtum* (Link) (Ietsw.) *Origanum onites* *Origanum minutiflorum*	Oregano	Carvacrol	Sivropoulou et al., 1996; Burt and Reinders, 2003; Baydar et al., 2004; Xu et al., 2008
Capsicum	Pepper	Capsaicin	Tellez et al., 1993; McElroy et al., 1994
Cinnamomum verum J. Presl *Cinnamomum osmophloeum*	Cinnamon	Cinnamaldehyde	Ouattara et al., 1997; Chang et al., 2007; Wong et al., 2008
Funicular vulgare	Fennel	Anethol Eugenol	Abed, 2007
Zingiber officinale	Ginger	Curcumin	Smith-Palmer et al., 1998
Syzygium aromaticum (L.) Merr. & Perry *Eugenia caryophyllata*	Cloves Fennel	Eugenol	Hammer et al., 1999; Burt and Reinders, 2003; Ozcan et al., 2006
Thymus vulgaris L. *Thymbra spicata*	Thyme Fennel	Thymol	Aktug and Karapinar, 1986; Marino et al., 1999; Dorman and Deans, 2000; Burt and Reinders, 2003; Baydar et al., 2004; Ozcan et al., 2006; Xu et al., 2008

Table 1.4. Plant extracts and their main components exhibiting anti-inflammatory activities

Scientific name	Common name	Main components	Anti-inflammatory effects	References
Allium saticum	Garlic	Allicin	Suppress NF-κB expression; Inhibit IL-1β, IL-8, IP-10, and MIG production; Inhibit iNOS activity	Dirsh et al., 1998; Schwartz et al., 2002; Aggarwal and Shishodia, 2004; Lang et al., 2004;
Syzygium aromaticum (L.) Merr. & Perry *Eugenia caryophyllata*	Cloves Fennel	Anethol Eugenol	Inhibit TNF-α and IL-1β secretion; Suppress NF-κB expression; Reduce PGE2 production; Suppress iNOS and COX-2 expression	Kim et al., 2003; Aggarwal and Shishodia, 2004; Li et al., 2006; Choi et al., 2007; Lee et al., 2007; Daniel et al., 2009; Dung et al., 2009
Capsicum	Pepper	Capsaicin	Suppress NF-κB expression	Aggarwal and Shishodia, 2004
Origanum vulgare	Oregano Thyme	Carvacrol	Suppress COX-2 and NF-κB expression	Kroismayr et al., 2008; Landa et al., 2009
Cinnamomum verum J. Presl *Cinnamomum osmophloeum*	Cinnamon	Cinnamaldehyde	Inhibit IL-1β & IL-6 production; Suppress iNOS and COX-2 expression; Inhibit lymphoproliferation	Koh et al., 1998; Lee et al., 2002; Chao et al., 2005; Lee et al., 2005; Chiang et al., 2006; Chao et al., 2008; Lin et al., 2008; Tung et al., 2008
Zanthoxylum schinifolium	Rutaceae	Citronellal *β*-Phellandrene	Suppress iNOS and COX-2 expression; Inhibit IL-1β secretion	Lee et al., 2009
Zingiber officinale	Ginger Turmeric	Curcumin Gingerol	Suppress NF-κB expression; Block NF-κB signal pathway	Jobin et al., 1999; Aggarwal and Shishodia, 2004
Punica granatum	Pomegranate	Ellagic acid	Suppress NF-κB expression	Aggarwal and Shishodia, 2004
Thymus vulgaris L. *Thymbra spicata*	Thyme Fennel	Thymol	Suppress NF-κB expression	Aggarwal and Shishodia, 2004

Table 1.5. Plant extracts and their main components exhibiting anti-oxidant activities

Scientific name	Common name	Main components	References
Thymus vulgaris *Thymus caoitatus* *Thymus floribundum* *Thymus munbyanus* *Thymus guyonii* *etc.*	Thyme	Thymol Carvacrol Terpinene	Aeschbach et al., 1994; Hazzit et al., 2006; Miguel et al., 2004; Farag et al., 1989a; Schwarz et al., 1996; Teissedre and Waterhouse, 2000
Zingiber officinalis	Ginger	Zingerone Gingerol	Aeschbach et al., 1994
Foeniculum vulgare	Fennel	Anethole	Anwar et al., 2009; Waterhouse, 2000
Capsicum annuum *Capsicum chinese*	Pepper	Capsaicin	Kogure et al., 2002; Oboh et al., 2007
Origanum acutidens *Origanum vulgare* *Origanum floribundum* *Origanum glandulosum* *etc.*	Oregano	Carvacrol	Hazzit et al., 2006; Sökman et al., 2004; Srihari et al., 2008; Waterhouse, 2000
Eugenia caryophyllus Spreng. *Eugenia caryophylata* Thunb	Clove	Eugenol	Gülçin et al., 2004; Waterhouse, 2000
Cinnamomum verum	Cinnamon	cinnamaldehyde	Frankič et al., 2010; Mathew et al., 2006

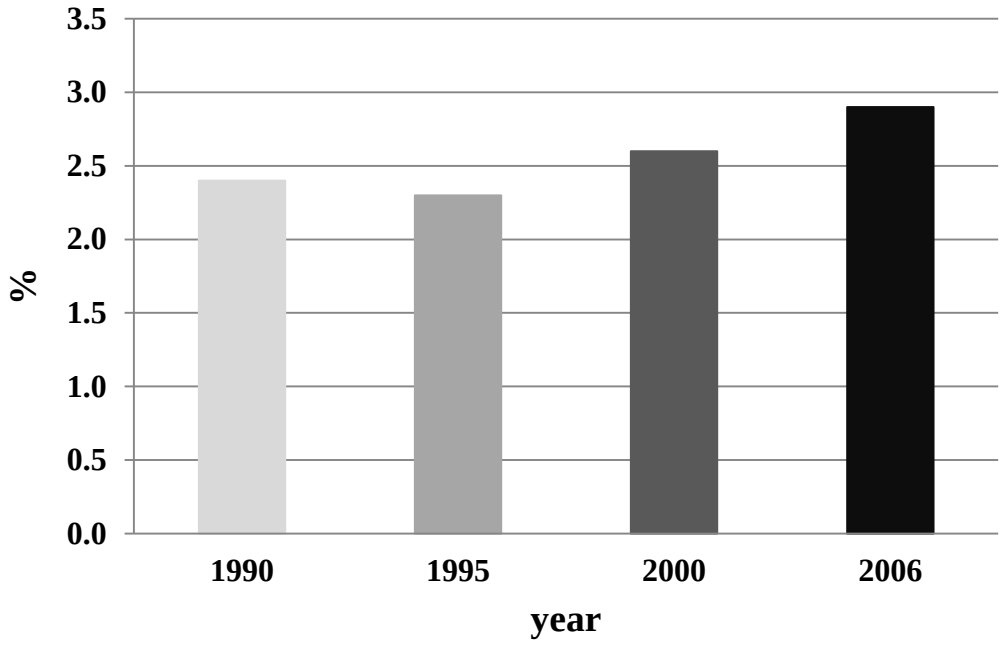


Figure 1.1. Post-weaning pigs mortality (Adapted from NAHMS, 2008)

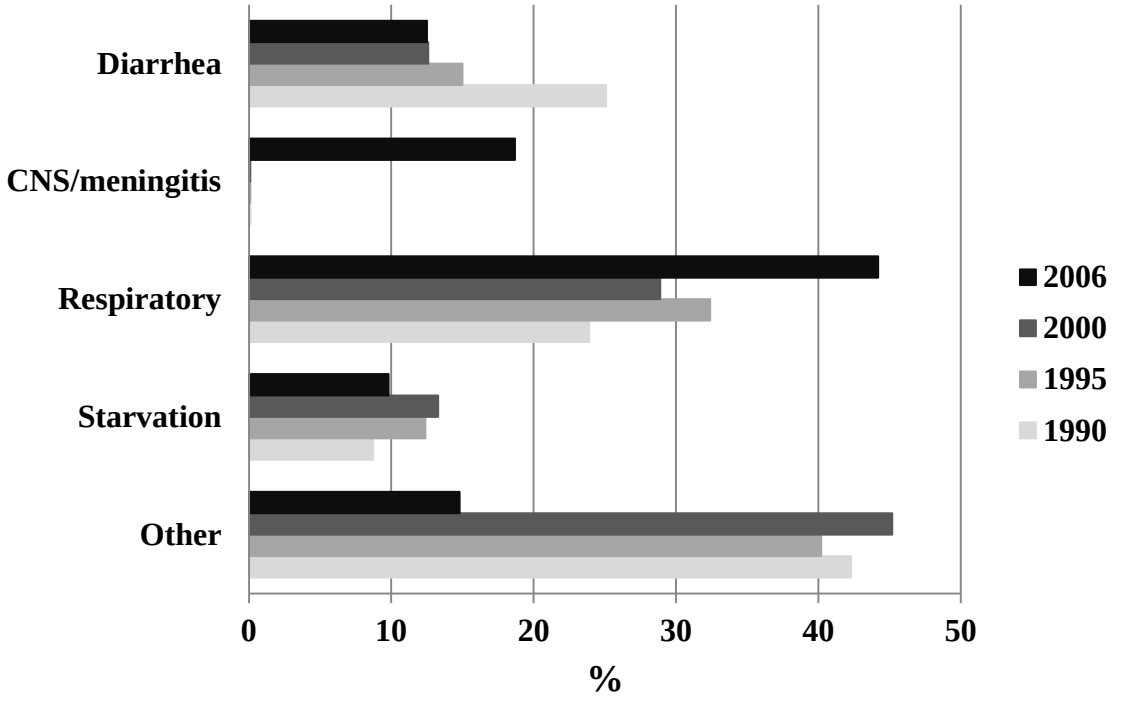


Figure 1.2. Causes of pig deaths in post-weaning period by producer (Adapted from NAHMS, 2008)

* Other includes other known and unknown problems.

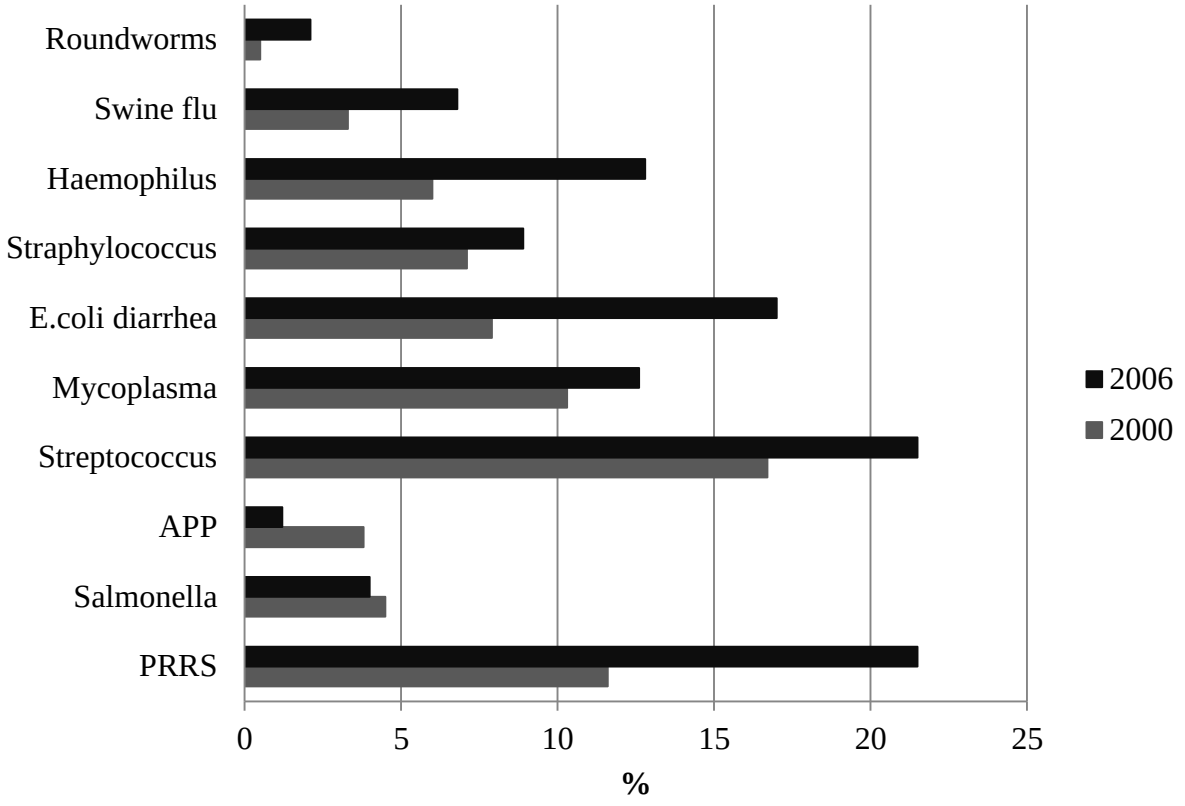


Figure 1.3. Causes of pig deaths in post-weaning period by veterinarian or laboratory (Adapted from NAHMS, 2008)

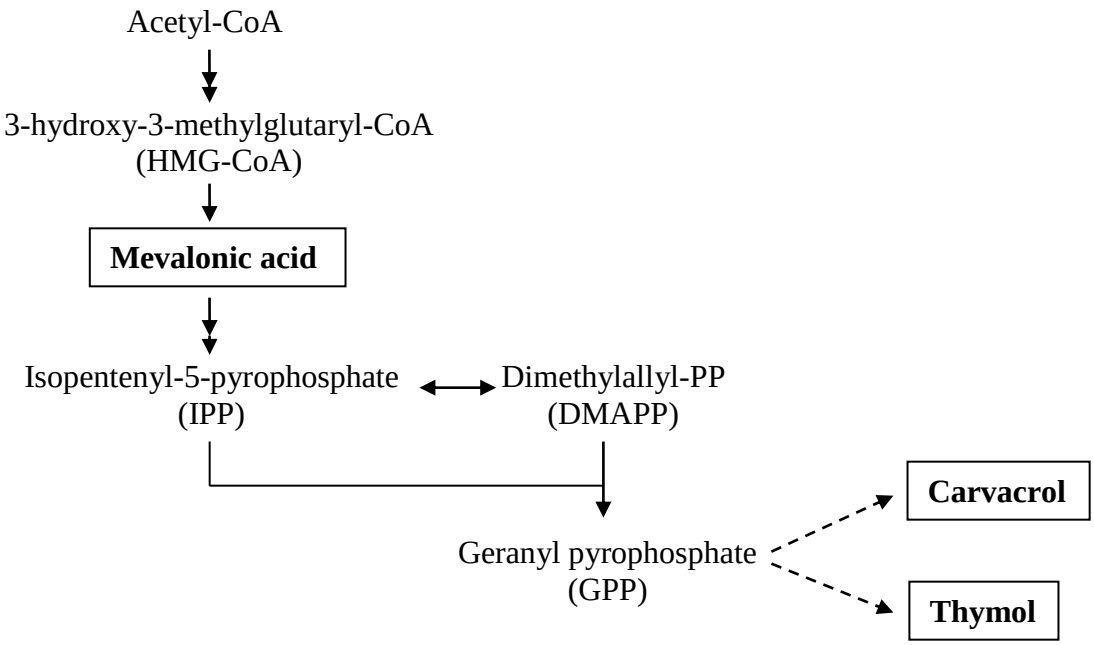


Figure 1.4. The mevalonic acid pathway (Miziorko, 2011)

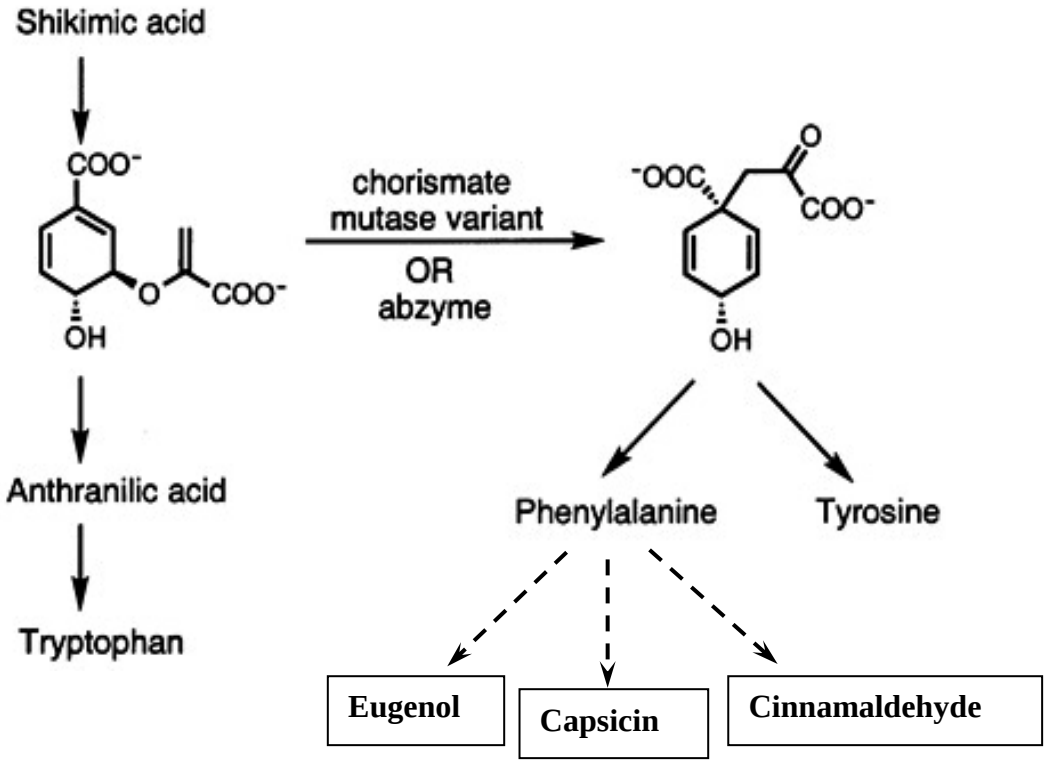


Figure 1.5. The shikimic acid pathway (Herrmann and Weaver, 1999)

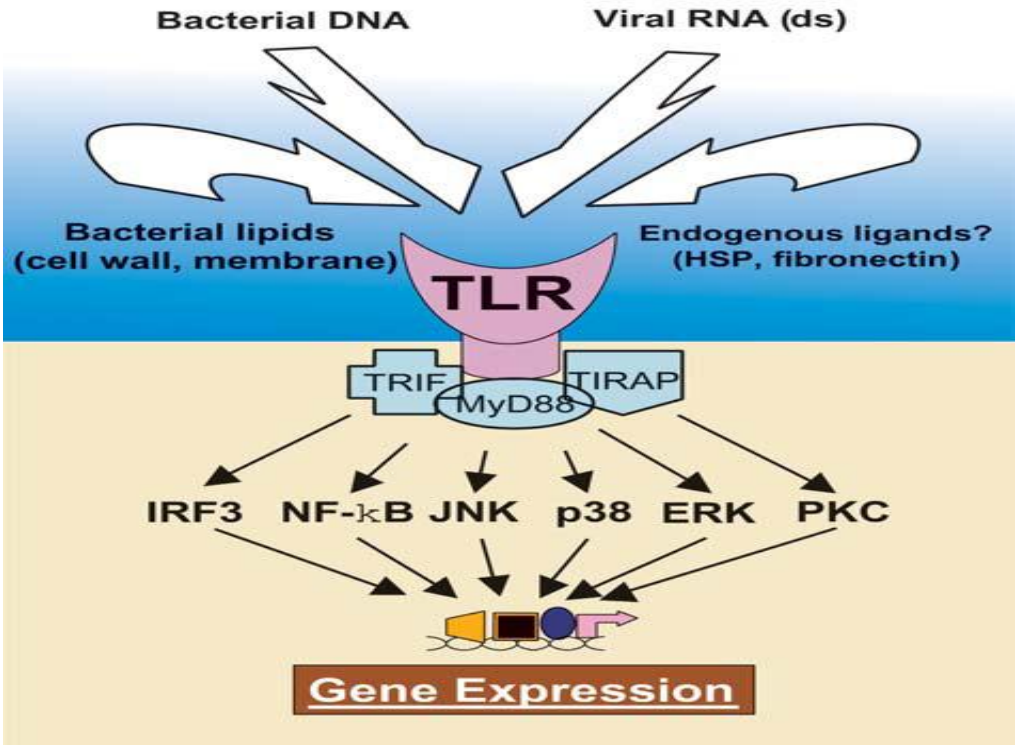


Figure 1.6. Schematic representation of the signaling pathways activated upon pattern recognition by TLRs. (Dempsey et al., 2003)

CHAPTER 2

ANTI-INFLAMMATORY EFFECTS OF SEVERAL PLANT EXTRACTS ON PORCINE ALVEOLAR MACROPHAGES IN VITRO

ABSTRACT

Plant extracts (PE) are bioactive substances of some foods or traditional herbs, known to possess antioxidant, antibacterial, and perhaps immunoregulatory effects. This study investigated the in vitro anti-inflammatory effects of 7 PE (anethol, capsicum oleoresin, carvacrol, cinnamaldehyde, eugenol, garlic, and turmeric oleoresin) on porcine alveolar macrophages (PAM) collected from weaned pigs (n = 6) by bronchoalveolar lavage. The experimental design for this assay was a 2 (with or without 1 μg lipopolysaccharide (LPS) /mL) × 5 (5 different levels of each PE) factorial arrangements in a randomized complete block design. The application levels of PE were 0, 25 50, 100, and 200 μg/mL, except for cinnamaldehyde and turmeric oleoresin, which were 0, 2.5, 5, 10, and 20 μg/mL. A colorimetric assay was used to determine the cell viability of PAM, Griess assay was applied to detect nitric oxide (NO) production from PAM, and ELISA was used to measure tumor necrosis factor-α (TNF-α), IL-1β, transforming growth factor β (TGF-β), and IL-10 in the cell culture supernatants of PAM. The LPS stimulation reduced (69 to 85%) cell viability and increased ($P < 0.001$) the secretion of TNF-α and IL-1β. Without LPS stimulation, anethol (35 to 52%) and capsicum oleoresin (39 to 59%) increased cell viability of PAM, whereas other PE reduced ($P < 0.05$) it. Anethol (12 to 34%), capsicum oleoresin (53 to 92%), or carvacrol (46 to 61%) enhanced ($P < 0.05$) the cell viability of LPS-treated PAM. Without LPS stimulation, anethol, capsicum oleoresin, cinnamaldehyde,

or turmeric oleoresin stimulated TNF-α secretion from PAM, whereas all PE except eugenol enhanced IL-1β secretion from PAM. However, all PE suppressed ($P < 0.05$, 15 to 100%) TNF-α, and carvacrol, cinnamaldehyde, eugenol, or garlic decreased ($P < 0.05$, 31 to 95%) IL-1β secretion from LPS-induced PAM. In addition, carvacrol, eugenol, garlic, or turmeric oleoresin suppressed ($P < 0.05$) anti-inflammatory cytokine TGF-β secretion from PAM with or without LPS stimulation. Only low levels of NO were detected in the supernatants of PAM with or without LPS stimulation, and the anti-inflammatory cytokine IL-10 was not detected in any supernatants. In conclusion, all PE may have potent anti-inflammatory effects to varying degrees.

KEY WORDS: alveolar macrophage, cell viability, cytokines, plant extracts, weaned pigs

INTRODUCTION

Macrophages are involved in the innate immune response through phagocytosis or production of a variety of compounds, like cytokines (Dempsey et al., 2003) or nitric oxide (NO). Among cytokines, tumor necrosis factor-α (TNF-α) and IL-1β are pro-inflammatory molecules whose secretion can be potently induced by lipopolysaccharide (LPS) (Alexander and Rietschel, 2001). Over-production of these cytokines might cause inflammatory diseases (Ferrero-Miliani et al., 2006). Macrophages also release anti-inflammatory cytokines, such as IL-10 and transforming growth factor beta (TGF-β) (Opal and DePalo, 2000). They also secrete NO which is an intercellular messenger produced through the activation of nitric oxide synthase (NOS; MacMicking et al., 1997). A high concentration of NO is associated with inflammatory diseases (Bogdan, 2001).

Certain plant extracts (PE) from foods or traditional herbs are considered to have antioxidant, antibacterial, and perhaps immunoregulatory effects. The active components can be synthesized in pure form and still be considered PE. Eugenol, garlic, and the PE from *C. operculatus* buds each had potential anti-inflammatory effects shown as inhibition of TNF-α and IL-1β secretion from LPS-induced human or rat cells (Lang et al., 2004; Lee et al., 2007; Dung et al., 2009). Previous studies from Lee et al. (2005) and Li et al. (2006) reported that cinnamaldehyde and eugenol can suppress NO release and inducible NOS expression in LPS-treated murine macrophages. Most of these in vitro experiments have been conducted in human, mouse, or rat cells. However, the potential anti-inflammatory effects of PE on porcine cells remain to be elucidated.

The purpose of this study was to investigate the effects of 7 PE on the inflammatory response in porcine alveolar macrophages (PAM). The results may indicate whether some of the PE may be useful as immunoregulators in pig diets.

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Materials

Seven PE (anethol, capsicum oleoresin, carvacrol, cinnamaldehyde, eugenol, garlic, and turmeric oleoresin) were provided by Pancosma, S. A., Geneva, Switzerland. Anethol, carvacrol, cinnamaldehyde, and eugenol are essential oils synthetically produced but identical to the natural compounds, and more than 95% pure. Capsicum and turmeric are extracted oleoresins, which were standardized to 6% capsaicin and dihydrocapsaicin, and 98% curcuminoides, respectively.

Garlic is a botanical extract from garlic, standardized to 40% propyl thiosulfonates. Before conducting the experiment, all PE were first dissolved in dimethyl sulfoxide (DMSO), and further diluted with the culture medium RPMI-1640 (Roswell Park Memorial Institute medium, HyClone Laboratories, Inc., Logan, UT) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT) and antibiotics, including 100 IU penicillin/mL and 100 μg streptomyci/mL n (Mediatech, Inc., Manassa, VA). The final concentration of DMSO in the medium did not exceed 0.05%. Lipolysaccharide (from *Escherichia coli* 0111:B4) was purchased from Sigma Co. (St. Louis, MO). Vybrant® MTT Cell Proliferation Assay Kits were purchased from Molecular Probes Inc. (Eugene, OR). The Griess Reagent System was bought from Promega Corp. (Madison, WI). Porcine TNF-α, IL-1β, TGF-β, and IL-10 ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN).

Collection of Porcine Alveolar Macrophages

Eighteen clinically healthy donor pigs (6 weeks old) were used to collect PAM. Each group of 6 pigs was used to test 2 or 3 PE. Pigs were anesthetized by intramuscular injection of a 1 mL combination of telazol, ketamine, and xylazine (2:1:1) per 23.3 kg body weight (BW). The final mixture contained 100 mg telazol, 50 mg ketamine, and 50 mg xylazine in 1 mL (Fort Dodge Animal Health, Fort Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection with 78 mg sodium pentobarbital (Sleepaway; Henry Schein, Inc., Indianapolis, IN) per 1 kg of BW.

Porcine alveolar macrophages from lungs were obtained by bronchoalveolar lavage by the following procedures. Lungs with intact trachea were removed immediately after euthanizing pigs and 150 mL PBS was poured into them through the trachea. After massaging the lungs for about 30 - 60 seconds, the lavage fluid was filtered through a double layer of sterile gauze into

50 mL conical centrifuge tubes and then pelleted by centrifuging at 400 × g for 15 min at room temperature. The pelleted cells were washed twice with Hank's Balanced Salt Solution and re-suspended in 5 mL of the culture medium RPMI-1640 with FBS and antibiotics. The live cells were stained by Trypan Blue dye exclusion (Sigma-Aldrich Co., St Louis, MO) and counted using a hemocytometer (Fisher Scientific Inc., Pittsburgh, PA). The final cell concentration was adjusted to 1×10^5 cells/mL. The viability of the cells was higher than 97%. In this manuscript, we use the term "PAM" because the majority (93 to 97%) of bronchoalveolar lavage fluid cells are macrophages (Dickie et al., 2009).

Cell Culture and Experimental Design

The PAM cells were cultured in 48- or 96-well plastic tissue culture plates at a density of 6×10^4 cells/well in 48-well plate and 1×10^4 cells/well in 96-well plate. All plates were incubated overnight at 37°C in a humidified 5% CO_2 incubator to allow PAM attach to the bottom. The non-adherent cells were washed away with warm Hank's balanced salt solution. Then adhered PAM cells were incubated with fresh medium containing different stimulators as described below. After 24 h more of incubation, the supernatants were collected and stored at -80°C for cytokine analysis.

This experiment contained 7 individual in vitro assays with the same experimental design, which was a 2 (without or with 1 μg of LPS/mL) × 5 (5 different levels of each PE) factorial arrangement in randomized complete block design. Therefore, there were a total of 10 treatments for each PE. The negative control was the treatment without either PE or LPS, and the positive control was the treatment without PE, but with LPS. All treatments were conducted with 3 wells, from which the supernatants were pooled for analysis. The concentrations of anethol, capsicum oleoresin, carvacrol, eugenol, and garlic tested in this experiment were 0, 25, 50, 100, and 200

μg/mL. The doses of cinnamaldehyde used in this experiment were adjusted to 0, 2.5, 5, 10, and 20 μg/mL according to Chao et al. (2008) and a preliminary experiment, in which doses of cinnamaldehyde of 50 μg/mL or more were found toxic to PAM cell viability. In addition, the levels of turmeric oleoresin used in this assay were reduced to 0, 2.5, 5, 10, and 20 μg/mL, due to the difficulty of dissolving turmeric oleoresin in both DMSO and culture medium.

Detection of Cell Viability

To determine the toxicity levels of PE on PAM, the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay was used, which measured the metabolic activity of cell cultures with a color reaction catalyzed by mitochondrial enzymes, to detect changes in cell viability. Briefly, after the 24 h incubation of cells in 96-well plates with stimulation and removal of the supernatants as described above, 100 μL of fresh culture medium RPMI-1640 was added to each well. Then 10 μL of 12 mM MTT solution was added to each well. After 4 h of incubation at 37^{o}C, 100 μL of the sodium dodecyl sulfate (SDS)-HCl (1 mg SDS with 10 mL of 0.01 M HCl) solution was added to each well and mixed thoroughly. The plates were incubated at 37^{o}C for 12 h in a humidified chamber. The optical density (OD) was measured at 570 nm. The background signal inherent to the plates when no cell was present was subtracted from the absorbance obtained from each sample. The OD of the cells in the negative control was taken as the standard and set to 100%. The relative viability was calculated by the formula: (OD of sample/OD of the control) × 100%.

Test of Nitric Oxide (NO)

The Griess assay was used to measure nitrite formed by the spontaneous oxidation of NO. Briefly, 50 μL of cell supernatant was added to each well of the 96-well microplate and incubated with 50 μL of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) at

room temperature for 5 to 10 min in darkness. Then, 50 μL of 0.1% N-1-napthylethylenediamine dihydrochloride in water was added to each well and incubated at room temperature for 5 to 10 min in darkness. The optical density was measured at 530 nm. Concentrations were calculated from a standard sodium nitrite curve. All samples were analyzed in duplicate.

Measurements of Cytokines

Protein levels of TNF-α, IL-1β, TGF-β, and IL-10 in the cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendation. Briefly, standard, control, and samples were added to the wells with coated monoclonal antibody specific for each cytokine. After incubation for 2 h, the unbound substances were washed away, and an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells to sandwich the cytokine immobilized during the first incubation. A further 2 h of incubation was followed by a wash to remove any unbound antibody-enzyme reagent, and then a substrate solution was added to the wells and color developed in proportion to the amount of the cytokine bound in the initial step. The color development was stopped by adding the stop solution and the intensity of the color was measured at 450 nm with the correction wavelength set at 530 nm. Concentrations were calculated from a standard curve. All samples were analyzed in duplicate. The intra-assay and inter-assay coefficients of variation provided by the kit manufactures were lower than 4.1 and 9.2, respectively.

Statistical Analysis

All data were analyzed by ANOVA using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The donor pigs were considered as random blocks and a pool of 3 wells was considered as an experimental unit. The model included the effects of block, LPS, various levels of PE, and LPS × PE interaction. The LSMEANS procedure was used to calculate mean values

and compare the differences between control and different doses of PE treated in this experiment with or without LPS. An alpha-value of 0.05 was used to assess significance among means.

RESULTS

Cell Viability

Cell viability of PAM responded differently to different PE, indicating a range of toxicity. For example, a high dose of capsicum oleoresin or anethol increased PAM cell proliferation ($P < 0.05$) in the absence of LPS stimulation (Table 2.1). Conversely, cells treated with 200 μg/mL of carvacrol, more than 100 μg/mL of garlic or even more than 50 μg/mL of eugenol, showed reduced cell viability ($P < 0.05$). Lower levels of cinnamaldehyde and turmeric oleoresin were used in this experiment as described above. Cinnamaldehyde reduced ($P < 0.05$) the cell viability without LPS stimulation, but still over 70% of PAM remained viable. Turmeric oleoresin at 20 μg/mL was severely toxic ($P < 0.05$) to PAM.

The stimulation of LPS inhibited the PAM cell viability ($P < 0.05$), except in the carvcarol and capsicum oleoresin group. The effects of PE were generally in the same direction as without LPS, with anethol, capsicum oleoresin, or carvacrol at some levels increasing ($P < 0.05$) the number of live cells. However, cinnamaldehyde, eugenol, garlic, and turmeric oleoresin did not increase cell viability of PAM with LPS stimulation.

Nitric Oxide Production

Very low levels of NO were detected in the supernatants of each treatment (Table 2.2). Compared with the negative control, at some levels capsicum oleoresin and turmeric oleoresin increased ($P < 0.05$) while carvacrol decreased ($P < 0.05$) NO production from PAM.

Lipopolysaccharide stimulation increased NO in the eugenol and garlic group, but no effect in other PE groups.

Compared with the positive control, high levels of capsicum oleoresin increased ($P < 0.05$) the secretion of NO, but 50 μg of garlic/mL reduced ($P < 0.05$) NO production in the presence of LPS. Anethol, cinnamaldehyde, eugenol, and garlic had no effect on NO production whether or not LPS was present.

Pro-inflammatory Cytokines

The inclusion of intermediate levels of anethol, capsicum oleoresin, cinnamaldehyde, and turmeric oleoresin enhanced ($P < 0.05$) the secretion of TNF-α from PAM in the absence of LPS (Figure. 2.1A, B, D, and G). However, the higher doses of cinnamaldehyde, eugenol, and garlic had opposite effects (Figure. 1C, E, and F). Stimulation by LPS tremendously increased ($P < 0.001$) the secretion of TNF-α from PAM, but all 7 PE tested dose-dependently inhibited ($P < 0.05$) the secretion of TNF-α from LPS-induced PAM (Figure. 2.1).

Six of the 7 PE tested increased ($P < 0.05$) the secretion of IL-1β from PAM in the absence of LPS, eugenol being the exception. The LPS sharply elevated ($P < 0.001$) the secretion of IL-1β from PAM. In the presence of LPS, individual PE affected secretion of IL-1β differently. The treatments with carvacrol, cinnamaldehyde, eugenol, garlic, and turmeric oleoresin suppressed ($P < 0.05$) the secretion of IL-1β from LPS-induced PAM in a dose-dependent manner (Figure. 2.2C, D, E, F, and G). However, anethol and capsicum oleoresin enhanced ($P < 0.05$) the secretion of IL-1β from LPS-induced PAM (Figure. 2.2A and B).

Anti-inflammatory Cytokines

Anti-inflammatory cytokines, IL-10 and TGF-β, were analyzed in this experiment, but IL-10 was not detectable in any supernatants of PAM treated with different stimulators. The detection limit of ELISA kit for IL-10 analysis was 1.76 pg/mL.

Compared with the negative control, eugenol, garlic, and turmeric oleoresin decreased ($P < 0.05$) the secretion of TGF-β from PAM in the absence of LPS (Figure. 2.3E, F, and G). There was no effect of LPS on the secretion of TGF-β from PAM. However, carvacrol, eugenol, garlic, and turmeric oleoresin suppressed ($P < 0.05$) the secretion of TGF-β from LPS-induced PAM (Figure. 2.3C, E, F, and G).

DISCUSSION

The present study shows for the first time that the addition of PE alters the secretion of cytokines by porcine cells with or without LPS stimulation. Notably, all 7 PE tested reduced the production of a pro-inflammatory cytokine by PAM stimulated by LPS.

Tumor necrosis factor-α and IL-1β are 2 important pro-inflammatory cytokines. The mediation of inflammation against infection by these pro-inflammatory cytokines is beneficial to the host, but over-expression of these cytokines might cause inflammatory diseases (Ferrero-Miliani et al., 2006). Previous studies related to mouse, human, and pig have reported that LPS stimulated the production of pro-inflammatory cytokines secreted from macrophages (Lee et al., 2007; Chao et al., 2008; Che et al., 2008). In the present study, it was also found that LPS stimulated sharply increased secretion of TNF-α and IL-1β from PAM. The results showed that all 7 PE inhibited the secretion of TNF-α from LPS-induced PAM in a dose dependent manner, consistent with previous studies in a human cell line model (Lee et al., 2007; Chao et al., 2008).

Interestingly, the IL-1β response differed from that of TNF-α in the presence of LPS. The treatments with carvacrol, cinnamaldehyde, eugenol, and garlic significantly suppressed the secretion of both TNF-α and IL-1β from LPS-induced PAM, but anethol and capsicum oleoresin stimulated or did not affect the secretion of IL-1β from LPS-induced PAM. The high dose of turmeric oleoresin (20 μg/mL) in the presence of LPS reduced both the number of live macrophages and cytokine secretion, suggesting the primary effect may be cytotoxicity rather than suppression of cytokine production. On the other side, anethol, capsicum oleoresin, cinnamaldehyde, garlic, and turmeric oleoresin stimulated the secretion of TNF-α, IL-1β, or both from PAM in the absence of LPS, which indicates that the PE may have the potential ability to enhance immune responses in the normal conditions.

One of the important anti-inflammatory cytokines found in the immune response is IL-10. It can suppress the secretion of pro-inflammatory cytokines from macrophages through several different ways (Opal and DePalo, 2000). The major part of IL-10 synthesis is stimulated by pro-inflammatory cytokines, such as TNF-α, and also requires the activation of other protein kinases or pathways (Wanidworanun and Strober, 1993; Meisel et al., 1996). However, the present study indicates that PAM failed to synthesize significant amounts of IL-10 in response to LPS stimulation. The results are consistent with Thomassen et al. (1996), Salez et al. (2000), and Daniels et al. (2011), who found a lack of IL-10 synthesis by human, murine, or porcine alveolar macrophages upon LPS stimulation. This failure of IL-10 synthesis was not due to an absence of PAM activation or to a lack of pro-inflammatory cytokines because TNF-α and IL-1β were detected as expected. The underlying mechanism accounting for the absence of IL-10 synthesis is not clear. Salez et al. (2000) suggested that the IL-10 protein expression is regulated at the

pretranscriptional level, and some unknown pulmonary environmental factors might suppress IL-10 mRNA expression by alveolar macrophages.

Transforming growth factor-β is another important cytokine involved in the immune response. Like many cytokines, TGF-β has both immune-suppressive and immune-enhancing activities (Opal and DePalo, 2000). As an anti-inflammatory cytokine, TGF-β can suppress the proliferation and differentiation of T and B cells, and deactivate monocyte/macrophage in a manner similar to IL-10 (Letterio and Roberts, 1997). However, as a pro-inflammatory cytokine, TGF-β in the presence of different cytokines can drive the differentiation of diverse T helper cells, which promote further tissue inflammation (Sanjabi et al., 2009). In the present study, the treatments with PE did not stimulate the secretion of TGF-β from PAM with or without LPS stimulation; on the contrary, they suppressed it. This result indicates TGF-β may have little or no effect on pro-inflammatory cytokine secretion from LPS-treated PAM by PE.

The viability test using the MTT assay was performed in order to make sure the influence of PE on the inflammatory mediators secreted from PAM resulted from mechanisms other than direct killing of cells. The high level (200 μg/mL) of carvacrol and garlic were cytotoxic to PAM, and very low levels of cinnamaldehyde and turmeric oleoresin significantly inhibited cell viability of PAM. However, anethol and capsicum oleoresin increased cell viability of PAM. These results indicated different effects of different PE on cell viability of PAM. Based on the MTT results, the data for the highest level of carvacrol (200 μg/mL), garlic (200 μg/mL), and turmeric oleoresin (20 μg/mL) were removed here due to the cytotoxic effect on PAM. In addition, normalization of all data by cell viability did not change the response pattern (data not shown). The MTT results in the LPS group also indicated that the inhibitory effects of PE on the

response to this pro-inflammatory mediator probably resulted from mechanisms other than direct killing of cells.

Nitric oxide is a very important molecule involved in a wide range of physiologic and pathologic processes in mammalian systems and its production by macrophages is fundamental for immune defense (MacMicking et al., 1997). Previous studies from Lee et al. (2002) and Li et al. (2006) reported cinnamaldehyde and eugenol suppressed NO production from LPS-treated murine macrophages. However, in the present study, the stimulation by 1 μg of LPS/mL did not affect the NO production of PAM, confirming the previous findings of Pampusch et al. (1998) and Zelnickova et al. (2008), who also failed to induce NO production from PAM with LPS stimulation. The LPS stimulation increased the secretion of pro-inflammatory cytokines, indicating that inability of PAM to produce NO was not caused by non-reactivity to stimulation with LPS. The fundamental differences among species in the abilities of macrophages to produce NO are not clear. In the present study, there were detectable effects of specific PE on NO production from PAM, but they were all small in magnitude and therefore not of clear importance.

In conclusion, the present results show the ability of all PE used in this study to inhibit LPS-induced production of the pro-inflammatory cytokine, TNF-α, by PAM. In addition, several PE can also suppress IL-1β secretion. These results indicate that all of these PE may have potent anti-inflammatory effects. Especially, carvacrol, cinnamaldehyde, eugenol and garlic might be the more powerful candidates because they block the secretion of both of the two pro-inflammatory cytokines measured, TNF-α and IL-1β. These observations require verification in vivo.

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TABLES AND FIGURES

Table 2.1. The relative cell viability of porcine alveolar macrophages treated with various concentrations of plant extracts (PE) in the absence or presence of 1 μg LPS/mL.[1]

Items	Without LPS					With LPS					SEM	LPS	PE	LPS × PE
	NC[2]	25	50	100	200	PC[2]	25	50	100	200				
Anethol	100	86	135*	114	154*	74	69	83†	69	99†	6.3	<0.001	<0.001	0.002
Capsicum oleoresin	100	110	139*	107	159*	86	108†	132†	127†	165†	6.4	0.818	<0.001	0.012
Carvacrol[3]	100	101	91	81	-	69	111†	101†	84	-	11.1	0.751	<0.001	0.047
Eugenol	100	102	85*	72*	67*	85	77	84	79	71†	4.6	0.034	<0.001	0.002
Garlic[3]	100	111	91	69*	-	85	87	89	78	-	5.3	0.051	<0.001	0.035
	NC	**2.5**	**5**	**10**	**20**	**PC**	**2.5**	**5**	**10**	**20**				
Cinnamaldehyde	100	83*	91	73*	70*	71	63	73	58†	74	5.3	<0.001	<0.001	0.006
Turmeric oleoresin[3]	100	87*	79*	68*	-	85	68†	67†	65†	-	4.9	<0.001	<0.001	0.384

[1] The unit for the cell viability was %, and the unit for the concentration of PE was μg/mL.

[2] NC = negative control, with no LPS or PE; PC = positive control, LPS but no PE.

[3] The high dose of the PE was toxic to cells and removed here.

* Significantly different ($P < 0.05$) from the negative control without LPS.

† Significantly different ($P < 0.05$) from the positive control stimulated with 1 μg/mL LPS.

Table 2.2. Nitric oxide production by porcine alveolar macrophages treated with various concentrations of plant extracts (PE) in the absence or presence of 1 μg/mL LPS.[1]

Items	Without LPS					With LPS					SEM	LPS	PE	LPS × PE
	NC[2]	25	50	100	200	PC[2]	25	50	100	200				
Anethol	1.10	1.04	1.14	0.96	1.04	1.06	1.00	1.16	1.12	1.18	0.060	0.191	0.171	0.241
Capsicum oleoresin	1.31	1.95*	1.97*	2.61*	3.14*	1.39	1.46	2.04†	2.77†	3.17†	0.163	0.773	<0.001	0.105
Carvacrol[3]	1.31	1.14	1.09*	1.19	-	1.17	1.32	1.35	1.37	-	0.119	0.120	0.271	0.082
Eugenol	0.81	0.89	0.89	0.92	0.93	0.93	0.94	1.01	1.02	1.04	0.054	0.004	0.208	0.961
Garlic[3]	0.81	0.79	0.74	0.77	-	0.93	0.84	0.81†	0.87	-	0.045	0.002	0.003	0.922
	NC	**2.5**	**5**	**10**	**20**	**PC**	**2.5**	**5**	**10**	**20**				
Cinnamaldehyde	1.15	0.97	1.16	1.04	1.23	0.99	1.11	1.03	1.13	1.16	0.105	0.597	0.333	0.238
Turmeric oleoresin[3]	0.81	0.94*	0.93*	0.86	-	0.93	0.95	0.89	0.90	-	0.057	0.520	0.033	0.492

[1] The unit for the cell viability was %, and the unit for the concentration of PE was μg/mL.

[2] NC = negative control, with no LPS or PE; PC = positive control, LPS but no PE.

[3] The high dose of the PE was toxic to cells and removed here.

* Significantly different ($P < 0.05$) from the negative control without LPS.

† Significantly different ($P < 0.05$) from the positive control stimulated with 1 μg/mL LPS.

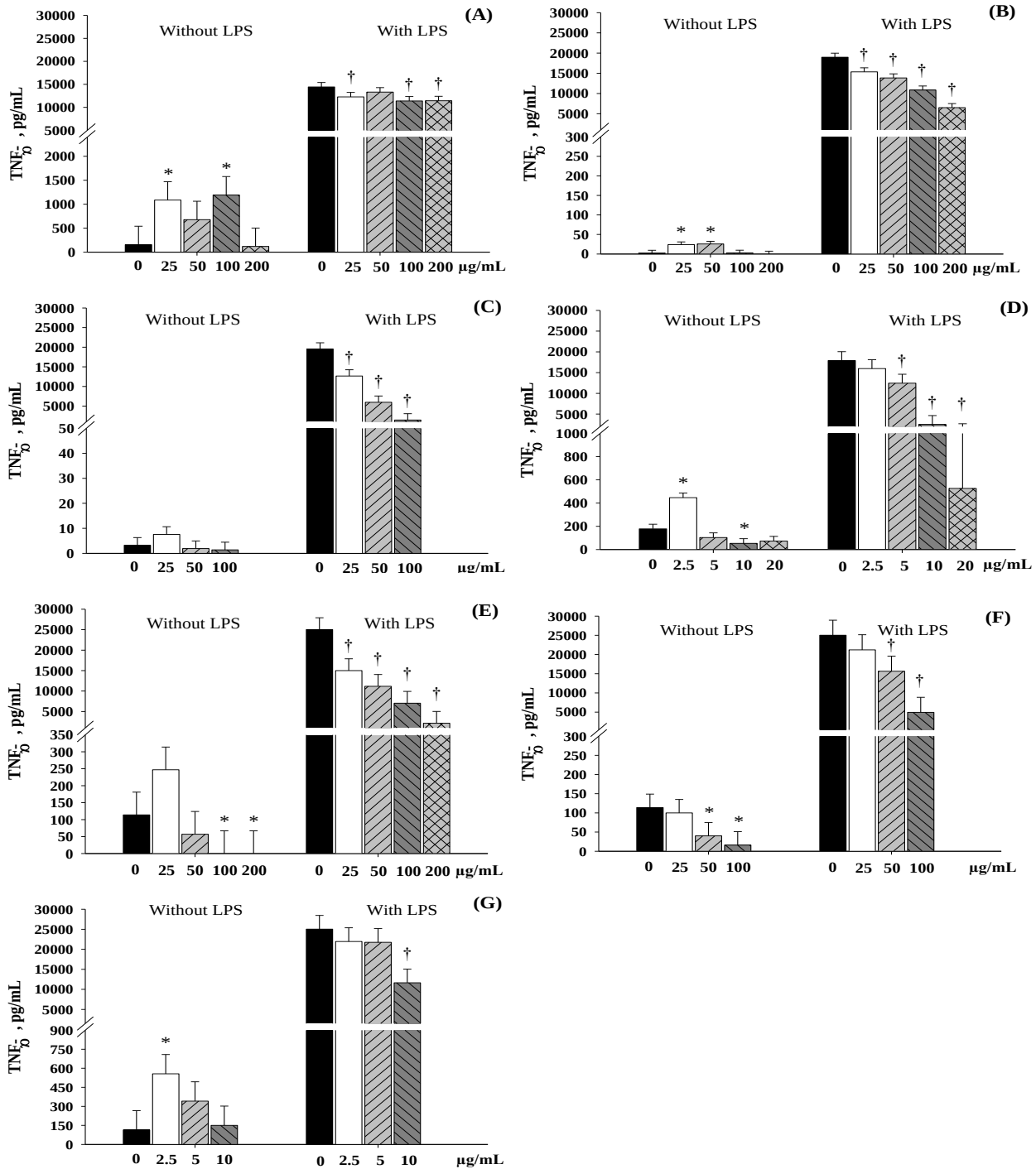

Figure 2.1. Plant extracts (PE) influence the production of TNF-α from porcine alveolar macrophage in the absence or presence of LPS. Cells were incubated with various concentrations (0, 25, 50, 100, and 200 µg/mL unless otherwise noted) of each PE in the absence or presence of

Figure 2.1. (cont.)

LPS (1 μg/mL) for 24 hours. The production of TNF-α by PAM treated with (A) Anethol, (B) Capsicum oleoresin, (C) Carvacrol, (D) Cinnamaldehyde (0, 2.5, 5, 10, and 20 μg/mL), (E) Eugenol, (F) Garlic, or (G) Turmeric oleoresin (0, 2.5, 5, 10, and 20 μg/mL) is presented as pg/mL. The results were means of values from 6 pigs. For anethol (A), LPS: $P < 0.001$; Level, $P = 0.720$; Interaction: $P = 0.466$. For all other PE, LPS: $P < 0.001$; Level, $P < 0.001$; Interaction: $P < 0.001$. *different ($P < 0.05$) from the negative control (0% PE) without LPS. †different ($P < 0.05$) from the positive control (0% PE) stimulated with 1 μg/mL LPS.

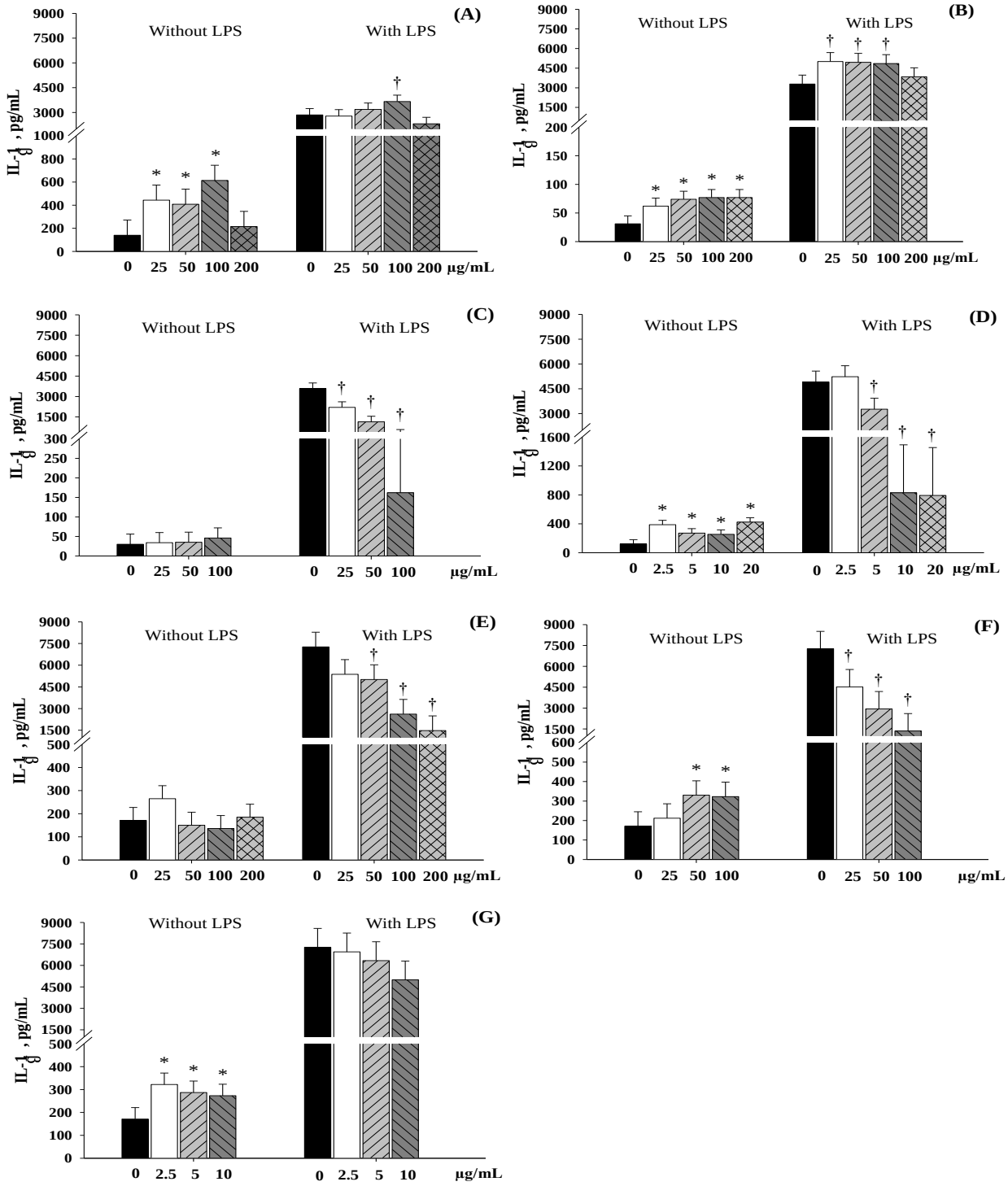

Figure 2.2. Plant extracts (PE) influence the production of IL-1β from porcine alveolar macrophage in the absence or presence of LPS. Cells were incubated with various concentrations

Figure 2.2. (cont.)

(0, 25, 50, 100, and 200 μg/mL unless otherwise noted) of each PE in the absence or presence of LPS (1 μg/mL) for 24 hours. The production of IL-1β by PAM treated with (A) Anethol, (B) Capsicum oleoresin, (C) Carvacrol, (D) Cinnamaldehyde (0, 2.5, 5, 10, and 20 μg/mL), (E) Eugenol, (F) Garlic, or (G) Turmeric oleoresin (0, 2.5, 5, 10, and 20 μg/mL) is presented as pg/mL. The results were means of values from 6 pigs. For anethol (A), LPS: $P < 0.001$; Level, $P = 0.016$; Interaction: $P = 0.349$. For capsicum oleoresin (B), LPS: $P < 0.001$; Level, $P = 0.249$; Interaction: $P = 0.280$. For all other PE, LPS: $P < 0.001$; Level, $P < 0.001$; Interaction: $P < 0.001$. *different ($P < 0.05$) from the negative control (0% PE) without LPS. †different ($P < 0.05$) from the positive control (0% PE) stimulated with 1 μg/mL LPS.

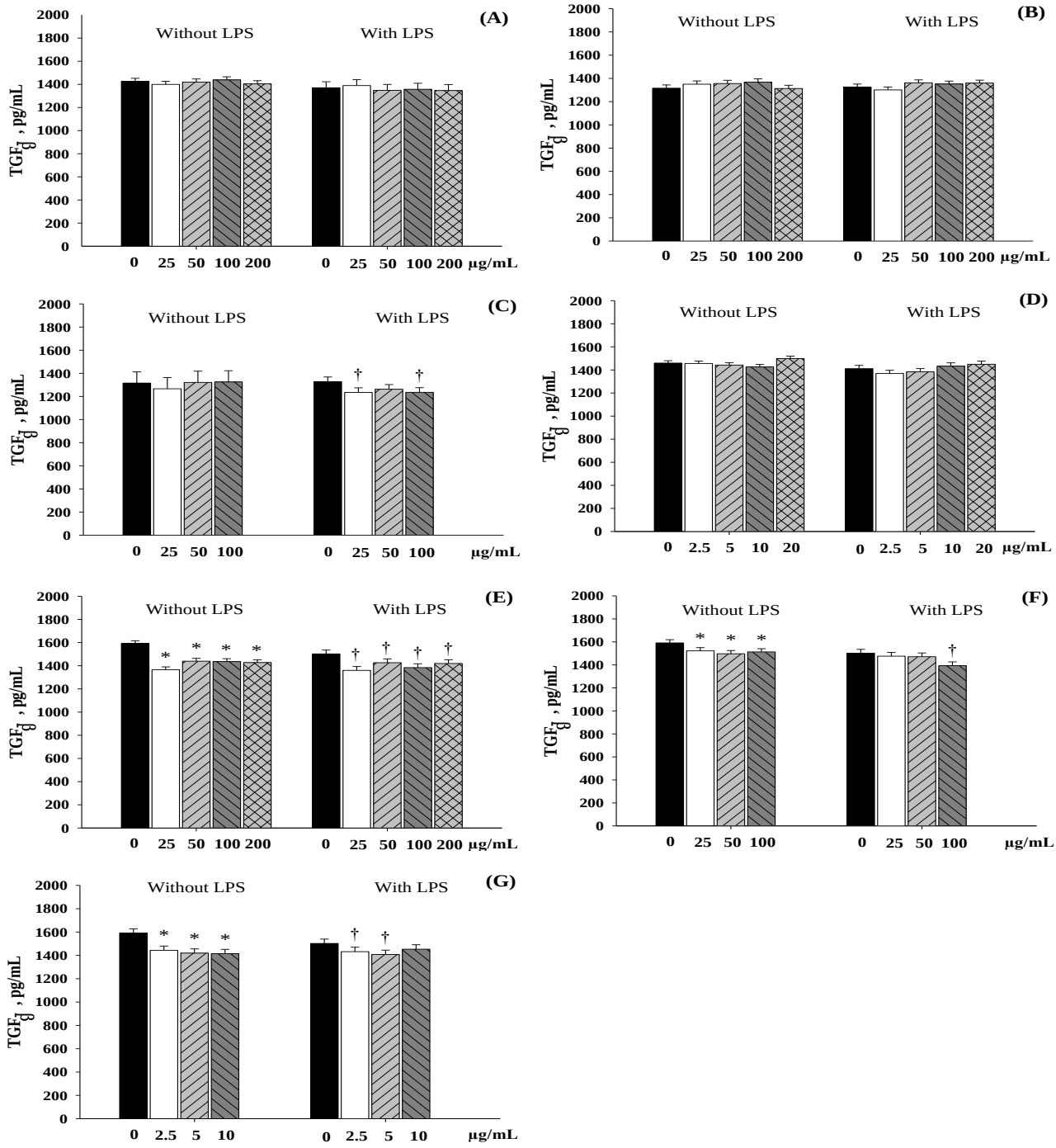

Figure 2.3. Plant extracts (PE) influence the production of TGF-β from porcine alveolar macrophage in the absence or presence of LPS. Cells were incubated with various concentrations

Figure 2.3. (cont.)

(0, 25, 50, 100, and 200 μg/mL unless otherwise noted) of each PE in the absence or presence of LPS (1 μg/mL) for 24 hours. The production of TGF-β by PAM treated with (A) Anethol, (B) Capsicum oleoresin, (C) Carvacrol, (D) Cinnamaldehyde (0, 2.5, 5, 10, and 20 μg/mL), (E) Eugenol, (F) Garlic, or (G) Turmeric oleoresin (0, 2.5, 5, 10, and 20 μg/mL) is presented as pg/mL. The results were means of values from 6 pigs. Cinnamaldehyde (D), Eugenol (E), Garlic (F), and turmeric oleoresin (G): Level: $P < 0.05$. No difference was from LPS stimulation. No interaction between LPS and level was observed. *different ($P < 0.05$) from the negative control (0% PE) without LPS. †different ($P < 0.05$) from the positive control (0% PE) stimulated with 1 μg/mL LPS.

CHAPTER 3

EFFECTS OF PLANT EXTRACTS ON DIARRHEA, IMMUNE RESPONSES, INTESTINAL MORPHOLOGY, AND GROWTH PERFORMANCE OF WEANED PIGS EXPERIMENTALLY INFECTED WITH A PATHOGENIC *ESCHERICHIA COLI*

ABSTRACT

A study was conducted to evaluate the effects of 3 different plant extracts on diarrhea, immune response, intestinal morphology, and growth performance of weaned pigs experimentally infected with a pathogenic F-18 *E. coli*. Weaned pigs (n=64, 6.3 ± 0.2 kg BW, 21 d old) were housed in individual pens in disease-containment chambers for 15 d: 4 d before and 11 d after the first inoculation (d 0). Treatments were in a 2 × 4 factorial arrangement: with or without an F-18 *E. coli* challenge (toxins: LT, STb, and SLT-2; 10^{10} cfu/3 mL oral dose; daily for 3 d from d 0) and 4 diets (a nursery basal diet (CON), 10 ppm of capsicum oleoresin (CAP), garlic (GAR), or turmeric oleoresin (TUR)). The growth performance was measured on d 0 to 5, 5 to 11, and 0 to 11. Diarrhea score (DS; 1, normal, to 5, watery diarrhea) was recorded for each pig daily. Frequency of diarrhea (FD) was the percentage of pig days with DS of 3 or higher. Feces were collected on d 0, 3, 5, 8, and 11 and plated on blood agar to calculate a ratio (RHT) of β-hemolytic coliforms to total coliforms by assessing the populations visually using a score (0, no bacterial growth, to 8, very heavy bacterial growth). Blood was collected on d 0, 5, and 11 to measure total and differential white blood cell (WBC) counts and serum tumor necrosis factor (TNF)-α, IL-10, transforming growth factor (TGF)-β, C-reactive protein (CRP), and haptoglobin

(Hp). On d 5 and 11, half of the pigs were euthanized to collect jejunum, ileum, and colon to measure villi height (VH), crypt depth (CDH), and their ratio (VH:CDH). In the challenged group, the PE treatments reduced ($P < 0.05$) average DS from d 0 to 2 and d 6 to 11 and FD, decreased ($P < 0.05$) TNF-α and Hp on d 5 and WBC, NEU, red blood cell counts, hemoglobin, and hematocrit on d 11, and increased ($P < 0.05$) ileal VH on d 5, and tended ($P = 0.10$) to increase jejunum VH and VH:CDH compared with the CON, but did not affect growth performance and RHT. In the unchallenged group, the PE treatments reduced ($P < 0.05$) average DS from d 3 to 5, overall FD, and Hp on d 5 compared with the CON. The *E. coli* infection increased ($P < 0.05$) WBC, TNF-α, and Hp, and reduced overall ADG, G:F, and VH of the small intestine as expected. In conclusion, the 3 PE tested reduced diarrhea, increased the VH of the small intestine, and affected total WBC, the populations of immune cells, and inflammatory mediators in *E. coli*-infected piglets, which may be beneficial to pig health.

KEY WORDS: blood, diarrhea, intestinal morphology, pathogenic *E. coli*, plant extracts, weaned pigs

INTRODUCTION

Plant extracts (PE) are secondary plant metabolites, which can be naturally obtained from plant materials or directly synthesized. Plant extracts have been of potential interest for a long time due to their antimicrobial (Baydar et al., 2004), anti-inflammatory (Lang et al., 2004), antioxidant (Dundar et al., 2008), and antiviral effects (Sökmen et al., 2004). Some previous studies reported that the supplementation of different PE improved growth performance, gut health, or reduced disease incidence of weaned pigs (Manzanilla et al., 2004; Michiels et al.,

2010; Sads and Bilkei, 2003), but other studies did not find beneficial effects from PE (Neill et al., 2006).

In our previous *in vitro* study all of 7 different PE tested, including the 3 PE tested here, had potential anti-inflammatory effects on porcine cells, as all of them inhibited the secretion of tumor necrosis factor-alpha (TNF-α) from lipopolysaccharide (LPS)-stimulated porcine alveolar macrophages (Liu et al., 2011c). Another experiment showed feeding 10 ppm of capsicum oleoresin, garlic, or turmeric oleoresin enhanced immune responses and growth efficiency of PRRSV-infected weaned pigs (Liu et al., 2011a, b).

Escherichia coli (*E. coli*) postweaning diarrhea is an important cause of death in weaned pigs. It is responsible for economic losses due to mortality, morbidity, decreased growth performance, and cost of medication (Nagy and Fekete, 2005). The toxins secreted from *E. coli* affect not only the physiological characteristics of the small intestine (Nagy and Fekete, 2005), but also the immune system of weaned pigs (Raetz and Whitfield, 2002; Bannerman and Goldblum, 2003). The objectives of this study were to investigate the effects of feeding PE-supplemented diets on growth performance and small intestinal morphology of weaned pigs challenged with a pathogenic *E. coli*, and to explore the effects of the PE on the disease resistance and immune responses of weaned pigs after *E. coli* challenge.

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. The experiment was conducted in the disease containment chambers of the Edwards R. Madigan Laboratory building at the University of Illinois at Urbana-Champaign.

Animals, Housing, Experimental Design, and Diet

A total of 64 weaned piglets (Pig Improvement Company (PIC) line C-22 female × PIC line 337 male) with same number of gilts and barrows and 6.3 kg of initial BW were selected from the Swine Research Center of the University of Illinois at Urbana-Champaign. The sows and piglets used in this experiment did not receive *E. coli* vaccines, antibiotic injections, or antibiotics in creep feed. After weaning, all pigs were transferred to the disease containment chamber and randomly assigned to treatment in a randomized complete block design with weight within sex as the blocks and pig as the experimental unit. Pigs were housed in the individual pens for 15 days (4 d before and 11 d after the first *E. coli* challenge (d 0)). There were a total of 64 individual pens, 4 in each of 16 chambers. There were 2 suites of 8 chambers, and each suite was used for either *E. coli* challenged or unchallenged pigs. The piglets had *ad libitum* access to feed and water.

The treatments were in a 2 × 4 factorial arrangement (with or without *E. coli* challenge; 4 dietary treatments). There were 8 replicates per treatment. In the *E. coli* challenge group, all pigs were inoculated orally with 3 mL F-18 *E. coli*/day for 3 consecutive days from d 0 post-infection (PI). The *E. coli* strain derived from a field disease outbreak contained heat-labile (LT), heat-stable (STb), and Shiga-like (SLT-2) toxins, and was provided at 10^{10} cfu per 3 mL dose in PBS. This dose has previous caused mild diarrhea (Song et al., 2011). In the unchallenged group, pigs were inoculated for the 3 consecutive days with 3 mL PBS/day as the sham control (Sham). The 4 dietary treatments were the complex nursery basal diet (CON) and the addition of 10 ppm of capsicum oleoresin (CAP), 10 ppm of garlic (GAR), or 10 ppm of turmeric oleoresin (TUR) to the CON respectively. All 3 plant extracts were obtained from Pancosma S. A. (Geneva, Switzerland). Capsicum and turmeric are extracted oleoresins, which were standardized to 6%

capsaicin and dihydrocapsaicin, and 98% curcuminoides, respectively. Garlic is a botanical extract from garlic, standardized to 40% propyl thiosulfonates. The CON diet was formulated to meet or exceed NRC (1998) estimates of requirements of weaned pigs (Table 3.1). Spray-dried plasma, antibiotics, or zinc oxide were not contained in the CON diet. The experimental diets were fed to pigs throughout the experiment.

Clinical Observations and Sample Collection

The procedures for this study were adapted from the methods of Song et al. (2011). Prior to weaning, feces from sows and all their piglets destined for this experiment were collected and plated on blood and MacConkey agars and verified to be free of detectable β-hemolytic *E. coli*. After *E. coli* inoculation, one pig from the infected control treatment and one pig from the infected TUR treatment were culled at d 2 and d 4 PI due to severe diarrhea and weight loss. During the experiment, clinical observations (diarrhea score and alertness score) were recorded daily from the first day of inoculation (d 0). Diarrhea score of each pig was assessed visually each day by 2 independent evaluators with a score from 1 to 5 (1 = normal feces, 2 = moist feces, 3 = mild diarrhea, 4 = severe diarrhea, and 5 = watery diarrhea). Frequency of diarrhea was calculated by counting pig days with diarrhea score of 3 or higher. Alertness score of each pig was assessed visually with a score from 1 to 3 (1 = normal, 2 = slightly depressed or listless, and 3 = severely depressed or recumbent). Every pig was alert throughout the experiment and was given an alertness score of 1 (normal) each day, so the data are not reported.

After inoculation, fecal samples were collected from the rectum of each pig using a fecal loop or cotton swab on d 0, 3, 5, 8, and 11 PI and kept on ice to test for β-hemolytic and total coliforms. Blood samples were collected in duplicate (Ethylenediaminetetraacetic acid (EDTA) blood and serum) from the jugular vein of each pig before *E. coli* challenge (d 0) and d 5 and 11

PI. Whole blood samples were used to measure the total and differential white blood cell (WBC) counts, and cytokines and acute phase proteins (APP) were measured in the sera.

Pigs and feeders were weighed on the day of weaning (d -4), the first inoculation day (d 0), d 5, and d 11. Growth performance (ADG, ADFI, and G:F) was measured for each interval from d 0 to 5, d 5 to 11, and d 0 to 11.

One-half of the pigs (2 males and 2 females from each treatment) were euthanized on d 5 PI at near the peak of infection and the remainder at the end of experiment (d 11 PI) during recovery. Before being euthanized, pigs were anesthetized by intramuscular injection of a 1-mL combination of telazol, ketamine, and xylazine (2:1:1) per 23 kg BW. The final mixture contained 100 mg telazol, 50 mg ketamine, and 50 mg xylazine in 1 mL (Fort Dodge Animal Health, For Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection with 78 mg sodium pentobarbital (Sleepaway) per 1 kg of BW (Henry Schein, Inc., Indianapolis, IN). Three 3-cm segments from the middle of jejunum, ileum (close to the ileocecal junction), and middle of colon were collected and fixed in 10% neutral buffered formalin for histological analysis.

Detection of β-Hemolytic Coliforms

The detection of β-hemolytic and total coliforms was based on Song et al. (2011). Briefly, fecal samples were plated on blood agar to distinguish β-hemolytic coliforms, which can complete by digest red blood cell contents surrounding the colony and appear as heavy gray and shiny colonies from non-hemolytic coliforms which appear as light gray colonies. Meanwhile, the same fecal samples were plated on MacConkey agar to support that hemolytic colonies on the blood agar were correctly identified as *E. coli* colonies, which are lactose fermenting bacteria and shown as flat pink colonies. All plates were incubated 24 h at 37^{o}C in a humidified 5% CO_2

incubator. Populations of both total coliforms and β-hemolytic coliforms on blood agar were assessed visually, with a score from 0 to 8 (0 = no bacterial growth, 8 = very heavy bacterial growth). The ratio of β-hemolytic coliforms to total coliforms was calculated. The uncertain colonies were sub-plated on new MacConkey and blood agars to identify whether they were β-hemolytic. The results of questionable isolate were further verified as the β-hemolytic *E. coli* challenge organism using triple sugar iron and lysine iron agars and F-18 *E. coli* by polymerase chain reaction.

Intestinal Morphology

The fixed intestinal tissues were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. The slides were scanned by NanoZoomer Digital Pathology System (Hamamatsu Co., Bridgewater, NJ), and all measurements were conducted in the associated NDP.view software. Fifteen straight and integrated villi and their associated crypts were selected for measurement. The intestinal morphological measurements included: villus height and crypt depth. Only crypt depth was measured in colon due to the lack of villi in this part of intestine. Mean values of each criteria and villus height:crypt depth ratio within each segment except colon were calculated for statistical analysis.

Determination of Differential Blood Leukocytes

Blood samples collected in EDTA were used to measure total and differential blood cell counts by the Veterinary Clinical Pathology Laboratory at the University of Illinois at Urbana-Champaign. Differential leukocyte proportions and concentrations were analyzed on a multiparameter, automated hematology analyzer calibrated for porcine blood (CELL-DYN 3700, Abbott Laboratories, Abbott Park, IL).

Measurements of Serum Cytokines and APP

The serum TNF-α, IL-10, and TGF-β concentrations were measured by ELISA according to the recommendation of the manufacturer (R&D Systems, Inc., Minneapolis, MN, USA). Briefly, standard, control, and samples were added to the wells with coated monoclonal antibody specific for each cytokine. After incubation for 2 h, the unbound substances were washed away, and an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells to sandwich the cytokine immobilized during the first incubation. A further 2 h of incubation was followed by a wash to remove any unbound antibody-enzyme reagent, and then a substrate solution was added to the wells and color developed in proportion to the amount of the cytokine bound in the initial step. The color development was stopped by adding the stop solution and the intensity of the color was measured at 450 nm with the correction wavelength set at 530 nm. Concentrations were calculated from a standard curve. All samples were analyzed in duplicate.

The serum C-reactive protein (CRP), and haptoglobin (Hp) levels were also measured by commercially available kits (GenWay Biotech, Inc., San Dieo, CA, USA). The procedures were similar to those described above, except for different incubation times. The intra-assay coefficients of variation for TNF-α, IL-10, TGF-β, CRP, and Hp were 5.2, 4.4, 3.5, 4.1, and 2.7%, respectively. The inter-assay coefficients of variation for TNF-α, IL-10, TGF-β, CRP, and Hp were 7.1, 5.8, 6.0, 5.6, and 6.2%, respectively. The results were expressed in picograms, nanograms, or micrograms per milliliter based on a standard curve for cytokines and acute phase proteins.

Statistical Analysis

Data were analyzed using the Proc Mixed procedure of SAS (SAS Inst. Inc, Cary, NC). Pig was the experimental unit. The statistical model included effects of *E. coli* challenge, diet,

and their interaction as fixed effects. Block was a random effect. Specific contrasts were used to test comparisons between the control and the PE treatments collectively within each challenge treatment. Differences among the PE treatments within each challenge treatment were tested by pair-wise comparisons when the overall main effects were significant. The Chi-square test was used for the frequency of diarrhea. Probability values of < 0.05 were considered to be significant.

RESULTS

Growth Performance

Several E. coli challenged pigs did not gain BW from d 0 to 5 PI, rendering the G:F values meaningless, so those data are not presented (Table 3.2). Before *E. coli* infection, dietary PE did not affect ($P > 0.05$) pig performance compared with the sham control (data not shown). Challenge by *E. coli* decreased ($P < 0.05$) the BW of pigs on d 5 and 11 PI, the ADG and G:F from d 0 to 5 and 0 to 11 PI. The PE treatments did not affect growth rate over the entire experimental period, but the unchallenged pigs fed the PE treatments grew faster ($P < 0.05$) than CON during the early part of the experiment, and more slowly later ($P = 0.051$). There were no differences among the PE treatments.

Diarrhea Score and Cultural Score

The *E. coli* challenge increased ($P < 0.05$) diarrhea scores from d 2 to 11 PI and increased ($P < 0.05$) the frequency of diarrhea (Table 3.3). The PE treatments in the *E. coli* challenged group reduced the diarrhea score ($P < 0.05$) from d 3 to 5 and d 9 to 11 PI, and the frequency of diarrhea ($P < 0.05$) for the entire period. In the sham group, the PE treatments reduced ($P < 0.05$) the diarrhea score from d 0 to 2 PI and the frequency of diarrhea for the entire period, compared with the CON. There were no differences among the PE treatments.

No treatment effects were found for the ratio of β-hemolytic coliforms to total coliforms on d 0, so data are not presented. The *E. coli* challenge increased ($P < 0.05$) the ratio of β-hemolytic coliforms to total coliforms on d 3, 5, 8, and 11 PI, compared with the sham group (Table 3.4). No PE effects were found on the culture score of feces in either the sham or challenged groups. The fecal β-hemolytic coliforms found in feces from the sham group were not F-18 *E. coli* when tested by PCR.

Complete Blood Counts

No treatment effects were found for the total and differential WBC counts and red blood cells (RBC) on d 0, so data are not shown here. The *E. coli* challenge increased the number of total WBC on d 5 (tendency; $P = 0.091$) and d 11 PI ($P < 0.05$), but the PE treatments in the *E. coli* challenged group decreased ($P < 0.05$) total WBC (Table 3.5). There were no differences among the PE treatments. Similarly, the *E. coli* challenge increased ($P < 0.05$) the number of neutrophils on d 11 PI, but the PE treatments in the *E. coli* challenged group decreased ($P < 0.05$) the number of neutrophils. There were no differences among the PE treatments.

The *E. coli* infection increased ($P < 0.05$) hemoglobin on d 5 and mean corpuscular hemoglobin concentration on d 5 and 11 PI (Table 3.6), but the PE treatments had no effect. In the *E. coli* challenged group, the supplementation of the PE in diets, reduced ($P < 0.05$) RBC, and hemoglobin, and packed cell volume on d 11 PI compared with the CON. No PE effects were observed in the sham group.

Serum Cytokines and Acute Phase Proteins

A pro-inflammatory cytokine, TNF-α, and 2 anti-inflammatory cytokines, IL-10 and TGF-β, were detected in the serum of all pigs (Table 3.7). There were no effects of dietary PE supplementation on serum TNF-α or IL-10 before the *E. coli* challenge, but PE reduced ($P < 0.05$)

TGF-β compared with the CON. The *E. coli* challenge increased ($P < 0.05$) serum TNF-α on d 5 PI, but the PE treatments in the *E. coli* challenge group reduced ($P < 0.05$) serum TNF-α as compared to the CON. There were no differences among the PE treatments. In addition, the *E. coli* challenge reduced ($P < 0.05$) TGF-β on d 11 PI, but the PE treatments did not show any effect. No differences were detected on IL-10 on either d 5 or 11 PI.

Two acute phase proteins, CRP and Hp were measured in the serum of all pigs (Table 3.7). The *E. coli* infection increased ($P < 0.05$) serum Hp levels on d 5 and 11 PI, but the PE treatments in the *E. coli* challenge group reduced ($P < 0.05$) Hp level on d 5 PI, and tended ($P = 0.074$) to reduce Hp level on d 11 PI compared with the CON. In the sham group, feeding PE decreased ($P < 0.05$) serum Hp concentration on d 5 PI as compared to the CON. The 3 PE tested here showed different effects on Hp, as pigs fed CAP and GAR had lower ($P < 0.05$) Hp than those fed TUR on d 5 PI and pigs fed GAR had lower ($P < 0.05$) Hp than those fed CAP and TUR. No differences were detected on CRP on either d 5 or 11 PI.

Intestinal Morphology

The *E. coli* challenge reduced ($P < 0.05$) the villi height of jejunum and ileum on d 5 PI, but the PE treatments in the *E. coli* challenged group tended to increase ($P = 0.093$) jejunum villi height and increased ($P < 0.05$) ileal villi height, compared with the CON (Table 3.8). In addition, *E. coli* infection decreased the villus height:crypt depth ratio of jejunum and ileum and the crypt depth of the colon on d 5 PI and the villus height:crypt depth ratio of jejunum on d 11 PI, but increased ($P < 0.05$) crypt depth of jejunum on d 11 PI. However, there were no PE effects for these measurements.

DISCUSSION

Escherichia coli that express F18 fimbria are a major cause of diarrhea and mortality of post-weaning pigs for pig producers (Nagy et al., 1997). The results reported here indicate that feeding PE to weaned pigs challenged with F18 *E. coli* improved the gut health as indicated by increasing villus height of the small intestine. In addition, the supplementation of the PE to the *E. coli* challenged pigs reduced diarrhea and inflammation caused by the *E. coli* challenge, as indicated by reducing WBC, pro-inflammatory cytokines, and acute phase proteins. These findings are in agreement with previous data showing an improvement of gut health and immune responses in piglets fed different PE (Manzanilla et al., 2004; Michiels et al., 2010; Wang et al., 2011).

The present experiment clearly shows a reduction of diarrhea by feeding PE to pigs challenged with *E. coli*. These results are supported by observations reported by Gutiérrez et al. (2007), Mukherjee et al. (1998) and Shoba and Thomas (2001), which indicated many plant extracts had antidiarrheal activity by inhibiting gastrointestinal mobility and PGE-induced enteropooling, and stimulating water absorption. Other potential mechanisms might be also involved in the reduced diarrhea. First, PE may directly affect the activity of pathogenic *E. coli* by killing or inhibiting bacterial proliferation or toxin secretion (Hammer et al., 1999). Second, PE may improve gut health by strengthening gut barrier function, resulting in some protection against bacteria and/or their toxins (Karmouty-Quintana et al., 2007; Park et al., 2011). Third, PE may enhance the gastrointestinal immune system and/or the systemic immune system, which indirectly improve pig health and reduce diarrhea of pigs (Manzanilla et al., 2004; Michiels et al., 2010; Wang et al., 2011).

Plant extracts have been proposed as alternatives for antibiotics due to their significant antimicrobial effects (Baydar et al., 2004; Sökmen et al., 2004). However, in the present experiment, the supplementation of PE did not affect the proportions of β-hemolytic coliforms in feces of *E. coli* challenged pigs, despite the reduction of diarrhea. These results indicate that the reduction of diarrhea may be caused by other mechanisms instead of the antimicrobial effect of PE. On the other hand, the supplementation level of the 3 PE tested in the current experiment was 10 ppm, much lower than the levels often tested, such as from 20 to 1000 ppm, depending on the source of plants and methods of extraction (Allan and Bilkei, 2005; Kim et al., 2008; Manzanilla et al., 2004). Therefore, the reduction of diarrhea and non-reduction of β-hemolytic coliforms from this experiment suggest that even low levels of PE may have beneficial effects on pig health, probably by regulation of the immune system. Moreover, the low supplemental level of PE may help swine producers to save feed cost when adding the PE in the diets.

Total WBC counts are used commonly to estimate the risk of serious bacterial infection, and an increase in the number of WBC suggests the presence of systemic inflammation (Gordon-Smith, 2009). The present study showed the *E. coli* infection induced marked increases in WBC and lymphocytes on d 5, and WBC, neutrophils, lymphocytes, and monocytes on d 11 PI. These results are consistent with Song et al. (2011) using the same strain and dose of *E. coli* in a challenge study with weaned pigs. The present study also showed the supplementation of PE decreased WBC, neutrophils, and lymphocytes, indicating the PE tested here may be beneficial for pig growth performance as the PE attenuate the systemic inflammation caused by *E. coli* infection. In agreement with these findings, Wang et al. (2011) reported that dietary fermented garlic powder reduced the number of WBC of pigs infected with LPS. Otherwise, in the normal

conditions, Czech et al. (2009) also observed that pigs receiving herbal extracts, such as garlic bulbs, had a better health condition with lower WBC.

Consistent with the increased WBC population in the *E. coli*-infected pigs, an increased TNF-α was observed in the serum of pigs in the *E. coli* challenge group. Inflammation is mediated by increased production of pro-inflammatory cytokines. Among these, TNF-α is one of most important cytokines released from a variety of porcine immune cells, especially macrophages/monocytes, in response to bacterial cell wall products and bacterial toxins. Therefore, systemic levels of TNF-α have been recorded as an indicator of the extent of inflammation in the circulation of pigs after intravenous entertoxin administration and during fatal gram-negative sepsis (Jesmok et al., 1992; Maeda et al., 1993; Frank et al., 2003). The reduction of serum TNF-α by feeding PE to *E. coli*-infected pigs in the present experiment suggests that PE might reduce the inflammation induced by *E. coli*. Wang et al. (2011) also reported that LPS-infected pigs fed fermented garlic powder had lower TNF-α than those fed the control diet. Otherwise, many in vitro studies have demonstrated anti-inflammatory effects of several PE (Lang et al., 2004; Lee et al., 2007; Dung et al., 2009). Our previous in vitro study also found all of 7 PE tested, including the 3 PE tested here, are anti-inflammatory as showed by inhibition of TNF-α secretion from LPS-stimulated porcine alveolar macrophage (Liu et al., 2011c). Both results of WBC and TNF-α in the present experiment clearly showed feeding dietary PE may alleviate the over-stimulation of the systemic immunity and early immune response of pigs infected with *E. coli*. The modes of action for the immune-regulating activity of PE are still not clear. The results from IL-10 and TGF-β in the current experiment indicates other mechanisms rather than the secretion of anti-inflammatory cytokines might contribute to the reduced TNF-α and WBC. However, in vitro evidence suggests that these effects are mediated, at

least in part, by blocking the NF-κB pathway (Aggarwal and Shishodia, 2004; Jobin et al., 1999; Youn et al., 2008).

The proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are important inducers of the synthesis of acute phase proteins, such as CRP and Hp by hepatocytes (Carroll et al., 2004, Jain et al., 2011). In the present study, the *E. coli* infection did not change serum CRP levels, but increased Hp compared with the sham group. The greater response of Hp than CRP has also been found in pigs infected with porcine reproductive and respiratory virus syndrome virus (Che et al., 2011; Liu et al., 2011b). Haptoglobin has also been reported to develop in pigs during infections with various other bacteria, such as *Bordetella bronchiseptica, Pasteurella multocida*, *Actinobacillus pleuropneumoniae,* and *Strepococcus suis* (Francisco et al., 2004; Hall et al., 1992; Knura-Deszczka et al., 2002). The increased levels of serum Hp in *E. coli*-infected pigs may play important roles in mediating immune responses during *E. coli* infection, such as suppression of T lymphocyte proliferation or inhibition of granulocyte chemotaxis, phagocytosis, and bactericidal activity (Arredouani et al., 2003; Rossbacher et al., 1999). Therefore, the reduction of serum Hp concentrations caused by the PE tested in the present experiment may be beneficial in controlling immune responses of *E. coli*-infected pigs.

It has been reported that STb toxin secreted by *E. coli* is able to induce partial villus atrophy in young pigs (Rose et al., 1987). In the present study, pigs infected with *F18* E. coli with LT, STb, and SLT-2 toxins had lower villus height in the jejunum and ileum compared with the sham group. Similarly, previous studies also observed the reduced villus height and volume of pigs infected with *E. coli* (Liu et al., 2010; Vijtiuk et al., 1995; Yi et al.,2005). The reduction of villus height related to the absorptive capacity of the mucus membrane (Buddle and Bolton, 1992) could induce a decreased absorption of nutrients, which may be responsible for the

reduced growth performance. However, the present study shows the pigs fed PE had higher villus height compared with the CON in the *E. coli* challenged group, which indicates the PE tested may be beneficial to gut health. In addition, plant extracts from garlic, Ginkgo biloba extract, and the mixture of carvacrol, cinnamaldehyde, and capsaicin have been reported to increase the tightening of tight junctions (Park et al., 2011), attenuate mucosal damage (Mustafa et al., 2006), and increase mucus synthesis (Jamroz et al., 2006), which may improve the gut health and prevent the villus atrophy in *E. coli*-infected pigs.

In the present experiment, *E. coli* infection significantly reduced growth rate and feed efficiency, which has also been shown in some other studies (Song et al., 2011; Yi et al., 2005). No beneficial effect on growth performance was observed from pigs fed PE in the *E. coli* challenged group; even though feeding PE increased villus height and reduced systemic inflammation. Several reasons may be involved in the failure of finding beneficial effects of PE on pig performance. First, the overall period for this challenge study was 15 days, and it is difficult to detect growth performance during this short period, and the immediate effect of weaning has dramatic effects on the growth performance. Second, the replication numbers for each treatment were large for a challenge study but small for detecting effects on growth performance.

In conclusion, feeding PE is effective in alleviating diarrhea of weaned pigs caused by an F-18 *E. coli* challenge. Feeding PE reduced serum TNF-α and haptoglobin, decreased white blood cells and lymphocytes, and mitigated villus atrophy and intestinal morphology disruption after *E. coli* challenge. We did not find clear differences among the 3 PE tested here, suggesting that all of 3 them may be beneficial in preventing pathogenic infection and maintaining normal intestinal integrity and function. This experiment clearly show evidence that a low level of

dietary PE can be used in pig diets to maintain pig health, especially alleviating post-weaning diarrhea. Further studies are needed to investigate the mechanisms through which the PE tested here exert these effects.

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TABLES AND FIGURES

Table 3.1. Ingredient composition of basal diet (as-fed basis).

Ingredient	Amount, %
Corn, ground	41.54
Whey dried	15.00
Soybean meal, 47%	10.82
Fishmeal	10.00
Lactose	10.00
Soy protein concentrate	5.00
Poultry byproduct meal	4.27
Soybean oil	2.67
Mineral premix[1]	0.35
Vitamin premix[2]	0.20
L-Lysine·HCl	0.05
DL-Met	0.05
L-Thr	0.03
L-Trp	0.02
Total	100.00
Calculated energy and nutrients	
ME, kcal/kg	3480
CP, %	22.67
Fat, %	6.34
Ca, %	0.80
P, %	0.72
Available P, %	0.49
Lys, %	1.50
Lactose, %	21.00

Table 3.1. (cont.)

[1]Provided as milligrams per kilogram of diet: 3,000 of NaCl; 100 of Zn from zinc oxide; 90 of Fe from iron sulfate; 20 of Mn from manganese oxide; 8 of Cu from copper sulfate; 0.35 of I from calcium iodide; 0.30 of Se from sodium selenite.

[2]Provided per kilogram of diet: 2,273 μg of retinyl acetate; 17 μg of cholecalciferol; 88 mg of DL-α-tocopheryl acetate; 4 mg of menadione from menadione sodium bisulfite complex; 33 mg of niacin; 24 mg of D-Ca-pantothenate; 9 mg of riboflavin; 35 μg of vitamin B_{12}; 324 mg of choline chloride.

Table 3.2. Effect of plant extract on growth performance of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
Body weight														
d 0, kg	6.66	6.46	6.63	6.66	6.51	6.59	6.43	6.63	0.20	0.63	0.90	0.83	0.71	0.89
d 5, kg	6.93	7.11	7.15	7.24	6.54	6.68	6.53	6.77	0.21	<0.05	0.62	0.94	0.30	0.64
d 11, kg	9.58	8.93	8.60	9.08	8.13	8.18	8.35	9.20	0.50	<0.05	0.34	0.25	0.095	0.38
d 0 to 5														
No. of pigs[5]	8	8	8	8	7	8	8	7						
No gain[6]	1	0	1	0	4	4	4	3						
ADG, g	53	130	105	115	5.7	18	20	29	23	<0.05	0.18	0.54	<0.05	0.55
ADFI, g	328	418	455	361	428	350	325	370	85	0.66	0.99	0.41	0.30	0.36
G:F	0.22	0.42	0.26	0.81	-	-	-	-	0.10	<0.05	0.44	0.58	0.27	0.81
d 5 to 11														
No. of pigs[5]	4	4	4	4	3	4	4	3						
No gain[6]	0	0	0	0	0	0	0	0						
ADG, g	470	384	292	350	278	263	321	345	76	0.10	0.69	0.25	0.051	0.68
ADFI, g	730	700	742	588	767	742	754	756	136	0.47	0.92	0.92	0.68	0.92
G:F	0.76	0.60	0.73	0.71	0.37	0.39	0.43	0.47	0.25	0.096	0.98	0.98	0.75	0.82

Table 3.2. (cont.)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	**CON**	**CAP**	**GAR**	**TUR**	**CON**	**CAP**	**GAR**	**TUR**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
d 0 to 11														
No. of pigs[5]	4	4	4	4	3	4	4	3						
No gain[6]	0	0	0	0	0	0	0	0						
ADG, g	276	251	212	246	149	157	182	218	38	<0.05	0.65	0.24	0.19	0.31
ADFI, g	540	512	596	421	615	568	561	545	70	0.23	0.42	0.64	0.65	0.48
G:F	0.53	0.50	0.38	0.61	0.25	0.31	0.34	0.40	0.09	<0.05	0.24	0.40	0.63	0.28

[1]n = 62 (7 or 8 pigs/ treatment) from d -4 to 5; n = 30 (3 or 4 pigs/treatment) from d 5 to 11.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or *E. coli* challenge group.

[5]No. of pigs = number of live pigs.

[6]No gain = number of pigs which did not gain BW.

Table 3.3. Effect of plant extracts on diarrhea score and frequency of diarrhea of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0 to 2[5]	1.96	1.17	1.21	1.29	1.93	1.67	1.35	1.36	0.36	0.32	<0.05	0.70	<0.05	0.11
d 3 to 5[5]	2.06	1.35	1.46	1.67	3.43	2.50	2.13	2.0	0.31	<0.05	<0.05	0.22	<0.05	<0.05
d 6 to 8[5]	1.44	1.18	1.17	1.21	2.86	2.58	2.83	2.90	0.52	<0.05	0.92	0.96	0.49	0.91
d 9 to 11[5]	1.09	1.04	1.08	1.00	3.51[a]	2.13[b]	1.21[c]	1.15[c]	0.26	<0.05	<0.05	<0.05	0.77	<0.05
Pig days[6]	64	64	64	64	53	64	64	53						
Frequency[7]	20	4	7	9	40	26	17	16		<0.05	<0.05	<0.05	<0.05	<0.05

[a, b, c] within a row, means without a common superscript differ ($P < 0.05$) within the *E. coli* challenge group.

[1]n = 62 (7 or 8 pigs/ treatment) on d 0, 3, and 5; n = 30 (3 or 4 pigs/treatment) on d 8 and 11.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or *E. coli* challenge group.

[5]Diarrhea score = 1, normal feces, 2, moist feces, 3, mild diarrhea, 4, severe diarrhea, 5, watery diarrhea.

[6]Pig days = number of pig x the number of days of diarrhea scoring.

[7]Frequency = number of pig days with diarrhea score ≥ 3. Statistical analysis was conducted by Chi-square test.

Table 3.4. Effect of plant extracts on culture score of feces from pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 3														
Coliform	5.3	5.5	6.0	6.6	6.5	7.0	6.4	6.1	0.43	<0.05	0.64	0.073	0.092	0.99
β-hemolytic	0	0	0	0	5.6	5.5	5.0	5.4	0.55	<0.05	0.94	0.94	1.00	0.60
β-hemo., ratio	0	0	0	0	0.86	0.79	0.77	0.89	0.07	<0.05	0.80	0.80	1.00	0.56
d 5														
Coliform	5.8	6.5	5.6	6.1	8.0	8.0	7.9	8.0	0.69	<0.05	0.88	0.93	0.66	0.96
β-hemolytic	0	0	0	0	7.5	6.8	7.0	7.9	0.48	<0.05	0.62	0.62	1.00	0.57
β-hemo., ratio	0	0	0	0	0.94	0.84	0.89	0.99	0.06	<0.05	0.65	0.65	1.00	0.63
d 8														
Coliform	5.8	7.3	6.0	5.8	6.8	7.8	7.8	7.3	0.60	<0.05	0.14	0.61	0.33	0.17
β-hemolytic	0.06	0.09	0	0	6.0	6.8	3.8	5.3	1.23	<0.05	0.48	0.54	0.98	0.50
β-hemo., ratio	0.05	0.07	0	0	0.85	0.87	0.50	0.70	0.15	<0.05	0.43	0.46	0.99	0.27

Table 3.4. (cont.)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	**CON**	**CAP**	**GAR**	**TUR**	**CON**	**CAP**	**GAR**	**TUR**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
d 11														
Coliform	4.6	7.0	5.8	4.8	5.8	7.0	6.3	5.3	1.00	0.38	0.15	0.93	0.20	0.67
β-hemolytic	0.13	0.21	0	0	2.5	1.8	0.5	1.9	1.03	<0.05	0.45	0.55	0.94	0.17
β-hemo., ratio	0.02	0.03	0	0	0.40	0.26	0.13	0.28	0.16	<0.05	0.61	0.71	0.94	0.18

[1]n = 62 (7 or 8 pigs/ treatment) on d 0, 3, and 5; n = 30 (3 or 4 pigs/treatment) on d 8 and 11.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or *E. coli* challenge group.

[5]Score of bacterial growth = 0, none, 1, rare, 2, a few, 3, light, 4, very light, 5, moderate, 6, very moderate, 7, heavy, 8, very heavy.

[6]Coliform = total coliforms; β-hemolytic = β-hemolytic coliforms; β-hemo., ratio = ratio of β-hemolytic coliforms score to total coliforms score.

Table 3.5. Effect of plant extracts on total and differential white blood cells of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 5														
WBC, $10^3/\mu L^5$	15.2	14.5	17.0	13.8	21.1	14.5	14.6	18.3	1.69	0.091	0.19	<0.05	0.98	<0.05
Neu, $10^3/\mu L^5$	10.22	7.96	10.37	8.40	10.16	7.66	8.59	9.15	1.32	0.70	0.28	0.79	0.36	0.27
Lym, $10^3/\mu L^5$	4.15	5.63	5.49	4.54	8.25	6.17	5.35	8.36	0.97	<0.05	0.71	0.050	0.32	0.15
Mono, $10^3/\mu L^5$	0.535	0.682	1.051	0.723	0.425	0.470	0.587	0.514	0.16	<0.05	0.14	0.66	0.10	0.58
Eos, $10^3/\mu L^5$	0.098	0.099	0.069	0.079	0.086	0.105	0.050	0.188	0.047	0.52	0.45	0.49	0.76	0.61
Baso, $10^3/\mu L^5$	0.031	0.062	0.032	0.002	0.028	0.054	0.029	0.060	0.032	0.55	0.62	0.56	0.97	0.53
Neu/Lym[5]	3.24	1.45	3.17	2.10	1.28	1.41	1.60	1.22	0.66	<0.05	0.36	0.44	0.17	0.86
d 11														
WBC, $10^3/\mu L^5$	16.7	15.8	19.0	14.4	32.2	21.6	23.0	24.3	3.44	<0.05	0.27	0.25	0.94	<0.05
Neu, $10^3/\mu L^5$	7.96	8.28	8.49	6.71	17.01	10.26	14.00	10.19	1.87	<0.05	0.099	0.20	0.95	<0.05
Lym, $10^3/\mu L^5$	8.57	6.66	9.86	6.99	13.48	9.43	7.63	12.30	2.43	0.062	0.45	0.20	0.74	0.10
Mono, $10^3/\mu L^5$	0.264	0.890	0.549	0.473	1.473	1.536	0.934	1.778	0.299	<0.05	0.29	0.28	0.23	0.85
Eos, $10^3/\mu L^5$	0.309	0.165	0.051	0.148	0.223	0.322	0.234	0.183	0.131	0.40	0.67	0.64	0.17	0.87
Baso, $10^3/\mu L^5$	0	0.151	0	0.054	0.039	0.026	0.127	0.128	0.070	0.53	0.64	0.26	0.34	0.45
Neu/Lym[5]	1.13	1.37	0.91	0.97	1.44	1.11	2.29	1.04	0.42	0.17	0.49	0.17	0.91	0.94

Table 3.5. (cont.)

[1]n = 62 (7 or 8 pigs/ treatment) on d 5; n = 30 (3 or 4 pigs/treatment) on d 11.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or *E. coli* challenge group.

[5]WBC = white blood cell; Neu = neutrophil; Lym = lymphocyte; Mono = monocyte; Eos = eosinophil; Baso = basophil; Neu/Lym = neutrophils/lymphocytes.

Table 3.6. Effect of plant extracts on red blood cell measures of pigs experimentally infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 5														
RBC, $10^6/\mu L$[5]	6.74	6.74	7.06	6.70	7.23	6.64	6.90	6.89	0.23	0.45	0.34	0.32	0.67	0.074
HGB, g/dL[5]	12.3	12.4	12.5	11.9	13.0	12.6	12.4	12.9	0.32	<0.05	0.88	0.23	0.92	0.31
HCT, %[5]	38.4	38.6	38.8	37.3	39.8	38.5	38.2	39.7	0.92	0.24	0.88	0.32	0.84	0.35
MCV, fL[5,6]	57.1	57.4	55.1	56.0	55.5	58.0	55.4	57.6	1.81	0.76	0.23	0.63	0.50	0.31
MCH, pg[5]	18.3	18.4	17.7	17.9	18.1	19.0	18.0	18.8	0.68	0.15	0.18	0.56	0.49	0.33
MCHC, g/dL[5]	32.0	32.1	32.1	32.0	32.6	32.6	32.5	32.6	0.29	<0.05	0.94	0.94	0.88	0.81
RDW, %[5]	28.7	28.5	28.3	29.3	27.5	28.0	33.3	26.4	2.69	0.92	0.21	0.089	0.99	0.35
d 11														
RBC, $10^6/\mu L$[5]	5.90	5.79	6.35	6.35	7.12	6.10	6.11	6.26	0.35	0.18	0.35	0.10	0.47	<0.05
HGB, g/dL[5]	11.1	10.7	11.1	11.5	12.8	11.0	10.9	11.7	0.44	0.10	<0.05	0.11	0.95	<0.05
HCT, %[5]	34.3	33.5	34.3	36.4	39.0	34.0	33.4	35.8	1.23	0.25	<0.05	0.064	0.75	<0.05
MCV, fL[5,6]	57.8	57.7	54.1	57.6	55.1	56.1	54.7	57.3	2.34	0.47	0.39	0.81	0.54	0.67
MCH, pg[5]	18.7	18.4	17.5	18.3	18.1	18.1	17.8	18.8	0.76	0.95	0.45	0.78	0.33	0.91
MCHC, g/dL[5]	32.4	31.9	32.3	31.7	32.9	32.3	32.4	32.7	0.35	<0.05	0.34	0.55	0.21	0.26
RDW, %[5]	28.0	26.9	25.6	25.0	25.7	25.3	30.8	24.7	2.46	0.86	0.39	0.21	0.33	0.58

Table 3.6. (cont.)

[1]n = 62 (7 or 8 pigs/ treatment) on d 5; n = 30 (3 or 4 pigs/treatment) on d 11.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or *E. coli* challenge group.

[5]RBC = red blood cell; HGB = hemoglobin; HCT = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red cell distribution width.

[6]fL= femtolitre (10^{-15} L).

Table 3.7. Effects of plant extracts on cytokine and acute phase protein concentrations in the serum of pigs infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 0														
TNF-α, pg/mL[5]	52.31	61.66	56.83	44.63	48.15	60.10	63.25	63.37	8.66	0.48	0.48	0.45	0.79	0.15
IL-10, pg/mL	0.34	1.08	3.73	0.88	0.36	0.55	0.37	0.86	1.02	0.19	0.44	0.36	0.19	0.84
TGF-β, ng/mL[5]	23.12	21.99	21.24	22.11	25.17	20.46	20.38	23.99	1.18	0.63	<0.05	0.25	0.30	<0.01
CRP, μg/mL[5]	23.96	18.15	20.19	22.24	21.20	25.11	21.76	20.75	2.88	0.60	0.96	0.35	0.26	0.69
HP, μg/mL[5]	603.4	587.0	735.6	568.3	536.1	596.8	513.8	616.6	83.5	0.33	0.93	0.38	0.78	0.68
d 5														
TNF-α, pg/mL[5]	58.97	68.27	56.59	58.06	79.55	61.82	72.2	63.08	5.76	<0.05	0.43	0.057	0.73	<0.05
IL-10, pg/mL	1.05	1.17	1.52	1.14	2.53	1.63	4.35	2.59	1.16	0.063	0.61	0.79	0.87	0.80
TGF-β, ng/mL[5]	22.64	24.11	21.71	21.78	23.40	21.22	21.66	21.03	1.33	0.41	0.52	0.50	0.94	0.15
CRP, μg/mL[5]	25.02	21.66	24.13	26.64	29.06	27.79	24.58	29.30	3.06	0.16	0.56	0.81	0.80	0.60
HP, μg/mL[5]	930.6	574.2	570.9	663.4	1399.5[a]	823.1[b]	887.5[b]	1269.6[a]	116.8	<0.05	<0.05	0.43	<0.05	<0.05

Table 3.7. (cont.)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	**CON**	**CAP**	**GAR**	**TUR**	**CON**	**CAP**	**GAR**	**TUR**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
d 11														
TNF-α, pg/mL[5]	68.00	66.35	88.58	61.53	87.61	80.91	65.62	66.23	14.44	0.67	0.73	0.35	0.78	0.27
IL-10, pg/mL	0.52	0	0	0.16	0.01	0.13	0.32	0.76	0.38	0.57	0.70	0.39	0.20	0.32
TGF-β, ng/mL[5]	30.44	27.55	24.61	25.74	23.04	18.72	25.01	25.87	2.47	<0.05	0.38	0.070	0.066	0.85
CRP, μg/mL[5]	34.78	30.18	31.52	25.05	26.60	32.15	21.13	31.96	8.24	0.60	0.85	0.51	0.39	0.82
HP, μg/mL[5]	407.1	304.0	507.6	236.3	1271.6[a]	937.8[a]	163.8[b]	1363.0[a]	291.6	<0.05	0.091	<0.05	0.81	0.074

[a, b] within a row, means without a common superscript differ ($P < 0.05$) within the *E. coli* challenge group.

[1]n = 62 (7 or 8 pigs/ treatment) on d 0 and 5; n = 30 (3 or 4 pigs/treatment) on d 11.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or *E. coli* challenge group.

[5]TNF-α = tumor necrosis factor-α, TGF-β = transforming growth factor-β, CRP = C-reactive protein, Hp = haptoglobin.

Table 3.8. Effects of plant extracts on histological characteristics of the small intestine of pigs infected with a pathogenic *E. coli*[1]

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR	SEM	*E.coli*	Diet	E*D	Sham	*E. coli*
d 5														
Jejunum														
Villi height, μm	315	334	320	259	259	271	317	320	21.6	<0.05	0.28	0.42	0.55	0.093
Crypt depth, μm	219	205	215	221	235	234	212	221	13.7	0.13	0.59	0.30	0.63	0.26
VH:CDH[5]	1.51	1.73	1.66	1.56	1.15	1.18	1.60	1.48	0.16	<0.05	0.20	0.27	0.39	0.10
Ileum														
Villi height, μm	334	385	319	333	263	305	316	326	25.6	<0.05	0.18	0.15	0.63	<0.05
Crypt depth, μm	183	214	193	213	202	206	196	205	14.0	0.87	0.47	0.74	0.16	0.99
VH:CDH[5]	1.92	1.93	1.79	1.64	1.39	1.55	1.70	1.69	0.16	<0.05	0.91	0.26	0.46	0.17
Colon														
Crypt depth, μm	335	333	389	361	338	318	315	332	19.6	<0.05	0.54	0.26	0.25	0.48
d 11														
Jejunum														
Villi height, μm	467	485	428	463	465	457	402	429	32.3	0.29	0.23	0.95	0.80	0.33
Crypt depth, μm	203	185	206	213	222	226	208	242	14.0	<0.05	0.34	0.48	0.93	0.85
VH:CDH[5]	2.43	2.75	2.13	2.25	2.19	2.09	2.05	1.83	0.21	<0.05	0.16	0.47	0.80	0.40

Table 3.8. (cont.)

	Treatment[2]									*P*-value				
	Sham				*E. coli*					Main effect[3]			PE vs. CON[4]	
Item	**CON**	**CAP**	**GAR**	**TUR**	**CON**	**CAP**	**GAR**	**TUR**	**SEM**	***E.coli***	**Diet**	**E*D**	**Sham**	***E. coli***
Ileum														
Villi height, μm	364	439	346	385	346	377	378	400	29.6	0.66	0.17	0.34	0.37	0.20
Crypt depth, μm	219	236	210	217	239	234	203	247	14.4	0.26	0.12	0.41	0.86	0.43
VH:CDH[5]	1.79	2.03	1.73	1.89	1.55	1.67	1.98	1.69	0.13	0.12	0.35	0.082	0.48	0.10
Colon														
Crypt depth, μm	383	373	408	381	371	393	406	400	27.7	0.73	0.62	0.89	0.86	0.32

[1]n = 32 (4 pigs/ treatment) on d 5; n = 30 (3 or 4 pigs/treatment) on d 11.

[2]Sham = unchallenged; *E. coli* = *E. coli* challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]*E. coli* = *E. coli* challenge effect; Diet = diet effect; E*D = interaction between *E. coli* and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or *E. coli* challenge group.

[5]VH:CDH = villi height:crypt depth.

CHAPTER 4

PLANT EXTRACTS IMPROVE IMMUNE RESPONSES AND GROWTH EFFICIENCY OF WEANED PIGS EXPERIMENTALLY INFECTED WITH PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

ABSTRACT

A study was conducted to evaluate the effects of 3 different plant extracts (PE) on growth performance and immune responses of weaned pigs experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV). Weaned pigs (n = 64, 7.8 ± 0.3 kg BW) were used in a 2 × 4 factorial arrangement with a randomized complete block design. The first factor was with or without PRRSV challenge (intranasal; 10^5 50% tissue culture infective dose). The second factor was 4 diets: a nursery basal diet (CON), 10 mg capsicum oleoresin (CAP), garlic (GAR), or turmeric oleoresin (TUR)/kg diet. Pigs were housed in disease containment chambers for 28 d: 14 d before and after the inoculation (d 0). Rectal temperature (RT) was measured every 3 or 4 d post-inoculation (PI). The ADG, ADFI, and G:F were measured on d -14 to 0, 0 to 7, and 7 to 14. Blood was collected on d 0, 7, and 14 to measure the total and differential white blood cells (WBC), and serum was collected to measure viral load (VL) by qPCR, PRRSV antibody titer (AT), tumor necrosis factor-alpha (TNF-α), IL-10, C-reactive protein (CRP), and haptoglobin (Hp) by ELISA. The PRRSV infection decreased ($P < 0.01$) ADG and ADFI from d 0 to 7, 7 to 14, and 0 to 14, and G:F from d 7 to 14 and 0 to 14, and increased ($P < 0.05$) RT on d 7, 9, and 11, VL on d 7 and 14, AT on d 14, and TNF-α, IL-10,

CRP, and HP on d 7 and 14, compared with the unchallenged group. In addition, the PRRSV infection reduced ($P < 0.01$) WBC and lymphocytes (LYM) on d 7, monocytes (MONO) on d 7 and d 14, and the ratio of neutrophils to LYM (NEU/LYM) on d 14, but increased NEU/LYM on d 7, WBC and LYM on d 14. In the PRRSV challenged group, CAP reduced ($P < 0.05$) RT on d 4 and VL, TNF-α, and CRP on d 7, and increased IL-10 and Hp on d 14; GAR reduced ($P < 0.05$) RT on d 4, and increased ($P < 0.05$) ADG from d 0 to 7 and Hp on d 14; TUR increased ($P < 0.05$) ADG from d 7 to 14, G:F from d 7 to 14 and from d 0 to 14, AT, and decreased VL on d 7 and 14 and TNF-α on d 7. The 3 PE tested did not influence the populations of peripheral immune cells. In the unchallenged group, all piglets were PRRSV negative during the overall period PI. The CAP increased ($P < 0.05$) ADFI from d 0 to 7 and overall period PI, and LYM on 7 compared with the CON. In conclusion, the 3 PE tested showed different effects on growth efficiency and immune responses, and TUR might strengthen the immune responses and efficiency of pigs infected with PRRSV.

KEY WORDS: growth, immune responses, plant extracts, PRRSV, weaned pigs

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS), caused by a positive single-stranded RNA virus (PRRSV), is one of most economically important diseases in the swine industry (Wardley et al., 1996; Neumann et al., 2005). The general clinical signs of PRRS in weaned pigs are fever, lethargy, respiratory depression, and decreased performance (Van Reeth et al., 1999; Hall, 2005). The PRRSV has a complex interaction with the immune system because the primary targets of PRRSV are immune cells, such as alveolar macrophages, monocytes,

anddendritic cells (Choi and Chae, 2002; Labarque et al., 2003). The PRRSV infection can impair immune responses (Lopez Fuertes et al., 1999; Mulupuri et al., 2007), which may increase the risk of secondary infection of pigs by other pathogenic bacteria or virus.

Plant extracts (PE) are secondary plant metabolites, which have been proposed as effective feed additives in swine production due to their in vitro antimicrobial (Sokmen et al., 2004), anti-inflammatory (Sosa et al., 2005), antioxidant (Dundar et al., 2008), or antiviral (Sökmen et al., 2004) effects, and potential beneficial effects on pig performance and health in vivo (Manzanilla et al., 2004; Allan and Bilkei, 2005). Our previous in vitro study indicated that 7 different PE had potential anti-inflammatory effects, as all PE tested inhibited the secretion of tumor necrosis factor-alpha (TNF-α) from LPS-stimulated porcine alveolar macrophages (Liu et al., 2011c).Another of our experiments showed capsicum oleoresin, garlic, and turmeric oleoresin reduced diarrhea and enhanced immune responses of *E. coli*-infected pigs (Liu et al., 2011a, b). However, it is not clear whether these 3 PE have beneficial effects on the immune system of pigs with viral infections. Therefore, the objective of this study was to investigate whether the 3 PE mentioned above alter growth performance, disease resistance, and immune responses of nursery pigs experimentally infected with PRRSV.

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign. The experiment was conducted in the disease containment chambers of the Edward R. Madigan Laboratory building at the University of Illinois at Urbana-Champaign.

Animals, Housing, Experimental Design, and Diet

A total of 64 weaned piglets (7.8 ± 0.3 kg BW, 21 d old; PIC C-22 female × PIC line 337 male) with same number of gilts and barrows were selected from the Swine Research Center of the University of Illinois at Urbana-Champaign. Prior to weaning, blood samples of all piglets destined for this experiment were tested to verify they were free of PRRSV. After weaning, all pigs were transferred to the disease containment chambers and randomly assigned to treatment in a randomized complete block design with weight within sex as the blocks and pig as the experimental unit. Pigs were housed in the individual pens for 28 days (14 d before and 14 d after the PRRSV challenge (d 0)). There were a total of 64 individual pens, 4 in each of 16 chambers. There were 2 suites of 8 chambers, and each suite was used for either PRRSV challenged or unchallenged pigs. The piglets had *ad libitum* access to feed and water.

The treatments were in a 2×4 factorial arrangement (with or without PRRSV challenge; 4 dietary treatments). There were 8 replicates per treatment. In the PRRSV challenge group, all 32 pigs were inoculated intranasally with 2 mL of high-virulence strain of PRRSV (Purdue isolate P-129) containing 10^5 50% tissue culture infective dose. In the unchallenged group, pigs were inoculated with 3 mL PBS as the sham control. The 4 dietary treatments were the complex nursery basal diet (CON), and the addition of 10 mg capsicum oleoresin (CAP), 10 mg/kg garlic (GAR), or 10 mg/kg turmeric oleoresin (TUR)/kg diet to the CON, respectively. All 3 plant extracts were obtained from Pancosma S. A. (Geneva, Switzerland). Capsicum and turmeric are extracted oleoresins, which were standardized to 6% capsaicin and dihydrocapsaicin, and 98% curcuminoides, respectively. Garlic is a botanical extract from garlic, standardized to 40% propyl thiosulfonates. The CON was formulated to meet or exceed NRC (1998) estimates of requirements of weaned pigs (Table 4.1). Spray-dried plasma, antibiotics, or zinc oxide were not

included in the diets, and the experimental diets were provided to pigs throughout the experimental period. After PRRSV inoculation, one pig from the infected GAR treatment was culled on d 5 post-inoculation (PI) due to difficult breathing, and other two pigs from infected CON and TUR treatments were euthanized on d 7 and 8 PI due to severe diarrhea and weight loss.

Sample Collection

Blood samples were collected in duplicate (Ethylenediaminetetraacetic acid (EDTA) blood and serum) from the jugular vein of each pig before the PRRSV challenge and d 7 and 14 PI. Whole blood samples were used to measure total and differential white blood cell counts. Sera were used for determination of viral load, antibody titer, cytokines, and acute phase proteins (APP).

Pigs and feeders were weighed on the day of weaning (d -14), the inoculation day (d 0), and d 7 and 14. Growth performance (ADG, ADFI, and G:F) was measured for each interval from d -14 to 0, 0 to 7, 7 to 14, and 0 to 14.

At the end of experiment (d 14 PI), all pigs were anesthetized by intramuscular injection of a 1 mL combination of telazol, ketamine, and xylazine (2:1:1) per 23 kg BW. The final mixture contained 100 mg telazol, 50 mg ketamin, and 50 mg xylazine in 1 mL (Fort Dodge Animal Health, For Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection with 78 mg sodium pentobarbital (Sleepaway, Henry Schein, Inc., Indianapolis, IN) per 1 kg of BW, and lung tissues were collected and fixed in 10% neutral buffered formalin for further histological analysis.

Clinical Signs and Histological Examination

Clinical observations were recorded daily PI. Rectal temperature (RT) for each pig was measured before PRRSV inoculation and on d 4, 7, 9, 11, and 14 PI.

The fixed lung tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. The slides were scanned by NanoZoomer Digital Pathology (NDP) System (Hamamatsu Co., Bridgewater, NJ), and viewed in the associated NDP.view software.

Determination of Differential Blood Leukocytes, Viral Load, and PRRSV Antibody

Blood samples collected in EDTA were used to measure total and differential blood cell counts by the Veterinary Clinical Pathology Laboratory at the University of Illinois at Urbana-Champaign. Differential leukocyte proportions and concentrations were analyzed on a multiparameter, automated hematology analyzer calibrated for porcine blood (CELL-DYN 3700, Abbott Laboratories, Abbott Park, IL).

The serum viral load and PRRSV-specific antibody were analyzed by the Indiana Animal Disease Diagnostic Laboratory at Purdue University. The viral load was measured by PCR, and the results are presented as cycle threshold (Ct) values. A higher Ct value indicates a lower viral load in the serum. The serum antibodies against PRRSV were measured by an ELISA method according to the procedures described by the manufacturer (IDEXX, Westbrook, ME). The results are shown as sample to positive ratio (S/P), calculated for each serum sample of the infected pigs. A ratio of 0.4 or greater is considered positive.

Measurements of Serum Cytokines and Acute Phase Proteins

The serum TNF-α and IL-10 concentrations were measured by ELISA according to the recommendations of the manufacturer (R&D Systems, Inc., Minneapolis, MN, USA). Briefly, standard, control, and samples were added to the wells coated with monoclonal antibody specific

for each cytokine. After incubation for 2 h, the unbound substances were washed away, and an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells to sandwich the cytokine immobilized during the first incubation. A further 2 h of incubation was followed by a wash to remove any unbound antibody-enzyme reagents, and then a substrate solution was added to the wells and color developed in proportion to the amount of the cytokine bound in the initial step. The color development was stopped by adding the stop solution and the intensity of the color was measured at 450 nm with the correction wavelength set at 530 nm. Concentrations were calculated from a standard curve. All samples were analyzed in duplicate. The intra-assay and inter-assay coefficients of variation provided by the manufacturer were lower than 4.4 and 7.1%, respectively.

The serum C-reactive protein (CRP) and haptoglobin (Hp) levels were measured by commercially available kits according to the recommendation of manufacturer (GenWay Biotech, Inc., San Diego, CA, USA). The detailed procedures were similar to those described above, except for different incubation times. The intra-assay and inter-assay coefficients of variation provided by the manufacturer were lower than 4.1 and 6.2%, respectively.

Statistical Analysis

Data were analyzed using the Proc Mixed procedure of SAS (SAS Inst. Inc, Cary, NC). The statistical model included effects of PRRSV challenge, diet, and their interaction as fixed effects. Block was a random effect. Specific contrasts were used to test comparisons between the control and the PE treatments collectively within each challenge treatment. Differences among the PE treatments within each challenge treatment were tested by pair-wise comparisons when the overall diet effect was significant. Probability values of < 0.05 were considered to be significant.

RESULTS

Growth Performance

Before the PRRSV challenge, dietary PE tended ($P = 0.075$) to decrease ADFI compared to the CON (Table 4.2). The PRRSV infection reduced ($P < 0.05$) ADG, ADFI, and G:F during the overall period PI ($P < 0.01$), but the supplementation of PE in the PRRSV infected group increased ($P < 0.05$) G:F during the overall period PI. There was a significant ($P < 0.05$) interaction between PRRSV infection and diet on ADFI for the overall period PI, as feeding CAP and TUR increased, and GAR decreased overall ADFI in the sham group, but the opposite pattern was found in the PRRSV-infected group. There were no differences among PE treatments.

Clinical Signs and Histopathology

After inoculation, the infected pigs showed the first signs of lethargy and anorexia on d 2 PI. Respiratory symptoms such as coughing and dyspnea were not detected. The PRRSV infection significantly increased ($P < 0.05$) RT on d 7, 9, and 11 PI compared with the sham group (Figure 4.1). An interaction between PRRSV infection and diet was observed ($P = 0.036$) on d 4 PI, as the PRRSV-infected pigs fed the 3 PE diets had lower RT than those fed the CON, but no difference between PE and CON was found in the sham pigs. All pigs in the sham group were clinically healthy during the entire experimental period.

The PRRSV infected pigs had lung lesions, whereas the PRRSV-negative pigs remained lesion-free (Figure 4.2). All PRRSV-infected pigs showed marked interstitial pneumonia characterized by a severe septal infiltration with macrophages, lymphocytes, and type II pneumocyte hypertrophy and hyperplasia. Also PRRSV-infected pigs showed accumulations of necrotic debris and mixed inflammatory cells in alveolar spaces.

Viral Load and Antibody Titer in Serum

All pigs were verified as PRRSV-negative by qPCR before starting the experiment. All pigs in the sham group were PRRSV-negative PI, whereas all pigs in the PRRSV-infected group were PRRSV-positive on d 7 and 14 PI. In the PRRSV-infected group, feeding CAP and TUR increased ($P < 0.05$) Ct values on d 7 PI, and TUR increased ($P < 0.05$) Ct value on d 14 PI (Figure 4.3), indicating these PE decreased viral load compared with the CON. The Ct values of serum virus on d 7 PI were lower than d 14 PI, which indicates serum viral load decreased during this period.

No PRRSV-specific antibody was detected in the sham group. In the PRRSV-infected group, the PRRSV-specific antibody was detected on d 7 PI, but the S/P ratios for all pigs were lower than 0.4 (Figure 4.4), considered negative. The antibody titers of all infected pigs on d 14 PI were positive and greater than on d 7 PI. There was no PE effect on PRRSV antibody.

Complete Blood Counts

The PRRSV infection induced ($P < 0.01$) leukopenia on d 7 PI but increased the number of white blood cells on d 14 PI (Table 4.3). In addition, the PRRSV infection decreased ($P < 0.01$) lymphocytes and increased ($P < 0.01$) the ratio of neutrophils to lymphocytes on d 7 PI, but, the reverse pattern was detected on d 14 PI compared with d 7 PI. The supplementation of PE tended to increase ($P = 0.057$) the number of lymphocytes on d 7 PI in the sham group. There was no PE effect in the PRRSV-infected group.

The PRRSV infection decreased ($P < 0.05$) the number of red blood cells (RBC), hemoglobin concentration (HGB), packed cell volume on d 7 and 14 PI, compared with the sham group (Table 4.4). There were only sporadic effects of PE on RBC measurements in the sham group, and none in the PRRSV-infected group.

Serum Cytokines and Acute Phase Proteins

A pro-inflammatory cytokine, TNF-α, and an anti-inflammatory cytokine, IL-10, were detected in the serum of all pigs (Table 4.5). There was no effect of dietary PE supplementation on serum TNF-α or IL-10 before the PRRSV infection. The PRRSV infection markedly increased ($P < 0.01$) TNF-α and IL-10 on d 7 and 14 PI, but the PE treatments in the PRRSV-infected pigs reduced serum TNF-α ($P < 0.05$) and increased ($P < 0.05$) serum IL-10 on d 7 PI as compared to the CON. However, this pattern was not found on d 14 PI. There were differences among the PE treatments on serum IL-10 concentration on d 7 PI, as PRRSV-infected pigs fed CAP had higher IL-10 concentration than those fed GAR.

Two acute phase proteins, CRP and Hp were detected in the serum of all pigs (Table 4.5). There was a significant ($P < 0.05$) diet effect on serum Hp on d 0 before PRRSV infection as GAR had higher Hp concentration than TUR. The PRRSV infection increased ($P < 0.01$) serum CRP and Hp levels on d 7 and 14 PI compared with the sham group, but the supplementation of PE in PRRSV-infected pig diets reduced ($P < 0.05$) serum CRP and Hp compared with the CON on d 14 PI. An interaction between PRRSV infection and diet was observed ($P < 0.05$) on d 14 PI, as the supplementation of PE decreased ($P < 0.05$) serum CRP of the PRRSV-infected pigs, but not of uninfected pigs.

DISCUSSION

Porcine reproductive and respiratory syndrome is one of main causes of mortality of post-weaning and growing pigs (NAHMS, 2008). The results reported here indicate that feeding PE to nursery pigs enhanced the pigs' immune responses to a PRRSV challenge and may help alleviate negative impacts of infection, as indicated by reducing viral load, pro-inflammatory cytokine,

acute phase proteins, and increasing feed efficiency. These findings are in agreement with previous data showing an improvement of immune responses in piglets fed different PE (Pu et al., 2009; Liu et al., 2011a, b).

In this experiment, the PRRSV infection model was achieved successfully, as all of the challenge pigs were PRRSV-positive, lethargic, and had increased rectal temperature and reduced feed intake, whereas the unchallenged pigs were PRRSV-negative and clinically healthy. Rectal temperatures of infected pigs were increased from d 4 PI and returned to normal at d 14 PI. The infected pigs fed the CON diet had a higher average RT at d 4 PI than those fed the PE diets, which indicates feeding PE may delay and shorten the appearance of pyrexia of infected pigs. Lung lesions were detected in the PRRSV infected pigs at d 14 PI. All PRRSV-infected pigs showed marked interstitial pneumonia and the accumulations of necrotic debris and mixed inflammatory cells in alveolar spaces, consistent with the observation that the immune cells in the lung are the main targets of PRRSV infection (Pol et al., 1991).

PRRSV infection resulted in a marked decrease in leukocyte populations at d 7 PI, but an increase at d 14 PI, which indicates PRRSV causes the infected pigs to undergo a state of immunosuppression for at least 1 week after infection. These results are consistent with those reported earlier (Lohse et al., 2004; Shi et al., 2008; Che et al., 2011). The decline in peripheral blood leukocyte populations may be explained by the apoptosis of immune cells at the early stage of PRRSV infection. Apoptosis is often considered as an innate defense mechanism that limits viral infection by elimination of infected cells. Therefore, many viruses inhibit apoptosis of infected cells to prevent premature cell death and increase viral infection and rep lication (Everett and McFadden, 1999). However, unlike other viruses, PRRSV induces the apoptosis of

monocytes/macrophages and other immune cells (Sur et al., 1998; Choi and Chae, 2002; Labarque et al., 2003).

In the present experiment, PRRSV infection significantly increased the viral load in the serum of infected pigs at d 7 PI, and the viral load decreased with time. Previous reports from Labarque et al. (2000), Pu et al. (2009), and Che et al. (2011) have indicated that PRRSV viremia peaks between 6 and 9 days post-infection. Feeding PE reduced viral load in PRRSV-infected pigs at d 7 and 14 PI, although no differences in clinical signs were observed during these days in the controlled conditions of this experiment. Pu et al. (2009) also reported that *Hypericum perforatum* extract reduced the viremia of pigs infected with PRRSV. The reduction of viremia indicates an anti-viral effect of PE.

Pro-inflammatory cytokines, such as TNF-α and IL-1β, primarily produced by monocytes or macrophages, are expressed rapidly following infection (Van Reeth et al., 2002; Thacker, 2006). Many of these cytokines are important in the development of inflammation within the respiratory tract. The TNF-α response to PRRSV in the present study was pronounced at d 7 and d 14 PI, suggesting that this cytokine was probably involved in the early immune response. Choi et al. (2001), Che et al. (2011), and Xiao et al. (2010) also found increased TNF-α mRNA expression in the macrophages and lung, and increased serum TNF-α concentration during PRRSV infection. The increased TNF-α in PRRSV infection may contribute to the macroscopic lung lesions and high fever of pigs in the challenged group, because of the recruitment of more immune cells to the site of infection (Che et al., 2011). The supplementation of PE in the diets reduced serum TNF-α production of infected pigs, suggesting PE may protect against continuing inflammation. This result is also consistent with an *in vitro* experiment from Liu et al. (2011b),

which found PE suppressed TNF-α secretion from LPS-treated porcine alveolar macrophages. Therefore, both in *vitro* and *in vivo* experiments indicate anti-inflammatory effects of these PE.

The anti-inflammatory cytokine IL-10 is a potent immunosuppressive cytokine, which can inhibit the activation of immune cells and the consequent production of proinflammatory cytokines such as TNF-α. The result is suppression of the inflammatory response (Moore et al., 2001). In the present experiment, PRRSV infection enhanced the production of IL-10, in agreement with previous studies (Genini et al., 2008; Che et al., 2011). Suradhat et al. (2003) and Charerntantanakul and Kasinrerk (2010) suggested that the up-regulation of IL-10 with PRRSV infection may contribute to the poor innate and adaptive immune responses, because the increased IL-10 at an early stage of PRRSV infection may enhance virus survival within the host and delay the development of protective immunity. However, in this experiment, the serum IL-10 production was not very high and both IL-10 and TNF-α levels were increased in PRRSV-infected pigs. These results suggest that IL-10 might not play a critical role in the suppression of immune responses at the early stage of PRRSV infection. The supplementation of PE enhanced IL-10 production at d 7 PI which, together with the reduced TNF-α, indicates that PE might alleviate the ongoing inflammation.

The production of TNF-α, IL-1β, and IL-6 triggers the synthesis of acute phase proteins, such as C-reactive protein and haptoglobin by hepatocytes (Cohen, 2002; Gómez-Laguna et al., 2010). Acute phase proteins induce a proinflammatory reaction and fever, but overexpression can lead to an anti-inflammatory response (Petersen et al., 2004). Increased CRP and Hp were detected in PRRSV infected pigs at d 7 and 14 PI in this experiment. The higher response of Hp than CRP for PRRSV infection is consistent with the results of Gómez-Laguna et al. (2010), who failed to find a difference in serum CRP concentrations between control and PRRSV-infected

pigs. Earlier studies also showed increased levels of serum Hp in PRRSV-infected pigs from d 5 to 21 PI, indicating its important role in mediating immune responses during PRRSV infection (Díaz et al., 2005; Gnanandarajah et al., 2008). Acute phase proteins are mainly secreted by IL-6-mediated hepatocytes; however, TNF-α has been shown to increase CRP production directly or indirectly through the induction of or in synergy with IL-6 (Tilg et al., 1997; Petersen et al., 2004). Thus, the reduced CRP production caused by feeding PE to PRRSV-infected pigs at d 14 PI may be partially explained by the reduced TNF-α. However, feeding PE enhanced the serum Hp production of PRRSV-infected pigs, which may indicate the anti-inflammatory effect of Hp if overexpressed.

In the present experiment, PRRSV infection significantly reduced growth rate and feed efficiency, which has also been shown in some other studies (Doeschl-Wilson et al., 2009; Escobar et al., 2004; Toepfer-Berg et al., 2004; Che et al., 2011). The proposed mechanisms from Doeschl-Wilson et al. (2009) explained the relationship between PRRSV infection and retarded growth of pigs: PRRSV infection leads to the loss of appetite and to competition for resources between growth and immunity of pigs. PRRSV infection increased the production of inflammatory mediators, such as pro-inflammatory cytokines and acute phase proteins. These enhanced immune responses not only directly affect growth by systematic regulation, but also compete for the resources need for growth. Feeding PE increased the feed efficiency of pigs infected with PRRSV, which may be at least partially explained by the modulated immune responses, as reducing TNF-α, CRP, and viral load.

In conclusion, the present results suggest that PE modulates the immune responses and enhances the growth efficiency of weaned pigs infected with PRRSV *in vivo*. The decreases in viral load, pro-inflammatory cytokine and C-reactive protein indicate that PE may help alleviate

the negative effects of PRRSV including inflammation. This result also confirms the anti-inflammatory activity of PE found in vitro. The increased antibody titers, anti-inflammatory cytokine, and haptoglobin also suggest that PE may enhance the immune responses of the host, and thereby suppress ongoing inflammation and prevent secondary infections. Further studies are needed to investigate the mechanisms behind these potential immunomodulatory effects of PE.

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TABLES AND FIGURES

Table 4.1. Ingredient composition of basal diet (as-fed basis)

Ingredient	Amount, %
Corn, ground	41.54
Whey dried	15.00
Soybean meal, 47%	10.82
Fishmeal	10.00
Lactose	10.00
Soy protein concentrate	5.00
Poultry byproduct meal	4.27
Soybean oil	2.67
Mineral premix[1]	0.35
Vitamin premix[2]	0.20
L-Lysine·HCl	0.05
DL-Met	0.05
L-Thr	0.03
L-Trp	0.02
Total	100.00
Calculated energy and nutrients	
ME, kcal/kg	3480
CP, %	22.67
Fat, %	6.34
Ca, %	0.80
P, %	0.72
Available P, %	0.49
Lys, %	1.50
Lactose, %	21.00

Table 4.1. (cont.)

[1]Provided as milligrams per kilogram of diet: 3,000 of NaCl; 100 of Zn from zinc oxide; 90 of Fe from iron sulfate; 20 of Mn from manganese oxide; 8 of Cu from copper sulfate; 0.35 of I from calcium iodide; 0.30 of Se from sodium selenite.

[2]Provided per kilogram of diet: 2,273 μg of retinyl acetate; 17 μg of cholecalciferol; 88 mg of DL-α-tocopheryl acetate; 4 mg of menadione from menadione sodium bisulfite complex; 33 mg of niacin; 24 mg of D-Ca-pantothenate; 9 mg of riboflavin; 35 μg of vitamin B_{12}; 324 mg of choline chloride.

Table 4.2. Effect of plant extract on growth performance of pigs experimentally infected with porcine reproductive and respiratory syndrome virus[1]

	Treatment[2]								SEM	*P*-value				
	Sham				PRRSV					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR		PRRSV	Diet	E*D	Sham	PRRSV
d -14, kg	7.79	7.90	7.85	7.85	7.82	7.80	7.82	7.82	0.35	0.85	1.00	0.99	0.74	0.97
d 0, kg	9.65	10.54	9.51	9.71	10.27	9.78	9.83	10.05	0.41	0.63	0.66	0.32	0.54	0.41
d 7, kg	12.79	13.89	12.33	12.55	11.96	12.72	12.14	12.06	0.63	<0.05	0.69	0.24	0.82	0.98
d 14, kg	17.2	19.2	16.7	17.4	14.3	14.2	14.5	15.3	0.87	<0.01	0.40	0.17	0.51	0.65
d -14 to 0														
ADG, g	133	188	119	133	177	139	145	158	24.4	0.48	0.54	0.19	0.59	0.28
ADFI, g	414	461	416	369	515	490	433	387	44.3	0.17	0.10	0.73	0.99	0.13
G:F	0.37	0.42	0.25	0.38	0.36	0.29	0.33	0.41	0.07	0.81	0.36	0.35	0.74	0.84
d 0 to 7														
ADG, g	448	479	402	405	239	278	328	288	49.0	<0.01	0.77	0.22	0.64	0.19
ADFI, g	745	943	714	807	600	604	729	625	67.2	<0.01	0.49	0.071	0.29	0.50
G:F	0.44	0.39	0.39	0.38	0.29	0.41	0.52	0.42	0.056	0.79	0.41	0.075	0.37	<0.05

Table 4.2. (cont.)

	Treatment[2]									*P*-value				
	Sham				PRRSV				SEM	Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR		PRRSV	Diet	E*D	Sham	PRRSV
d 7 to 14														
ADG, g	636	761	625	693	333	359	339	469	49.9	<0.01	0.090	0.32	0.29	0.33
ADFI, g	1009	1246	1004	1100	765	682	757	682	77.1	<0.01	0.65	0.11	0.20	0.51
G:F	0.64	0.62	0.63	0.64	0.42	0.54	0.53	0.70	0.062	0.055	0.13	0.17	0.93	<0.05
d 0 to 14														
ADG, g	542	620	513	549	286	318	334	378	42.7	<0.01	0.31	0.20	0.64	0.18
ADFI, g	877	1095	859	954	683	643	743	653	62.3	<0.01	0.49	<0.05	0.18	0.97
G:F	0.63	0.58	0.60	0.59	0.42	0.51	0.45	0.58	0.052	<0.01	0.45	0.14	0.48	0.065

[1]n = 60 (7 or 8 pigs/treatment).

[2]Sham = unchallenged; PRRSV = PRRSV challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]PRRSV = PRRSV challenge effect; Diet = diet effect; E*D = interaction between PRRSV and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or PRRSV challenge group.

Table 4.3. Effect of plant extracts on differential leukocyte counts of pigs experimentally infected with porcine reproductive and respiratory syndrome virus[1]

	Treatment[2]								SEM	*P*-value				
	Sham				PRRSV					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR		PRRSV	Diet	E*D	Sham	PRRSV
d 0														
WBC, $\times 10^3$/uL[5]	14.32	14.29	15.22	13.21	15.60	14.12	14.56	13.35	1.17	0.83	0.32	0.82	0.95	0.20
Neu, $\times 10^3$/uL[5]	8.29	7.74	9.52	6.06	9.13	7.82	7.14	7.22	1.16	0.89	0.10	0.17	0.61	0.10
Lym, $\times 10^3$/uL[5]	5.56	5.79	4.90	6.37	6.01	5.54	6.92	5.23	0.73	0.58	0.99	0.15	0.88	0.90
Mono, $\times 10^3$/uL[5]	0.63	0.70	0.72	0.74	0.35	0.52	0.53	0.68	0.17	0.12	0.59	0.92	0.63	0.24
Neu/Lym[5]	1.55	1.54	2.10	0.99	1.54	1.67	1.08	1.57	0.38	0.72	0.69	0.09	0.97	0.78
d 7														
WBC, $\times 10^3$/uL[5]	13.81	14.30	15.75	12.78	8.26	9.69	10.57	9.72	1.06	<0.01	0.20	0.62	0.71	0.14
Neu, $\times 10^3$/uL[5]	6.84	6.12	6.54	5.75	4.95	6.50	6.19	5.63	1.02	0.45	0.87	0.64	0.52	0.26
Lym, $\times 10^3$/uL[5]	6.01	7.59	7.88	6.36	3.00	2.89	3.86	3.52	0.61	<0.01	0.12	0.29	0.057	0.49
Mono, $\times 10^3$/uL[5]	0.37	0.40	0.56	0.45	0.16	0.11	0.27	0.27	0.10	<0.01	0.23	0.89	0.34	0.53
Neu/Lym[5]	1.20	0.82	0.90	0.94	1.83	2.30	2.05	1.73	0.37	<0.01	0.91	0.58	0.43	0.59
d 14														
WBC, $\times 10^3$/uL[5]	18.68	16.32	15.04	15.99	23.68	20.52	26.28	22.17	2.03	<0.01	0.35	0.20	0.15	0.73
Neu, $\times 10^3$/uL[5]	7.65	7.62	7.46	7.10	9.05	8.06	9.62	8.71	1.16	0.077	0.90	0.89	0.83	0.84
Lym, $\times 10^3$/uL[5]	9.14	7.16	6.45	7.43	13.39	11.04	15.27	12.14	1.61	<0.01	0.36	0.24	0.16	0.71
Mono, $\times 10^3$/uL[5]	1.66	1.40	0.96	1.14	0.56	0.54	0.49	0.70	0.30	<0.01	0.60	0.59	0.13	0.97
Neu/Lym[5]	0.92	1.13	1.30	1.12	0.74	0.74	0.72	0.81	0.28	<0.01	0.76	0.71	0.19	0.96

Table 4.3. (cont.)

[1]n = 60 (7 or 8 pigs/treatment).

[2]Sham = unchallenged; PRRSV = PRRSV challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]PRRSV = PRRSV challenge effect; Diet = diet effect; E*D = interaction between PRRSV and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or PRRSV challenge group.

[5]WBC = white blood cell; Neu = neutrophil; Lym = lymphocyte; Mono = monocyte; Neu/Lym = the ratio of neutrophils to lymphocytes.

Table 4.4. Effect of plant extracts on red blood cell measures of pigs experimentally infected with porcine reproductive and respiratory syndrome virus[1]

	Treatment[2]									*P*-value				
	Sham				PRRSV				SEM	Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR		PRRSV	Diet	E*D	Sham	PRRSV
d 0														
RBC, $\times 10^6$/uL[5]	6.34	6.29	6.49	6.86	6.68	6.86	6.52	6.59	0.27	0.37	0.81	0.37	0.51	0.94
HGB, g/dL[5]	12.18	11.33	11.94	12.31	12.25	12.05	11.58	12.11	0.43	0.85	0.39	0.53	0.51	0.49
HCT, %[5]	36.45	34.44	36.36	37.34	37.65	36.44	36.60	37.00	1.33	0.39	0.47	0.77	0.79	0.53
MCV, fL[5, 6]	57.32	54.59	55.73	56.45	56.45	56.29	56.05	56.55	1.81	0.99	0.33	0.75	0.24	0.55
MCH, pg[5]	19.08	17.98	18.24	18.12	18.40	17.64	17.75	18.50	0.69	0.49	0.41	0.79	0.17	0.53
MCHC, g/dL[5]	33.34	32.97	32.86	33.01	32.55	33.01	31.74	32.73	0.42	0.045	0.24	0.40	0.38	0.89
RDW, %[5]	28.68	30.12	28.54	29.98	26.38	29.14	27.05	29.34	1.69	0.24	0.39	0.96	0.65	0.28
d 7														
RBC, $\times 10^6$/uL[5]	5.89	6.48	6.32	6.39	6.00	5.82	5.97	5.83	0.20	<0.01	0.63	0.16	<0.05	0.53
HGB, g/dL[5]	11.16	11.88	11.23	11.18	10.43	10.25	10.34	10.55	0.40	<0.01	0.80	0.36	0.46	0.88
HCT, %[5]	33.37	35.40	33.78	33.46	32.68	31.71	32.04	32.75	1.25	<0.05	0.93	0.44	0.49	0.66
MCV, fL[5, 6]	56.63	54.60	53.60	52.71	54.68	54.44	53.55	56.14	1.98	0.74	0.50	0.22	0.063	0.98
MCH, pg[5]	18.98	18.32	17.80	17.63	17.45	17.63	17.30	18.13	0.66	0.060	0.46	0.093	<0.05	0.60
MCHC, g/dL[5]	33.49	33.57	33.23	33.45	32.00	32.36	32.36	32.28	0.30	<0.01	0.87	0.76	0.83	0.28
RDW, %[5]	27.79	27.47	27.97	29.54	25.30	27.09	26.66	26.20	1.91	<0.05	0.76	0.67	0.73	0.34

Table 4.4. (cont.)

	Treatment[2]								SEM	*P*-value				
	Sham				PRRSV					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR		PRRSV	Diet	E*D	Sham	PRRSV
d 14														
RBC, $\times 10^6$/uL[5]	6.22	6.70	6.46	6.37	6.03	6.01	6.27	5.54	0.26	<0.01	0.086	0.18	0.17	0.66
HGB, g/dL[5]	11.73	11.93	11.76	11.67	10.75	10.51	10.76	10.22	0.32	<0.01	0.69	0.78	0.87	0.48
HCT, %[5]	35.57	36.57	36.09	35.46	33.00	32.28	33.47	31.40	0.99	<0.01	0.52	0.70	0.66	0.57
MCV, fL[5, 6]	57.39	54.63	56.12	55.82	55.09	53.83	53.53	56.58	1.82	0.18	0.30	0.53	0.20	0.76
MCH, pg[5]	18.92	17.85	18.27	18.37	17.95	17.52	17.23	18.40	0.59	<0.05	0.13	0.49	0.11	0.62
MCHC, g/dL[5]	32.97	32.64	32.57	32.90	32.56	32.52	32.23	32.57	0.30	0.14	0.56	0.96	0.41	0.72
RDW, %[5]	25.84	27.35	26.55	28.48	25.97	26.33	26.95	26.06	2.57	0.56	0.88	0.85	0.42	0.81

[1]n = 60 (7 or 8 pigs/treatment).

[2]Sham = unchallenged; PRRSV = PRRSV challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]PRRSV = PRRSV challenge effect; Diet = diet effect; E*D = interaction between PRRSV and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or PRRSV challenge group.

[5]RBC = red blood cell; HGB = hemoglobin; HCT = packed cell volume; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW = red cell distribution width.

[6]fL= femtolitre (10^{-15} L).

Table 4.5. Effects of plant extracts on cytokine and acute phase protein concentrations in the serum of pigs infected with porcine reproductive and respiratory syndrome virus[1]

	Treatment[2]									*P*-value				
	Sham				PRRSV					Main effect[3]			PE vs. CON[4]	
Item	CON	CAP	GAR	TUR	CON	CAP	GAR	TUR		PRRSV	Diet	E*D	Sham	PRRSV
d 0														
TNF-α, pg/mL[5]	67.8	52.7	54.6	55.9	60.5	55.4	60.2	52.4	6.75	0.88	0.33	0.72	0.086	0.51
IL-10, pg/mL	8.9	7.6	6.4	5.5	7.7	6.4	7.2	8.0	1.76	0.86	0.79	0.69	0.24	0.81
CRP, μg/mL[5]	22.9	16.8	18.4	24.8	24.5	26.0	27.4	22.3	5.34	0.26	0.97	0.63	0.64	0.91
HP, μg/mL[5]	257	194	280	154	221	376	367	167	62.8	0.11	<0.05	0.22	0.47	0.21
d 7														
TNF-α, pg/mL[5]	56.5	47.7	44.3	54.2	179.5	146.2	159.6	139.4	10.95	<0.01	0.12	0.23	0.48	<0.05
IL-10, pg/mL	11.9	12.1	15.0	9.2	18.9[a]	36.4[b]	22.9[a]	31.4[ab]	4.62	<0.01	0.34	0.16	0.97	<0.05
CRP, μg/mL[5]	23.2	19.9	29.8	26.0	52.1	42.5	45.0	42.0	5.16	<0.01	0.49	0.45	0.69	0.14
HP, μg/mL[5]	302	204	436	388	1312	1572	1563	1322	173.0	<0.01	0.69	0.57	0.82	0.36
d 14														
TNF-α, pg/mL[5]	42.2	41.3	33.4	46.8	201.0	172.0	207.8	177.8	15.27	<0.01	0.69	0.34	0.91	0.40
IL-10, pg/mL	4.5	4.1	4.0	6.5	15.2	15.4	16.5	17.2	2.15	<0.01	0.71	0.97	0.87	0.65
CRP, μg/mL[5]	16.8	18.4	27.6	23.4	41.0	27.9	32.8	34.4	3.63	<0.01	0.15	<0.05	0.087	<0.05
HP, μg/mL[5]	150	234	492	249	890	1503	1485	1035	152.9	<0.01	0.038	0.40	0.37	<0.05

Table 4.5. (cont.)

[a, b] within a row, means without a common superscript differ ($P < 0.05$) within the sham or PRRSV challenge group.

[1]n = 60 (7 or 8 pigs/treatment).

[2]Sham = unchallenged; PRRSV = PRRSV challenged; CON = control diet; CAP = control diet plus 10 g capsicum oleoresin / ton; GAR = control diet plus 10 g garlic / ton; TUR = control diet plus 10 g turmeric oleoresin / ton.

[3]PRRSV = PRRSV challenge effect; Diet = diet effect; E*D = interaction between PRRSV and diet effects.

[4]Contrast between CON and the 3 plant extract treatments within the sham or PRRSV challenge group.

[5]TNF-α = tumor necrosis factor-alpha; CRP = C-reactive protein; Hp = haptoglobin.

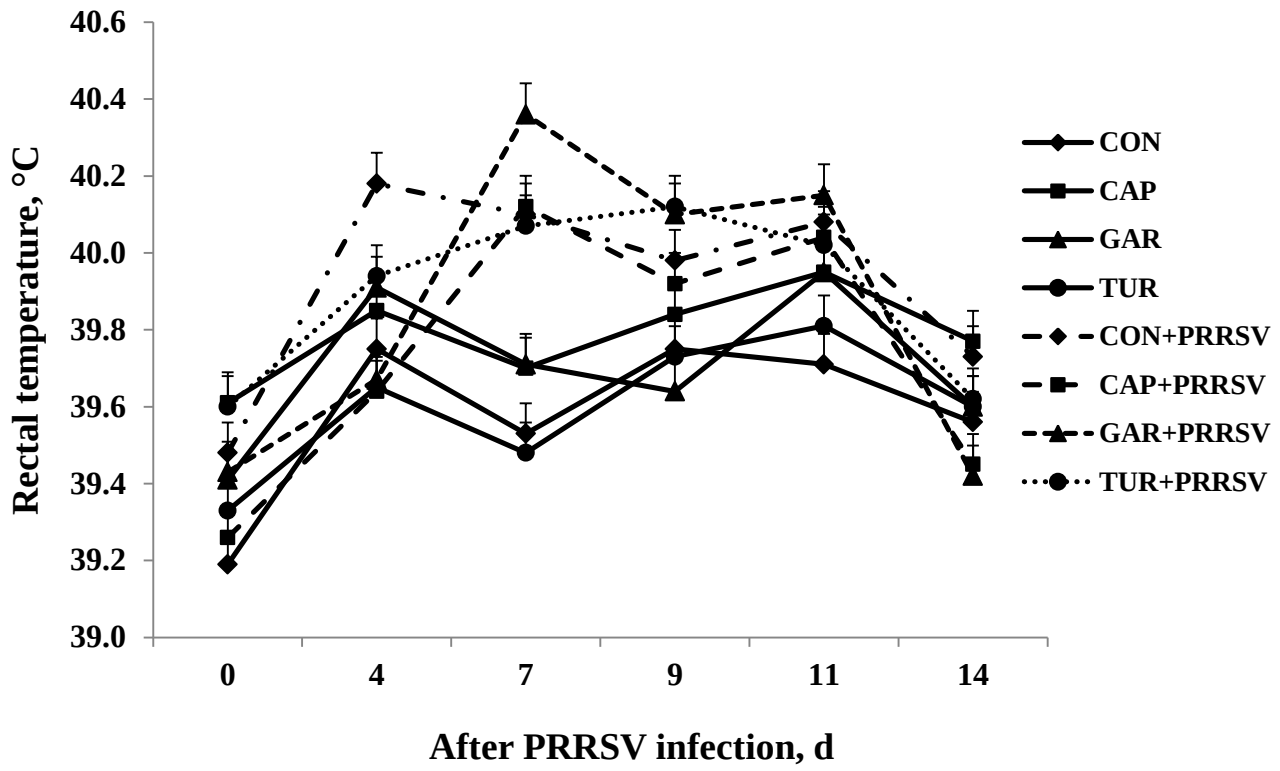

Figure 4.1. Rectal temperature (RT) in pigs fed control (CON) or capsicum oleoresin (CAP), garlic (GAR), or turmeric oleoresin (TUR) diets with or without porcine reproductive and respiratory syndrome virus (PRRSV) infection. The RT of PRRSV-infected pigs at d 7 ($P < 0.01$), 9 ($P < 0.01$), and 11 ($P < 0.05$) postinfection (PI) was greater than that of uninfected pigs. There was a PRRSV×diet interaction ($P < 0.05$) on RT at d 4 PI, as the PRRSV-infected pigs fed the 3 PE diets had lower RT than those fed the CON, but no difference between PE and CON was found in the sham pigs. Value were means ± pooled SEM, n = 7 or 8. Individual pig was an experimental unit.

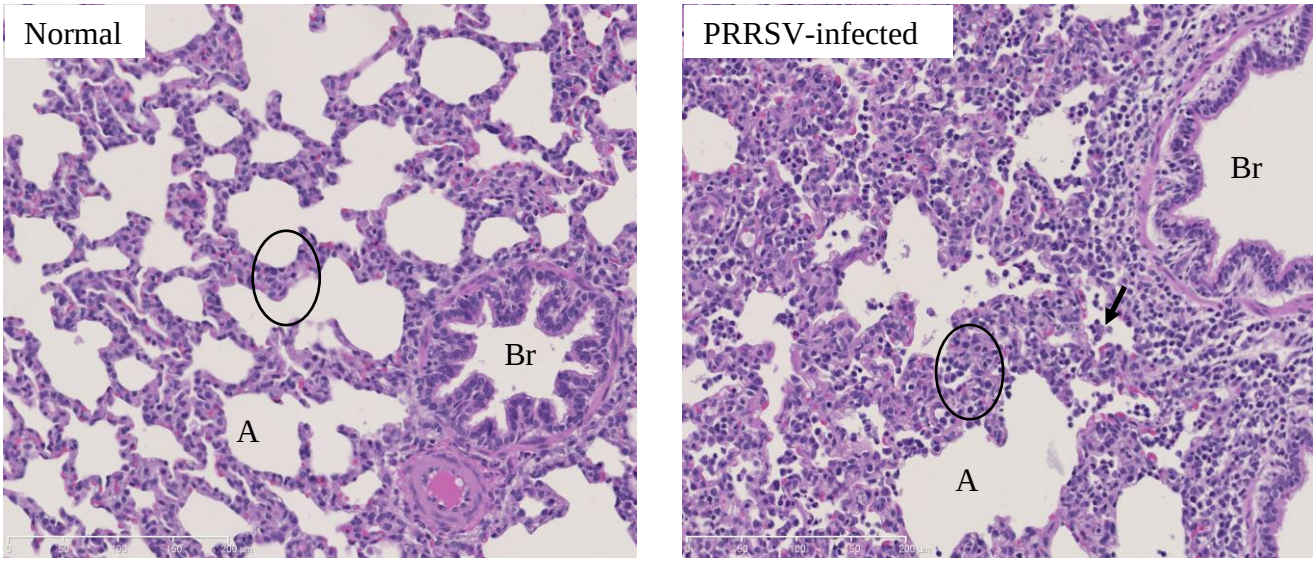

Figure 4.2. Microscopic section of lung from pig not infected (left) and infected (right) with porcine reproductive and respiratory syndrome virus. In the uninfected lung (left panel), there was no exudate in alveoli (A) or bronchioles (Br) and alveolar septa (circle) were normal thickness. However, in the infected lung (right panel), there was interstital pneumonia characterized by a severe septal infiltration with macrophages, lymphocytes, and type II pneumocyte hypertrophy and hyperplasia (circle). Necrotic debris and mixed inflammatory cells (arrow) accumulated in alveolar spaces (Hemotoxylin and Eosin stain, 200 × magnification).

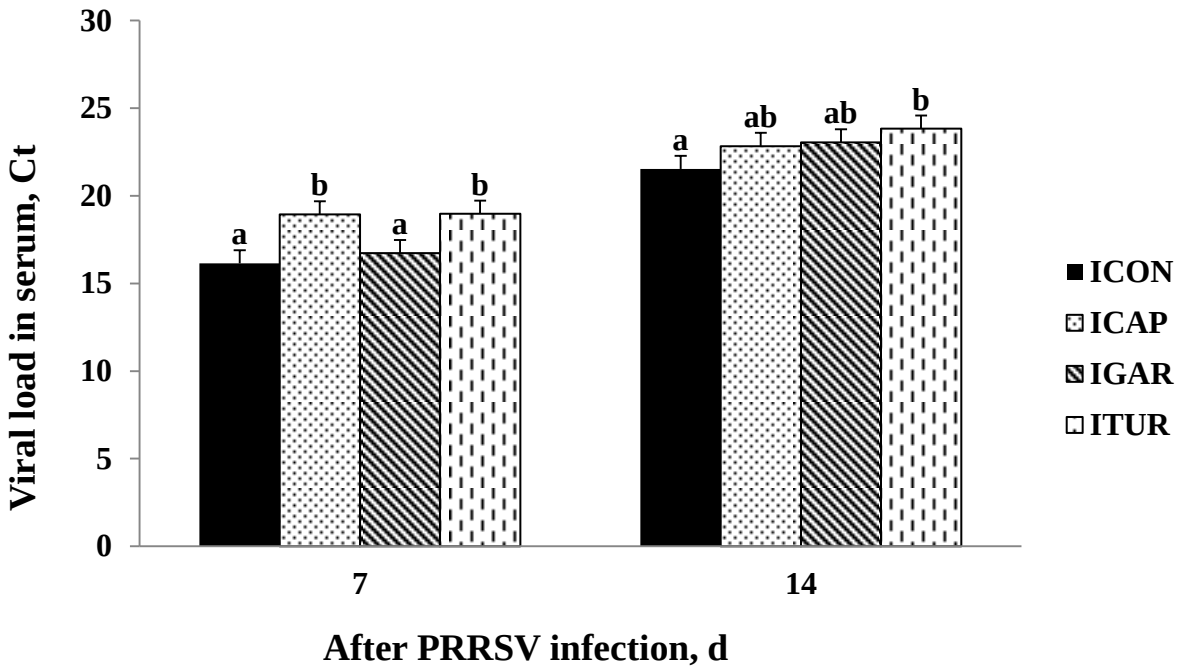

Figure 4.3. Serum viral load in control- or plant extracts (PE)-fed pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). ICON: pigs fed control diet in the PRRSV-infected group; ICAP: pigs fed capsicum oleoresin diet in the PRRSV-infected group; IGAR: pigs fed garlic diet in the PRRSV-infected group; ITUR: pigs fed turmeric oleoresin diet in the PRRSV-infected group. Viral load is presented as cycle threshold (Ct) values, where Ct value is inverse to the amount of virus. Capsicum oleoresin and turmeric oleoresin increased Ct value at d 7 PI, and turmeric oleoresin increased Ct value at d 14 PI, which indicates these 2 PE reduce the viral load in the serum of PRRSV-infected pigs. Values were means ± pooled SEM, n = 7 or 8. The individual pig was the experimental unit. $^{a, b}$ means without a common superscript differ ($P < 0.05$) within the PRRSV challenge group.

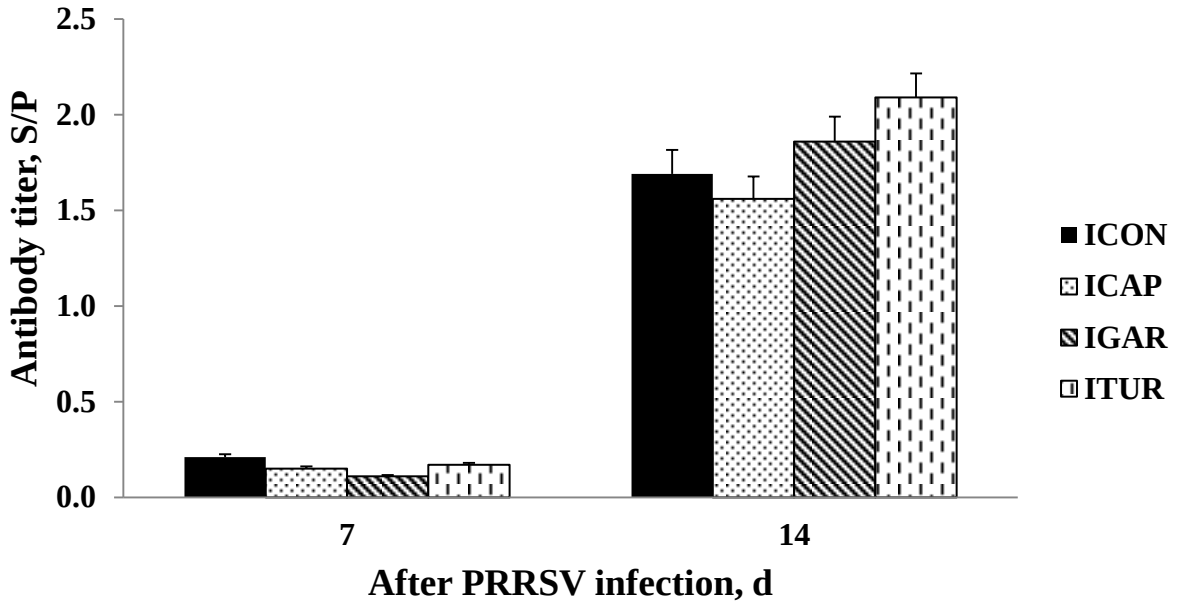

Figure 4.4. Antibody titer in control- or plant extracts (PE)-fed pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). ICON: pigs fed control diet in the PRRSV-infected group; ICAP: pigs fed capsicum oleoresin diet in the PRRSV-infected group; IGAR: pigs fed garlic diet in the PRRSV-infected group; ITUR: pigs fed turmeric oleoresin diet in the PRRSV-infected group. Antibody titer is presented as sample to positive control (S/P) ratios of all infected pigs, where a ratio of 0.4 or greater is considered positive. Plant extracts did not affect ($P > 0.05$) the S/P ratios of the infected pigs postinfection. Values were means ± pooled SEM, n = 7 or 8. The individual pig was the experimental unit.

CHAPTER 5

GENERAL SUMMARY, DISCUSSION, AND CONCLUSION

Many of the feed additives now available for use as "alternatives to antibiotics" in the pig industry either alter microbial populations in the gastrointestinal tract or influence the immune system of the pig. Plant extracts (PE) are extracted from parts of plant materials are among the interesting additives, and have been largely employed for both human nutrition and the livestock industry. Plant extracts are of potential interest due to their antiviral, antimicrobial, antioxidant, anti-inflammatory, and other biological effects. This may lead to the ability to these PE, instead of antibiotics, in diets to improve performance and health of animals. However, because of the large variation in composition, the biological or practical effects of different batches of the same PE may differ. Therefore, it is necessary to select pure principles for evaluating their possible role as alternatives to antibiotics in livestock production. The important aim of these studies was to evaluate the effects of several different PE on immune function and disease resistance of pigs. This research contained 3 studies: (1) anti-inflammatory properties of 7 different PE on porcine alveolar macrophage (PAM) in vitro; (2) the effects of dietary supplementation of 3 different PE on growth performance, intestinal morphology, and immune responses of pigs infected with a bacterial model; (3) the effects of dietary supplementation of the same 3 PE on growth performance and immune responses of pigs infected with a viral model.

The first study tested the in vitro anti-inflammatory effects of 7 PE (anethol, capsicum oleoresin, carvacrol, cinnamaldehyde, eugenol, garlic, and turmeric oleoresin) on PAM collected from weaned pigs by bronchoalveolar lavage. Lipopolysaccharide (LPS) sharply stimulated the

secretion of 2 pro-inflammatory cytokines: TNF-α and IL-1β. The inclusion of each of the 7 PE suppressed the secretion of TNF-α from LPS-treated PAM in a dose dependent manner. But, the IL-1β response differed from that of TNF-α in the presence of LPS. The treatments with carvacrol, cinnamaldehyde, eugenol, and garlic significantly suppressed the secretion of both TNF-α and IL-1β from LPS-induced PAM, but anethol and capsicum oleoresin stimulated or did not affect the secretion of IL-1β from LPS-induced PAM. These results indicate that all of PE may have potent anti-inflammatory effects to varying degrees. Several PE increased the secretion of TNF-α or IL-1β from PAM in the absence of LPS, which indicated that the inclusion of these PE in the normal condition may be beneficial to the host, since the inflammatory mediators can protect the host against infection. Plant extracts did not increase production of the 2 anti-inflammatory cytokines, IL-10 and TGF-β, which suggests other mechanisms may be involved in the reduction of pro-inflammatory cytokines from PAM by PE. In addition, the cell viability test found different influences of 7 PE on PAM. The high level (200 μg/mL) of carvacrol and garlic were cytotoxic to PAM, and very low levels of cinnamaldehyde (20 μg/mL) and turmeric oleoresin (10 μg/mL) significantly inhibited cell viability of PAM. The cell viability results indicate that the inhibitory effects of PE on the production of pro-inflammatory cytokines resulted from mechanisms other than direct killing of cells. Otherwise, these results also suggest different dose effects of these 7 PE and the importance of dose selection when we use PE in the future.

Based on the results from the first study, capsicum oleoresin (CAP), garlic (GAR), and turmeric oleoresin (TUR) were selected to perform the second and third experiments with bacterial or viral challenge, because they showed different effects on the production of pro-inflammatory cytokine IL-1β and cell viability of PAM. In the presence of LPS stimulation, CAP

increased the secretion of IL-1β and cell viability of PAM, GAR inhibited IL-1β production and cell viability of PAM, TUR inhibited the cell viability of PAM but did not affect IL-1β production.

In the second study, 10 mg CAP, GAR, or TUR was added per kg diet to investigate the effects of supplementation of these PE on the immune responses, growth performance, and intestinal morphology of weaned pigs infected with a pathogenic F-18 *Eshcherichia coli* (*E. coli*). The supplementation of PE improved the gut health as indicated by increased villi height and the ratio of villi height to crypt depth. In addition, feeding PE to *E. coli* challenged pigs reduced diarrhea score, frequency of diarrhea, total white blood cell number, serum pro-inflammatory cytokine TNF-α, and serum concentration of the acute phase protein haptoglobin, all of which indicate that PE may alleviate the inflammation caused by *E. coli* infection. However, feeding PE did not affect the proportions of β-hemolytic coliforms in feces of *E. coli* challenged pigs, which indicates the reduction of diarrhea may be induced by mechanisms rather than an antimicrobial effect of PE. The reduction of serum TNF-α, haptoglobin, and white blood cells suggests feeding dietary PE may attenuate the over-expression of the systemic immunity and inflammatory mediators of pigs infected with *E. coli*. On the other hand, these results also indicate that the supplementation of low level of PE, 10 ppm, still has beneficial effects on pig health probably by modulating the immune system of pigs. The 3 PE tested here showed different effects on several measurements. Future studies are needed to investigate the mechanisms through which different PE exert these effects.

The third study was conducted with a similar experimental design as the second study, with same dietary treatments but a different challenge model, porcine reproductive and respiratory syndrome virus (PRRSV). Similar to the post-weaning *E. coli* infection, PRRS is an

economically important disease in the swine industry. In this study, as expected, PRRSV infection induced increased rectal temperature, serum viral load, serum specific antibody titer, serum TNF-α, IL-10, C- oreactive protein, and haptoglobin, and reduced growth performance of pigs. In addition, PRRSV infection reduced total white blood cells on d 7 post-inoculation (PI), but increased total white blood cells on d 14 PI, which indicates the delayed immune responses of pigs typical of PRRSV infection. In the PRRSV challenge group, the supplementation of PE reduced viral load and serum TNF-α and C-reactive protein, which indicate that PE may alleviate the negative effects of PRRSV, such as over-stimulation of immune responses and poor growth rate. In addition, adding PE in pig diets increased growth efficiency, serum specific antibody titer, and serum IL-10 and haptoglobin, which also suggest that PE may have the ability to enhance the immune response of host, and thereafter may suppress ongoing inflammation and prevent the secondary infection. The 3 PE tested here also showed some different effects on the measurements, and TUR may be the most powerful candidate to strengthen the efficiency and immune responses of pigs infected with PRRSV.

In overall conclusion, both in vitro and in vivo studies show evidence of potential anti-inflammatory effects of PE, as PE reduced the diarrhea of pigs infected with *E. coli* and reduced the serum viral load of pigs infected with PRRSV. Dietary PE can be supplemented as alternatives to antibiotics, not to treat sick animals but to provide some prophylaxis to pigs. The inclusion of PE in pig diets shows benefits in the face of both a bacterial challenge model and a viral challenge model, which indicates the supplementation of PE may potentially improve pig health and/or growth performance by modulating the immune system of pigs. Future research, including measurement of gene expression in specific tissue or immune cells, should be conducted to detect the mechanism through which different PE show different effects. Combined

infections of bacterial and viral pathogens should be investigated to detect the effect of PE on the immune responses of pigs to combined diseases as pigs often encounter a complex of pathogens. In addition, combinations of different PE may be also interesting in the future research since the several PE tested in this project showed different influences in vitro and in vivo.

AUTHOR'S BIOGRAPHY

Yanhong Liu was born in Jiaozuo City, Henan Province, China. She earned her Bachelor's degree in Biological Sciences (2004) at China Agricultural University, Beijing, China. After the completion of her Bachelor's degree, she enrolled in the Ministry of Agriculture Feed Industry Centre, also in China Agricultural University and received her Master's degree under the guidance of Dr. Defa Li, Spring 2007. During her Master program, she conducted several different experiemnts, most of which were related to nutrition and immunology. The title of the Master's thesis was "Effects of particle size and physical form of diets on mast cell numbers, histamine, and stem cell factor concentration in the small intestine of broiler chickens".

After she accomplished her Master's degree, she joined the swine nutrition group in Department of Animal Sciences at the University of Illinois, Urbana-Champaign, IL, Spring 2008, in pursuit of Ph.D. degree under the guidance of Dr. James E. Pettigrew, mainly focusing on the dietary effects on pig health. Her Ph.D. program is related to the plant extracts on immune function and disease resistance in pigs with the goal of looking for some additives for nursery pigs to improve their performance and disease resistance. During the Ph.D. program, she led three research projects as co-investigator, made 5 research manuscripts, 9 abstracts with oral or poster presentations, 1 research article, and 1 conference proceedings as a first author as well as 6 research manuscripts, 5 abstracts, and 2 conference proceedings as a co-author, received 2 graduate research paper award (3^{rd} in Ph.D. oral presentation competition and 2^{nd} in poster competition).

Yanhong got married to Peng in June 2011, and will have their baby in Febrary 2012. Upon the completion of her Ph.D. program, Yanhong will work as a postdoctoral research associate in Dr. Pettigrew lab.