THE EFFECT OF ESSENTIAL OILS ON LIPOPOLYSACCHARIDE-INDUCED OXIDATIVE STRESS IN INTESTINAL EPITHELIAL CELLS

By

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ABSTRACT

Maintaining gut health is important in animal growth and production. Gastrointestinal infection can lead to oxidative stress in the gut and impair animal growth. Oxidative stress is a detrimental process associated with reduced nutrient absorption and growth performance. Essential oils have received attention as a potential antibiotic alternative to control infection in animals. Lipopolysaccharide (LPS) is an endotoxin produced by gram-negative bacteria that can induce oxidative stress. The objective of this study was to examine the effect of essential oils (citral, and cinnamaldehyde) on LPS-induced oxidative stress in an intestinal epithelial cell model (Caco-2 cells). LPS caused a significant increase in oxidative stress biomarker malondialdehyde (MDA, end product of lipid peroxidation) and a reduction of endogenous antioxidants: superoxide dismutase (SOD) and reduced glutathione (GSH). Such an effect was time and dose dependent. Preincubation of cells with citral (25 μM), cinnamaldehyde (12.5 μM) or combination of both compounds attenuated LPS-induced lipid peroxidation and restored SOD and GSH levels. These results suggest that citral and cinnamaldehyde have a protective effect against LPS-induced oxidative stress through down-regulation of lipid peroxidation and improvement of the endogenous antioxidant system. Essential oils supplementation in feed may exert a beneficial effect against oxidative stress-induced gut injury in animals during infection.

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LIST OF ABBREVIATIONS

•NO	Nitric oxide
•OH	Hydroxyl radical
$^1O_2$	Singlet oxygen
AGP	Antibiotic growth promoters
AP-1	Activator protein-1
CA	Cinnamaldehyde
CAT	Catalase
CD14	Glycerophosphatidylinositol-anchored protein
COS	Chito-oligosaccharide
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DMSO	Dimethyl sulfoxide
E.coli	*Escherichia coli*
EO	Essential oil
ETEC	Enterotoxigen-*Escherichia coli*
FDA	Food and Drug Administration
FOS	Fructooligosaccharide
GI	Gastrointestinal
GIT	Gastrointestinal tract
GSH	Reduced glutathione
GSH-Px	Glutathione peroxidase
GlcN	N-acetyl glucosamine
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
Hep	L-glycero-D-manno heptose
HO-1	Heme oxygenase-1
IBD	Inflammatory bowel disease
ICE-6	Intestinal epithelial cell line
IFNs	Interferons
IKK	Inhibitory κB kinase

IL-1b	Interleukin 1b
IL-6	Interleukin 6
IL-8	Interleukin 8
IRAKs	IL-1 receptor associated kinases
IRF3	Interferon regulatory factor-3
IRF-3	Interferon regulatoryfactor-3
Kdo	3-deoxy-D-manno-octulosonic acid
LAB	Lactic acid bacteria
LPS	Lipopolysaccharides
MD-2	Myeloid differentiation 2
MDA	Malondialdehyde
MOS	mannan-oligosaccharide
MPAK	Mitogen-activated protein kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation factor 88
N_2O	Nitrous oxide
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-B
NO-	Nitroxyl anion
NOX	NADPH oxidase
NQO1	NAD(P)H dehydrogenase quinone 1
Nrf2	Nuclear factor erythroid 2-related factor 2
O^-_2	Superoxide anion
ONOO−	Peroxynitrite anion
PAMPs	Pathogen-associated molecular patterns
PRRs	Pathogen recognition receptors
RIP1	Receptor-interacting protein-1
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT	Room temperature
SCFA	Short chain fatty acids

SE	Standard Error
SOD	Superoxide dismutase
TAK1	Transforming growth factor-β-activated kinase 1
TANK	TRAF family member-associated NF-kB activator
TBARS	Thiobarbituric acid reactive substance
TBK1	TANK binding kinase 1
TIR	Toll-interleukinn-1 receptor
TLR4	Toll like receptor 4
TNF-α	Tumor necrosis factor-α
TRAF-6	TNF receptor associated factor 6
TRAM	TRIF-related adaptor molecules
TRIF	Interferon-β
UBC13	Ubiquitin-conjugating enzyme13
UEV1A	Ubiquitin-conjugating enzyme E2 variant1 isoform A
WHO	World Health Organization

CHAPTER I

INTRODUCTION

1.1. Intestinal health in animals

The concept of "gut health" has become an important topic in animal production. The three major components of gut health in animals are the diet, mucosa and microbial community. All these components play critical roles in physiology, health status, welfare and performance of animals (Celi et al., 2017). In medicine, the healthy gut is defined as effective digestion and absorption function, absence of gastrointestinal illness, normal and stable intestinal microbial community, effective immune status and in a status of wellbeing (Bischoff, 2011). According to this definition, the presence of diseases as well as the changes in structure and functions can lead to unhealthy gut in animals.

Although gastrointestinal tract (GIT) represents a small weight of total body weight, it plays the main role in animal growth (Adewole et al., 2016). The mammalian intestine is considered as the largest immune organ in the body as it plays an important role in overall health, other than its functions of digestion and absorption of nutrients. In pigs and poultry, GIT contains more than half of total body immune cells (Jha et al., 2019). GIT exhibits direct immune responses, as well as acts as a physical barrier between the body and external environment, and provides resident for gut microbes (Aw, 1999; Celi et al., 2017). Therefore, healthy intestine is critical not only in production performances, but also in overall health and welfare of animals (Celi et al., 2017). The GIT of pigs is a complex environment and its health status is affected by various factors. The major factors influencing intestinal health of pigs include weaning, gut microbiota, diet, environment and infections (Amarakoon, 2017). Particularly, weaned piglets are highly affected, which causes considerable economic losses in swine industry. Therefore, various feeding and management practices are adopted to enhance intestinal health in pigs around weaning (Lalles et al., 2007).

To investigate different approaches to improve intestinal health in piglets, *in vivo* and *in vitro* challenge models are used. In *in vivo* studies, disease and sanitation challenge models are commonly used. The main causative agent of post weaning diarrhea (PWD), enterotoxigenic *Escherichia coli* (ETEC) and a virulent factor of gram-negative bacteria, lipopolysaccharides are widely used to stimulate the infections (Adewole et al., 2016). In *in vitro* studies, intestinal epithelial cell models are used.

1.1.1. Physiological function of intestine

The gut of monogastric animals is physiologically divided into three parts: stomach, small intestine and large intestine. The small intestine of swine is composed of duodenum, jejunum and ileum, which accounts for one third of total volume of the GIT (Mosenthin, 1998). It is made up of four layers: serosa, the muscularis, the submucosa and the mucosa. The innermost tissue layer, mucosa consists of three sublayers: muscularis mucosa, the lamina propria and the epithelium. The epithelium which has a direct contact with luminal content, is composed of continuous monolayer of epithelial cells called enterocytes. Goblet and enteroendocrine cells are interspersed among enterocytes. Goblet cells act as major secretory cells which produce mucus to protect and lubricate the mucosa layer. Enteroendocrine cells produce hormones such as secretin and cholecystokinin (Lærke and Hedemann, 2012). Intestinal epithelial cells play important roles in digestion, absorption and defensive functions. For the efficient nutrient digestion and absorption, epithelial surface area is increased by folding to form villi (Mosenthin, 1998). The villi are the finger-like projections extended to intestinal lumen. The microscopic cellular membrane projections present on the epithelium cells in villi are called microvilli. They form the brush border membrane and further increase the surface area. Brush border membrane contains wide range of enzymes which are important in the terminal stage of digestion (Mosenthin, 1998; Lærke and Hedemann, 2012). The crypts which are located around the bases of villi facilitate renewal of the intestinal epithelium (Lærke and Hedemann, 2012).

In the small intestine, carbohydrates, proteins and lipids are digested and absorbed. Duodenum is the major digestive site in the small intestine while jejunum and ileum predominantly involve in absorption of nutrients (Volk and Lacy, 2017). Carbohydrates in the diet are first digested in luminal phase by α-amylase secreted from the pancreas and further digested in the membranous phase by enzymes located in the apical membrane of duodenal and jejunal enterocytes. The monosaccharides are then absorbed either by active transport or facilitated diffusion in both jejunum and upper ileum. Protein digestion starts in the stomach and continues in small intestine. Pancreatic proteolytic enzymes and brush border peptidases play important roles in the latter part of protein digestion. The end products are then absorbed by enterocytes through active transport, mainly in the jejunum and upper ileum. Digestion of lipids is a multistep process which starts in the stomach and proceeds in small intestine. Absorption of lipids occurs through passive diffusion

and carrier mediated active transport, mainly in the jejunum (Lærke and Hedemann, 2012; Volk and Lacy, 2017). Apart from these major nutrients, small intestine absorbs most of the vitamins important for body functions. For instance, folate absorbance occurs by carrier mediated active process in the duodenum and upper jejunum, vitamin B12 and bile acids are absorbed in terminal ilium (Volk and Lacy, 2017).

The role of intestinal cells in innate immune defence system is important in overall health of animals. The intestine continuously exposes to foreign materials such as toxins, oxidants and pathogens. The single cell layer of epithelium forms physical barrier between the body and external environment to protect the body from pathogens and potentially toxic materials. The tight junctions located between cells mainly involve in maintaining of barrier integrity (Oswald, 2006). Moreover, intestinal epithelial cells have different mechanisms to fight against invasion of pathogens. Upon infections, cells produce signalling molecules such as cytokines and chemokines to initiate innate and adaptive immune responses (Arce et al., 2010). Mucus secreted by goblet cells also participate in defence mechanism against pathogenic infections. Furthermore, antimicrobial peptides produced by intestinal epithelial cells show different protective mechanisms. Except their direct antimicrobial activities, they stimulate recruitment of immune cells and promote the production of pro-inflammatory cytokines (Oswald, 2006).

The large intestine of pig consists of cecum and colon. The caecum is relatively short and located at the proximal end of the colon. Colon absorbs water and other remaining nutrients which are not absorbed in small intestine. Importantly, most of the mineral absorption is completed in the large intestine such as sodium, potassium and chloride (Lærke and Hedemann, 2012). Other functions of large intestine include conversion of luminal content into feces, temporary storage of feces and to provide site for the microflora. It is characterized by large number of bacteria ranging from 10^{10}-10^{11}per g or mL of content, low redox potential and high concentration of short chain fatty acids (SCFA) (Gaskins et al., 2002). The microbial community in large intestine helps to digest complex carbohydrates including dietary fiber and synthesize certain vitamins such as vitamin K, biotin, cobalamin, folates, nicotinic acid, panthotenic acid, pyridoxine, riboflavin, and thiamine (Hill, 1997).

1.1.2. Factors affect intestinal health in piglets

The common factors that are known to affect intestine health in piglets include weaning, gut microbiota, diet, environment and infections. Weaning has a direct impact on the health status of intestine in terms of physiology, microbiology and immunology. At the weaning, the piglets are abruptly separated from mothers and mixed with other piglets. Their diet changes from liquid (milk) form to more complex solid feed with the absence of interactions with sows (Lalles et al., 2007; Heo et al., 2013). As a result of weaning, they are exposed to various stressors including environmental, social, nutritional and psychological. Importantly, weaning disturbs the structure and function in small intestine. Morphologically, the changes in villus height (VH) and crypt depth (CD) occur during post weaning period (Heo et al., 2013). Furthermore, GIT undergoes alterations in digestion and absorption rate, microbiota composition, and immunological functions. (Lalles et al., 2007). The digestion and absorption functions are affected through the changes in brush-border enzyme activities, and small intestinal secretions. However, these effects are segment specific within the intestine (Heo et al., 2013). Post weaned piglets are susceptible to enteric diseases such as diarrhoea. Poor immunity, higher gastric pH, and changes in gut microbial community facilitate the colonization of ETEC bacteria. In addition, gut inflammation and oxidative stress associated with weaning impair the gut health in piglets (Lalles et al., 2007; Heo et al., 2013).

Gut microflora has profound influence on gut health in piglets as it directly interacts with the intestinal tract. The microflora population consists of both detrimental organisms such as *Escherichia coli* (*E.coli*), *Salmonella*, and Clostridia, and beneficial microorganisms such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Fouhse et al., 2016). In host-microbial relationship, they provide energy substrate to the intestinal epithelial cells in the form of SCFA and thereby cell proliferation and differentiation are promoted. Indeed, constant supply of energy is required to maintain the physical barrier to prevent the entry of pathogens. Also. commensal gut flora restricts the pathogen adhesion to the intestinal epithelium, and facilitates the mucin production (Che et al., 2014; Fouhse et al., 2016). Moreover, they have ability to produce antimicrobial peptides which could impede the growth and adhesion of pathogenic microorganisms (Celi et al., 2017). Different factors including the stress associated with weaning, diet and management practices change the balance of intestinal microbiota in piglets. The imbalance of microbiota increases the risk of diseases, in particular post weaning diarrhoea (Fouhse et al., 2016).

The diet also plays an important role in intestinal health. Diet has direct impacts on gut microbiota, immune and intestinal barrier functions (Celi et al., 2017). The effects are varied depending on the nutritional composition, feed additives, processing method, and presence of pathogens and mycotoxins. With respects to the nutrient composition, it is crucial to provide adequate nutrients to meet the requirement of animals, but not in excess. The excessive supply of some nutrients can lead to gastrointestinal disturbances. For example, dietary protein is important for the optimum immunity function. Nevertheless, excessive supply of dietary protein causes the production of potentially toxic metabolites such as ammonia, biogenic amines, and hydrogen sulphide. Accumulation of those metabolites promote the growth of pathogenic bacteria and development of gastro intestinal disorders (Gao et al., 2019). Feeding contaminated feed also might lead to serious issues in animal health. The feed can be contaminated during the processing, transportation and feeding. Among the contaminants, mycotoxins are the most common contaminants in cereal based pig feed. Furthermore mycotoxin contaminated feed has been shown to induce oxidative stress in weaned piglets (Van Le Thanh et al., 2016). Considering the role of the diet in gut health in piglets, various nutritional interventions are implemented to improve the overall health in animals and thereby increased production performances. Recently, the usage of bioactive compounds as feed additives, in terms of functional feed ingredients and nutraceuticals have attracted more attention in maintaining gut health in piglets. For instance, supplementation of antioxidants has shown to attenuate the production of reactive oxygen species in weaned piglets (Van Le Thanh et al., 2016; Celi et al., 2017).

To maximize the production performances and maintain healthy gut in piglets, it is essential to provide optimum environment conditions along with other management practices. The temperature, humidity, ventilation and housing condition are the factors to be considered.

1.1.3. Infections in piglets

Weaned piglets are more susceptible to infections and causes large economic losses in pig industry. Indeed, PWD which is characterised by frequent discharge of watery faeces is the most common gastrointestinal infection observed in weaned piglets (Heo et al., 2013; Fouhse et al., 2016). *E. coli* is a gram-negative bacteria, which express several virulent factors such as fimbria, enterotoxins (exotoxins), and endotoxins to cause diarrhoea. However, not all strains of *E. coli* are harmful. ETEC is the main pathotype of post weaning diarrhoea or post-weaning enteric colibacillosis (Luppi, 2017). The strains of ETEC possess adhesive fimbriae called F4 (K88) and F18. In the pathogenesis process, fimbriae bind to glycoprotein receptors on small intestinal enterocytes and facilitate the colonization. The degree of colonization determines the severity of the infection, ranging from mild to severe condition. Following the colonization, ETEC synthesize and secrete several types of enterotoxins which cause diarrhoea as a result of loss of fluid homeostasis in the small intestine (Dubreuil et al., 2016; Luppi, 2017). The enterotoxins produced by porcine ETEC are either heat-labile or heat-stable. In the family heat-stable enterotoxins, three different sub groups have been identified in ETEC, based on the structure and biochemical properties: heat stable enterotoxin a, heat stable enterotoxin b and enteroaggregative *E.coli* heat stable enterotoxin (De et al., 1956). Similarly, two distinct toxin types have been described in heat-liable group: LTI and LTII. Further, L1 is found in two different forms as LT1h and LT1p, produced respectively by human and porcine ETEC (Luppi, 2017). Both heat-labile and heat-stable enterotoxins stimulate the secretion of electrolytes and water to the intestinal lumen and decrease their absorption which cause dehydration and acidosis. In addition, heat-stable enterotoxin a is known to reduce the intestinal barrier function which leads to leaky gut. Those mode of actions result in diarrhoea, impaired feed intake and reduced nutrient digestibility, reduced growth and even sudden death (Heo et al., 2013; Dubreuil et al., 2016).

Other than enterotoxins, endotoxins are also involved in pathogenesis of diseases in piglets. Lipopolysaccharide (LPS), endotoxin produced by gram negative bacteria is used to model gram-negative bacterial infections experimentally in piglets.

1.2. Oxidative stress

Oxidative stress is the imbalance between production of free radicals and their neutralization mechanism by antioxidants. Free radicals are unstable, highly reactive molecules which contain one or more unpaired electrons. These molecules can either be reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Amarakoon, 2017). In living organisms, ROS are generated from molecular oxygen as by-products of cellular metabolism. They are present in two different forms: free radicals and nonradicals. Free radicals can be generated by donating or up taking electrons from nonradicals while nonradicals can be formed by sharing unpaired electrons in between free radicals. This reactive nature is induced by different factors and leads to proceed as chain reactions (Phaniendra et al., 2015). The major types of ROS are superoxide anion (O^-_2), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and singlet oxygen ($^1O_2$), whereas RNS include nitric oxide (•NO), nitrous oxide (N_2O), peroxynitrite anion (ONOO−), and nitroxyl anion (NO-) (Amarakoon, 2017). At low or natural production level, these reactive molecules are involved in beneficial functions in the biological system. Particularly, they can act as regulatory mediators in signalling transductions which are important in various physiological functions such as activation of immune system, regulation of redox and vascular homeostasis, and regulation of cell proliferation and apoptosis (Patel et al., 2018).

However, over production of reactive species causes oxidative stress. The excessive amount of ROS reacts with cellular components and lead to lipid peroxidation, DNA alternation, protein degradation, functional changes of various molecules and tissue damages (Birben et al., 2012). Lipids are more likely to be oxidized by free radicals. Oxidation of cell membrane lipids alters cellular structure and functions. This process leads to increased cellular permeability, and formation of cytotoxic and mutagenic compounds such as malondialdehyde (MDA) and conjugated diene compounds (Pham-Huy et al., 2008). MDA is a secondary oxidation product formed during the lipid peroxidation and used as an indirect biomarker of oxidative stress. Cellular protein molecules are also subjected to degradation by ROS in several mechanisms, including the fragmentation of the peptides, modification of the molecules through the alteration of electrical charges and formation of cross links (Kelly and Mudway, 2003). In the situation of oxidative stress, DNA molecules are also modified by ROS through the degradation of bases, single or double stranded breaks, modifications of bases and other associated molecules, mutations, deletions and

formation of cross-linking with proteins. Most of these modified DNA molecules become risk factor for the pathogenesis of cancer, neurodegenerative, cardiovascular, and autoimmune diseases (Birben et al., 2012). Apart from the indirect effects of oxidative stress through the interactions with cell components, those reactive radicals directly become detrimental to the host and become risk factors for various diseases including cancers, cardiovascular, metabolic and inflammatory diseases (Caradonna et al., 2000; Griendling and FitzGerald, 2003; Ceriello, 2006; Valko et al., 2006).

1.2.1. Sources of reactive oxygen species

Different enzymes and non-enzymatic molecules are also involved in the formation of ROS. The enzymes involved in enzymatic pathways of ROS generation include NADPH oxidase (NOX), xanthine oxidase, cytochrome P-450 and peroxidases. Among them, NOX which represents a class of enzymes is the major enzyme involved in ROS generation. However, ROS can either be generated by endogenous or exogenous sources. Cellular organs which consume high level of oxygen such as mitochondria, peroxisomes and endoplasmic reticulum are the major endogenous sources of generating ROS (Phaniendra et al., 2015). Living organisms produce ROS from molecular oxygen in the process of normal cellular metabolism. The electron transport chain of the mitochondria is the major source of producing O^-_2. It has been estimated that 1-3% of all the electrons which are transforming during the normal cellular respiration are leaked to produce O^-_2 (Birben et al., 2012). This process is non-enzymatic and O^-_2 are produced in several sites of mitochondria and released into the matrix. Importantly, complexes I and III are known to be major sites of superoxide production. This mitochondrial ROS production is increased by different factors such as tumor necrosis factor-α (TNF-α), hypoxia, serum deprivation, and various diseases (Bae et al., 2011; Patel et al., 2018).

The ROS produced in peroxisomes include H_2O_2, O_2-, •OH and •NO. Interestingly, H_2O_2 are formed during metabolic process in peroxisomes. In endoplasmic reticulum also, ROS are formed through the enzymatic pathways. Mainly, cytochrome p-450 and b5 enzymes and diamine oxidase contribute to the generation of ROS. In addition, prostaglandin synthesis, auto-oxidation of adrenalin, phagocytic cells, reduced riboflavin, immune cell activation, inflammation, mental stress, excessive exercise, cancer, aging, and ischemia are considered as endogenous sources of

ROS generation. Furthermore, ROS are generated endogenously as a result of activation of NOX enzyme complex during pathogenic infections (Kallapura et al., 2014).

The highly reactive nature of superoxide leads to form various ROS and RNS and continue the reactions. At the initial reactions, O^-_2 are converted to H_2O_2 by enzyme called superoxide dismutase (SOD). Although H_2O_2 is non-radical derivate, it participates in Fenton and Haber-Weiss reactions to produce highly reactive and toxic •OH, in the presence of transition metal ions (Birben et al., 2012; Borza et al., 2013). In addition, H_2O_2 is converted to hypochlorous acid by neutrophil-derived enzyme called myeloperoxidase in the presence of chloride ions. Hypochlorous acid is highly oxidative and plays important role in infections (Klebanoff, 2005). Singlet oxygen is also considered as highly reactive ROS and produced as a result of several reactions such as reaction between H_2O_2 or •OH with superoxide anions, enzyme mediated reactions, decomposition of endoperoxides and degradation of hydroperoxides in liver microsomes (Borza et al., 2013). Furthermore, lipid peroxidation initiated by ROS also facilitates the production of more free radicals (Birben et al., 2012).

The exogenous sources of ROS include ionizing radiation, heavy metals, cigarette smoke, alcohol, ozone and nitrogen oxide, certain drugs and industrial solvents and certain foods (Pham-Huy et al., 2008; El, 2012). Apart from being a source of ROS, these molecules can stimulate cells to generate reactive molecules by different mechanisms.

1.2.2. Antioxidants

Antioxidants are defined as compounds that protect biological systems against the potentially harmful effects of processes or reactions that can cause excessive oxidations (Krinsky, 1992). They are also known as reducing agent. Antioxidants have ability to neutralize the free radicals, to protect cellular components against their toxic effects and to prevent the initiation or progression of oxidative stress associated diseases (Pham-Huy et al., 2008). The body has endogenous antioxidant system to maintain the redox homeostasis.

Endogenous antioxidants can either be enzymatic or nonenzymatic. The major antioxidant enzymes include SOD, glutathione peroxidase (GSH-Px) and catalase (CAT). SOD is the most powerful antioxidant in the cells. It catalyses the dismutation of O_2^- into H_2O_2 by reduction (Santos-Sánchez et al., 2019). It is a metalloenzyme which requires metal as a cofactor in its activity. Therefore, there are different forms of SOD in the cells depending on the type of metal it

requires. Among them, CuZn-SOD and Mn-SOD are known to act as bulk scavengers (Birben et al., 2012; Santos-Sánchez et al., 2019). CAT is a highly efficient enzyme, mainly found in peroxisomes. It breaks down H_2O_2 to water and molecular oxygen. Therefore, CAT plays an important role in detoxification during the lipid peroxidation (Krinsky, 1992; Santos-Sánchez et al., 2019). Similarly, GSH-Px also removes the cellular hydrogen peroxides. Glutathione is the major nonenzymatic antioxidant produced in the cells. It is a cysteine containing peptide and the thiol group resents on cysteine molecule is involved in antioxidant activities. Owning to a high electron donating capacity, reduced glutathione (GSH) exerts antioxidant activity through several mechanisms. GSH converts H_2O_2 into H_2O and O_2 by donating electrons. This reaction is catalysed by GSH-Px while oxidized glutathione (GSSG) is formed. GSSG is potentially toxic to the cells. To avoid the accumulation of GSSH in the cells, GSSG is reduced into GSH by enzyme called glutathione reductase using the reducing power of NADPH (Birben et al., 2012; El, 2012). In addition, GSH protects cell membrane from the damages by ROS and plays a role in restoring vitamin C and vitamin E into their reduced forms (Birben et al., 2012). Based on the reactive nature, the ratio between GSH and GSSH is used as a parameter to determine the oxidative stress.

Exogenous antioxidants include vitamins (vitamin C and E), glutathione, carotenoids, polyphenols and trace elements. Vitamin E is a fat-soluble and chain breaking antioxidant. It plays an important role in protecting the cell membranes from oxidation by free radicals. Among the available forms, α-tocopherol is the most efficient antioxidant. Being an electron donor, it acts as a peroxyl radical scavenger and prevents the propagation of lipid peroxidation (Pham-Huy et al., 2008; Traber and Stevens, 2011; El, 2012). The resulted oxidised α-tocopheroxyl radicals can be recycled back to α-tocopherol by other antioxidants such as ascorbic acid and retinol (El, 2012). Vitamin C or ascorbic acid is a water-soluble antioxidant. Other than its synergistic action with vitamin E in preventing lipid peroxidation, vitamin C performs as a reducing agent to neutralize ROS such as H_2O_2 (Krinsky, 1992; El, 2012).

Although human and animal body has antioxidant defensive system, its capacity reduces with aging, diseases, and oxidative stress. Therefore, it is important to provide adequate dietary antioxidants to maintain healthy life. Phytochemicals including phenolic compounds and carotenoids shows antioxidant activities.

1.2.3. Impact of oxidative stress on intestinal health

GI tract is more susceptible for oxidative stress as it is frequently exposed to different diet-derived oxidants. For example, ingestion of diet high in unsaturated fat causes luminal accumulation of lipid peroxides which promote generation of ROS and propagation by increased lipid peroxidation (Aw, 1999). In addition, changes in gut microorganisms, infections, nonsteroidal anti-inflammatory drugs lead to the production of excessive free radicals. Importantly, pathogens and their products activate the epithelial and immune cells to produce cytokines and other mediations which contribute further to oxidative stress (Bhattacharyya et al., 2014). Other than the effects of ROS on the physiological, immune and metabolic functions of the gut, they are associated with pathogenesis of gastrointestinal diseases such as inflammatory bowel disease (IBD), colon cancer (Tian et al., 2017). IBD is a chronic inflammatory disorder which is mainly in two forms: crohn's disease and ulcerative colitis. crohn's disease can occur in any region of the GI tract while ulcerative colitis occurs only in the lower part of the GI tract. Increased ROS level and decreased antioxidant level which induce inflammation and disruption of gut barrier function are the contributing factor in the development of IBD (Bhattacharyya et al., 2014).

Excessive ROS can attack cell components, and thereby cellular function and viability is affected. Mild oxidative stress activates intestinal cell proliferation while, higher oxidant stress causes intestinal cell death by apoptosis or necrosis (Cepinskas et al., 1994). Therefore, it is clear that oxidative stress leads to the disruption of gastrointestinal barrier. Among the ROS, hydrogen peroxide and nitric oxide are the major molecules involved in tight junction disruption and barrier dysfunction. There are different mechanisms involved in disruption of gut epithelial barrier including the inhibition of interaction between tight junction proteins to form protein complex, disruption of adherent junctions, and modification of the protein molecules (Rao, 2008). It still remains unclear whether inflammation or oxidative stress occurs first. Anyway, overproduction of ROS stimulated by dietary factors causes intestinal inflammation (Aw, 1999). To investigate the role of oxidative stress in inflammation, different stimuli are used. The most commonly used stimuli are cytokines, bacterial toxins, ischemia-reperfusion, and lipid mediator. Those stimuli induce oxidative stress in the experimental model and allow to examine the effect of oxidative stress in different aspects including the pathogenesis of diseases(Aw, 1999; Erridge et al., 2002). Effect of oxidative stress on inflammation of the gut has been studied *in vivo* and *in vitro*. In

addition, the main function of the intestine and the nutrient absorption have been shown to be affected by oxidative stress.

1.3. Lipopolysaccharide

Lipopolysaccharide (LPS), also called endotoxin is a component of the outer membrane of gram-negative bacteria. It is widely used as an immune stimulant in disease challenge model of gut health related studies (Adewole et al., 2016). LPS plays an important role in the phenomena of the activation of innate immune system as a response to gram-negative bacterial infections (Erridge et al., 2002). As a result of the activation of immunity system, host is protected by eliminating the pathogens.

Gram negative bacteria which are resident in the gut also produce LPS and it is crucial to detoxifying them to maintain the host health. It occurs in two ways either by enzymatic degradation or complement mediated degradation (Sampath, 2018). The enzyme called acyloxyacyl hydrolase, modifies the structure of LPS by removing the secondary acyl chains from lipid A portion. The potency of resulted deacylated LPS is 0.2–1% of acylated LPS and modified structure interrupts its recognition through signalling pathways (Munford and Hall, 1986; Kitchens et al., 1992). Alkaline phosphatase is also a detoxifying enzyme which has ability to dephosphorylate the lipid A and produce monophosphoryl lipid A. The modified LPS is less toxic than that of intact LPS (Bates et al., 2007).

Many studies have showed that LPS causes significant physiological and behavioural changes in farm animals (Kanno et al., 1996; Webel et al., 1997; Yi et al., 2016). Excessive exposure to LPS causes serious health issues including septic shock in the animals (Beutler and Rietschel, 2003). Proinflammatory cytokines induced by LPS, mainly tumour necrosis factor alpha (TNF-α), interleukin 6 (IL-6) and interleukin 1b (IL-1b) showed effects on metabolic pathways which lead to reduce the feed intake and growth parameters of the animals (Webel et al., 1997).

1.3.1. Structure of lipopolysaccharide

Endotoxin consists of three structural components: lipid A, core region, and O antigen repeats (hydrophilic polysaccharides). Immunogenicity or the ability of LPS to provoke the immunity system is associated with polysaccharide components, whereas lipid A portion is highly hydrophobic and responsible for toxicity of the molecule (Sampath, 2018). Apart from that, lipid A acts as an anchor to attach to the bacterial outer membrane. Structurally, lipid A consist of a N-acetyl glucosamine backbone which is phosphorylated and acylated at position 1 and 4 of the saccharides and positions 2 and 3 of each monosaccharide, respectively (Homma et al., 1985; Galanos and Freudenberg, 1993). In general, six acyl chains are esterified with the disaccharide backbone. Acyl chains which are directly esterified with the sugar moiety are called as primary acyl chains and those which are esterified to hydroxyl groups of primary acyl chains are known as secondary acyl chains (Steimle et al., 2016). However, lipid A structure varies among species and sometime among strains of same species (Raetz and Whitfield, 2002). These variations exists in terms of the esterified positions, length and number of the acyl chains, and the molecules bind to the disaccharide backbone (Raetz et al., 2007).

Core region which is made up of sugar molecules is joined to the lipid A by covalent bonds. It has two structurally distinct regions as inner region and outer region. The inner core (lipid A proximal) contains unconventional sugar molecules such as 3-deoxy-D-manno-octulosonic acid and L-glycero-D-manno heptose (Erridge et al., 2002). This region is highly conserved and more anionic in nature as a result of high degree of phosphorylation. In contrast, the outer core consists of conventional sugars namely hexoses and hexosamines. The main hexoses find in this region are D-glucose, D-galactose, D-glucosamine, and N-acetylgalactosamine (Alexander and Rietschel, 2001). O antigen or O polysaccharide is linked to the outer core of LPS providing unique properties. However, it presents only in smooth type of LPS producing bacteria. This outermost part of LPS is made up of repeating subunits of sugars where each unit consists of one to eight sugar molecules (Caroff and Karibian, 2003). It is one of the most variable components of LPS, and thereby used as basis for serotype classification of LPS producing bacteria. Variations are mainly caused by types of sugar present, their arrangement patterns in O unit and the bonds within and between O units (Wang et al., 2010). In the development of vaccines, those variations are taken into consideration.

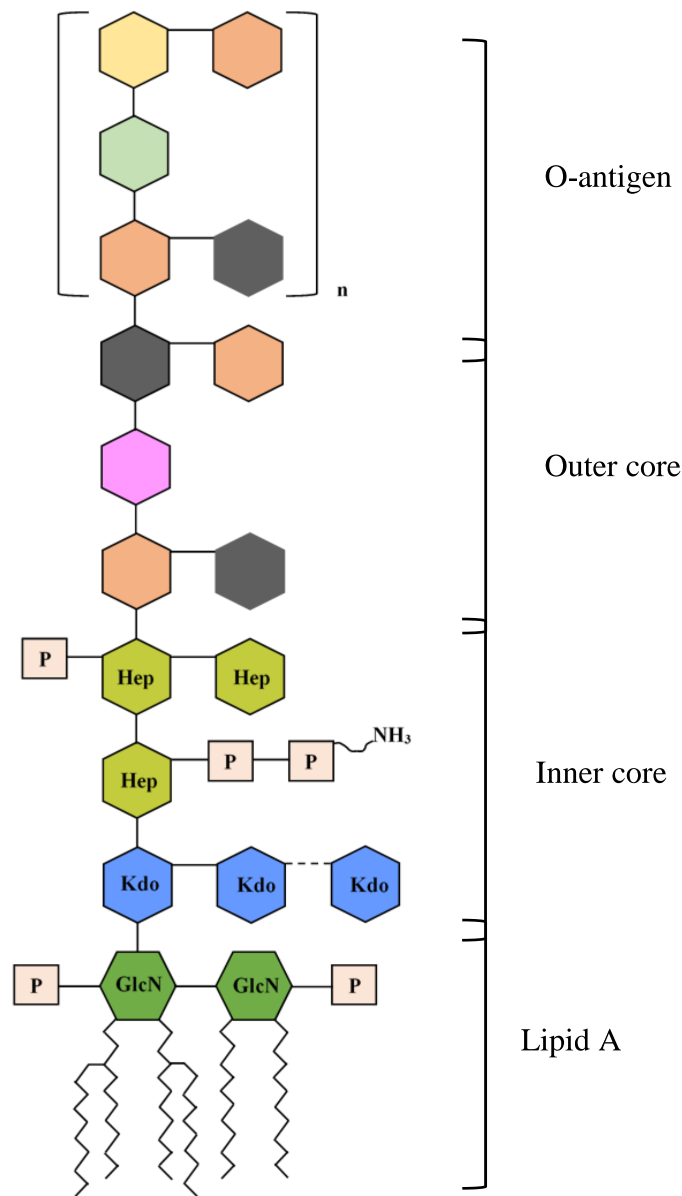


Figure 1.1. The chemical structure of lipopolysaccharide from gram-negative bacteria

It consists of three main components: lipid A, core region, and O antigen repeats. Lipid A is made up of N-acetyl glucosamine (GlcN) backbone which is phosphorylated and acylated at position 1 and 4 of the saccharides and positions 2 and 3 of each monosaccharide, respectively. Lipid A region is linked to core region by covalent bonds. Inner core region contains 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep), while outer core contains common sugars molecules such as hexoses and hexosamines. Outer core is joined to the o-antigen which is consists of repeating subunits of sugars.

1.3.2. Mechanisms of lipopolysaccharide action

Upon the microbial infections, the innate immunity system is activated. It involves serious of interactions, starting from the recognition of pathogens or pathogenic compounds. Pathogen recognition receptors (PRRs) present on the cells detect the pathogen-associated molecular patterns (PAMPs) (Mogensen, 2009). LPS, a structural motif, is one of the PAMPs which is recognized by specific group of PRRs called toll-like receptors. Toll like receptor-4 (TLR4) is the key receptor for LPS (Poltorak et al., 1998). However, the activation of TLR4 signalling pathway requires interactions of LPS with other proteins. The first protein assisted in the signalling pathway is LPS-binding protein (LBP) which is produced in the liver. LBP directly binds to freely available LPS or outer membrane of intact bacteria to extract LPS, and then LPS is transferred to Glycerophosphatidylinositol-anchored protein (CD14) (Peri et al., 2010). CD14 exists either in soluble form (sCD14) or membrane bound form (mCD14). In order to recognise LPS, TLR4 form a complex with myeloid differentiation 2 (MD-2) protein (Hung and Suzuki, 2017). The purpose of CD14 is to transfer LPS to MD2-TLR4 complex in order to form a dimer of the ternary structure. With the binding of LPS, TLR4 undergoes oligomerization and recruits Toll-interleukinn-1 receptor (TIR) domains and transduces signals to adaptor proteins. This is important for the initiation of downstream signalling transduction. Depending on the type of recruited adaptor protein, TLR4 signalling cascade has been divided into two pathways: myeloid differentiation factor 88 (MyD88) dependent and MyD88-indipendent pathways (Lu et al., 2008; Hung and Suzuki, 2017).

MyD88 pathway is mainly important in the early stage of acute inflammation which mediates the production of proinflammatory cytokines through the downstream signalling. MyD88 molecule consists of TIR domain and a death domain at the positions of carboxyl-terminal and amino-terminal respectively. MyD88 is induced upon LPS recognition by MD-2-TLR4 complex, resulting in recruitment of IL-1 receptor associated kinases (IRAKs) to the receptor complex. The death domain of MyD88 involves in this reaction. Although IRAK-4 is a main mediator involves in downstream signalling, there are other IRAKs namely IRAK-1 and IRAK-2 are known to be involved (Swantek et al., 2000; Lu et al., 2008). Activation of IRAK-1 facilitates the recruitment of TNF receptor associated factor 6 (TRAF-6) to the receptor complex. The TRAF domain of TRAF-6 links to the IRAK-1 and then dissociate from receptor complex. Subsequently, TRAK-6

binds with two enzymes namely ubiquitin-conjugating enzyme13 (UBC13) and ubiquitin-conjugating enzyme E2 variant1 isoform A (UEV1A) and activates transforming growth factor-β-activated kinase 1 (TAK1) (Lomaga et al., 1999; Gohda et al., 2004). As a result of this, both mitogen-activated protein kinase (MPAK) and inhibitory κB kinase (IKK) pathways are activated. Activation of those pathways causes activation of protein-1 (AP-1) and nuclear factor kappa B (NF-κB) transcription factors respectively, to produce proinflammatory cytokines and chemokines (Lu et al., 2008).

MyD88 independent (TRIF dependent) pathway induces the expression of Type 1 interferons and interferon-inducible genes which plays important roles in the late stage of acute inflammation. TIR domain-containing adaptor inducing interferon-β (TRIF) is the key adaptor protein in this signalling transduction. TLR4 induced by LPS recruits TRIF and TRIF-related adaptor molecules (TRAM). Those activated TRIF recruit TRAF-3 (Lu et al., 2008). In order to continue the downstream signalling, this IRAF-3 molecule forms a complex with TANK (TRAF family member-associated NF-kB activator), TBK1 (TANK binding kinase 1) and IKKi (IkB Kinase i) (Oganesyan et al., 2006). This leads to phosphorylation and activation of interferon regulatory factor-3 (IRF3). Apart from the IRF3 activation, TRIF interacts with receptor-interacting protein 1 (RIP1) and in return NF-κB transcription factor is activated. Both activations induce nuclear translocation of interferon regulatoryfactor-3 (IRF-3) and IRF-7 to lead to the production of interferons (IFNs) (Fitzgerald et al., 2003; Lu et al., 2008; Hung and Suzuki, 2017).

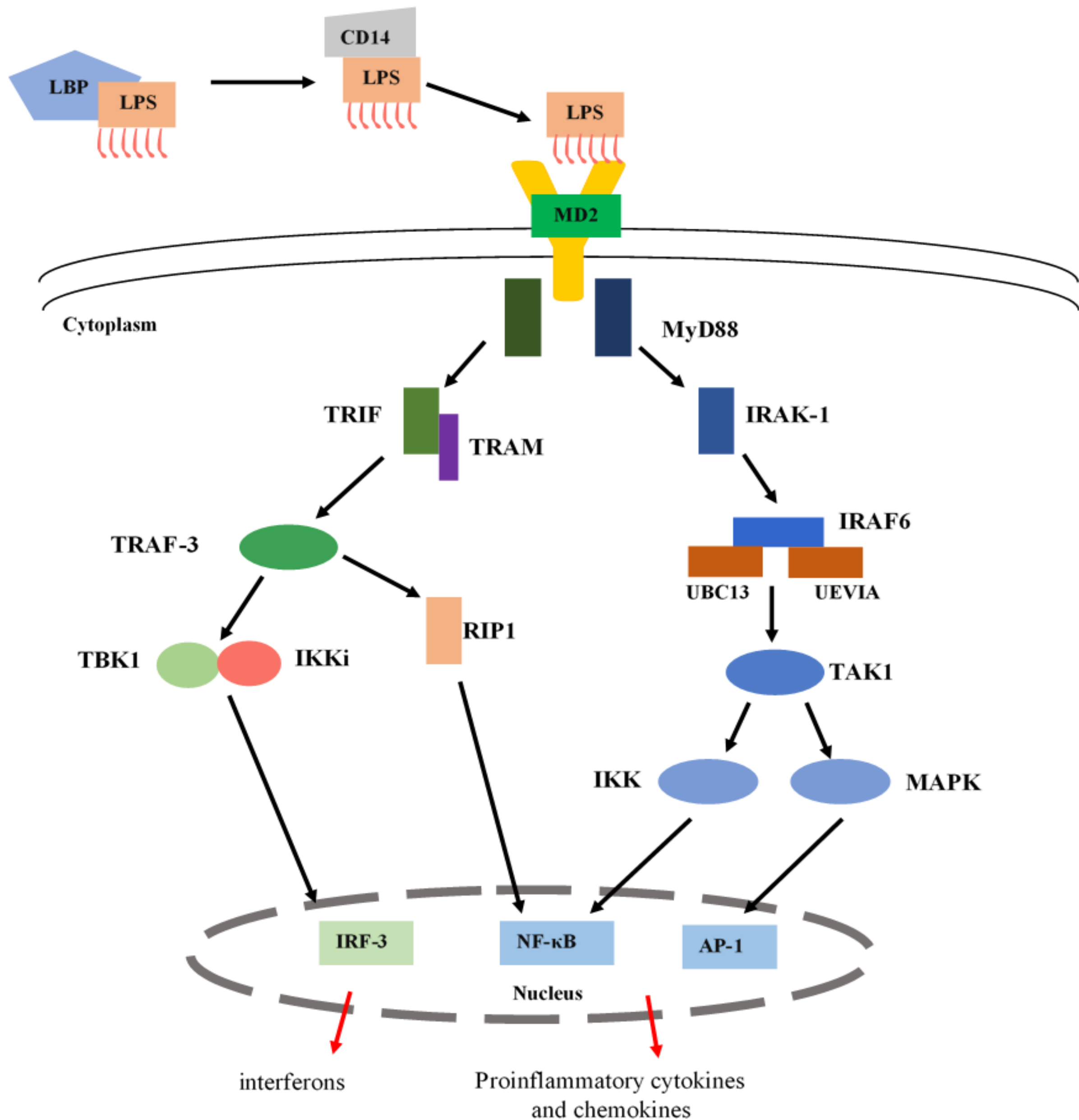


Figure 1.2. TLR4 signalling pathways

LPS-binding protein (LBP) directly binds to lipopolysaccharide (LPS) and deliver to Glycerophosphatidylinositol-anchored protein (CD14). Then CD14 transfer LPS to myeloid differentiation 2- toll like receptor-4 (MD2-TLR4) complex. Upon recognition, TLR4 undergoes oligomerization and recruits adaptor proteins. Subsequent signalling pathways can be either myeloid differentiation factor 88 (MyD88) dependent or MyD88-indipendent pathways.

1.3.3. Effect of lipopolysaccharide on intestinal health

To find out the effect of LPS on intestinal health, *in vivo* as well as *in vitro* studies have been conducted (Wang et al., 2013; Yi et al., 2016). Once the intestine is exposed to LPS, different signalling pathways are activated. It leads to impairment of intestinal health in different ways. Many studies have shown the importance of LPS in the disruption of intestinal tight junction barrier. In vitro study done by Guo et al., (2013), showed that LPS increased tight junction permeability is associated with the activation of TLR-4/FAK/MyD88 signal-transduction axis. Furthermore the activation of TLR4 signalling pathway by LPS leads to intestinal inflammation by releasing inflammatory mediators such as TNF-α, IL-6 and IL-1b and interleukin 8 (IL-8) (Erridge et al., 2002; Wang et al., 2013). In the previous study, it has been shown that Caco-2 cells treated with LPS at the concentration of 1µg/mL significantly increased the mRNA expression of TNF-α, IL-1b, and IL-8 (Wang et al., 2013).

Importantly, weaning piglets are mostly affected by LPS as a result of infectious diseases following the weaning. LPS affects the expression of genes important in intestinal immunity, absorption and metabolism, and integrity of gut barrier function. The effect of LPS on jejunum and ileum is greater than that on duodenum and colon. In the previous study, decreased mRNA level of epidermal growth factor in the duodenum, jejunum, and colon and for mammalian target of rapamycin in the jejunum and ileum were observed after LPS challenge in weaned piglets. This indicates the effect of LPS on growth, proliferation and regeneration of intestinal enterocytes (Yi et al., 2016). Exposure to LPS might lead to morphological and physiological changes in intestine. Indeed, the effects of LPS on morphology of the villi and intestinal glands were reported in a previous study. According to that study, decreased height and surface area and increased width of intestinal villi were observed in weaned piglets up on oral administration of LPS. Furthermore, it has shown that LPS increased depth and width of intestinal glands. (Parra et al., 2011). Villus height and gland's depth, especially crypt depth are important parameters to estimate digestive and absorptive capacity in intestine. Reduced villus height and increased crypt depth are associated with a decline in digestion and absorption in intestine (Mosenthin, 1998; Parra et al., 2011).

Moreover, it has been showed that LPS down regulate the expression of aquaporins 8 gene in weaned piglets. This gene is the major water transporter in the gut. Therefore, down regulation of this gene causes decreased absorption of water and development of diarrhoea (Yi et al., 2016).

Furthermore, An *in vivo* study indicated that LPS cause significant decrease in intestinal absorption of electrolytes (Na+, and Cl-), and glucose in piglets (Kanno et al., 1996).

1.4. Management of gut health in swine

1.4.1. Use of antibiotics

The antibiotics can be used for both therapeutic and nontherapeutic purposes in animal production. Originally, they are used to treat diseases at therapeutic levels. However, they have been used at nontherapeutic level in animal feed with the discovering of their benefits in animal production. Interestingly, they are known as antibiotic growth promoters (AGP) due to their significant effects on growth performance parameters such as growth rate and feed utilization. The Food and Drug Administration (FDA) has approved 12 antibiotics to be used in swine feed including Penicillin, Apramycin, Oxytetracycline, and Bacitracin methylene disalicylate. The effectiveness of those compounds has been studied widely (Cromwell, 2002).

There are four main mechanisms that have been proposed to explain the actions of antibiotics on growth performance: (i) reduction of sub-clinical infections, (ii) decreasing the production of growth-depressing microbial metabolites, (iii) reduction of microbial use of nutrient and (iv) improving the absorption of nutrient in the intestine (Gaskins et al., 2002). AGP are widely used in swine production, particularly in nursery diet, in order to improve the gut health and thereby to control the infectious diseases associated with weaning and to improve growth performances. (Omonijo et al., 2018). However, their effectiveness on growth performances is reduced with the age of pigs. It has been reported that antibiotics improve growth rate and efficiency of feed utilization by 16.4% and 6.9% respectively in young pigs (7-25 kg), while pigs in growing and finishing stage (24-89 kg) improved growth rate only by 4.2% and feed efficiency by 2.2%. In addition, AGP are known to improve reproductive performances and reduces mortality and morbidity in pigs (Cromwell, 2002).

Regardless of the benefits of using AGP in animal production, there are certain safety issues were emerged. The major concerns are the development of antibiotic resistant bacteria, particularly pathogenic bacteria in animals, and remaining of drug residues in animal products which can be transmitted to human and cause a potential public health risk. Indeed, antibiotics used in both

human and animals were highly concerned. Sweden was the first country to ban some of the in-feed antibiotics in 1986. Following Sweden, other countries in European Union prohibited the use of AGP in animal production. Canada also has implemented policies to restrict the use of AGP in animal production (Cromwell, 2002; Omonijo et al., 2018). However, the restriction on AGP in animal feed caused an increase in the frequency of infectious diseases and negatively affected animal production. Therefore, the amount of therapeutic antibiotic used in animal industry has been increased over the time (Cheng et al., 2014).

1.4.2. Alternatives to antibiotics

The emerged concern on antibiotic resistance and prohibition of AGP in livestock production has increased the demand of alternatives for AGP. In the past few decades, studies were conducted to discover the feed additives which have similar effects like conventional antibiotics. Prebiotics, probiotics, essential oils, organic acids, enzymes, antimicrobial peptides and medium chain fatty acids have been identified as potential antibiotic alternatives to be used in swine production.

Prebiotic is defined as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid, 1995). The definition itself indicates that the beneficial effects of prebiotics is mainly through the modification of microbial community in the colon. Most of the prebiotics are nondigestible carbohydrates such as fructooligosaccharide (FOS), mannan-oligosaccharide (MOS), and oligofructose (Cheng et al., 2014). Different experimental models have been used to investigate the impacts of prebiotics and prebiotic containing feed ingredients on pigs. Interestingly, those non-digestible compounds stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacill and reduce the growth of detrimental bacteria in the gut. Yang et al., (2012) indicated that dietary chito-oligosaccharide (COS) supplementation increased the population of bifidobacteria and lactobacilli, and decreased the population of *S. aureus* in weaned piglets. In another study, it was shown that MOS product reduced the colonization of coliforms in duodenum, jejunum, cecum, and colon and increased the serum immunoglobulin G (IgG) in young pigs. The toxins produced by coliform bacteria contribute to the development of diarrhoea and other intestinal disorders. Therefore, decreased number of coliform bacteria is more desirable in gut health (White et al., 2002). Application of

prebiotics has more advantages over probiotics as prebiotics are less expensive, lower risk and easy to handle. However, it is possible to use prebiotic together with probiotics in animal feed.

The World Health Organization (WHO) has defined probiotic as live microorganisms which, administered in adequate amounts confers a health benefit on the host. Administration of probiotic helps to maintain gut microbiota balance (Lalles et al., 2007). The main functions of probiotics in swine diet are to stimulate the growth of beneficial bacteria, prevent colonization of pathogenic bacteria, improve the digestion and absorption of nutrients, improve intestinal immunity, and to maintain intestinal barrier (De Lange et al., 2010). Once probiotics colonized in the gut, they produce antimicrobial compounds such as bacteriocins and organic acids to inhibit the growth of pathogenic bacteria. Also, probiotics compete with pathogens for the nutrients which leads to reduce the growth of pathogens. Furthermore, their adherence to intestinal mucosa inhibits pathogen's adhesion to mucosa (Cheng et al., 2014). Lactic acid bacteria (LAB), especially *Lactobacillus* and *Bifidobacterium* species which are resident microflora in the gut of pigs, are mainly used as probiotics in swine feed. They can be used individually or in combination, as well as together with prebiotics. The antimicrobial property of LAB is associated with their ability to produce lactic acid through the fermentation. The acidic condition created by LAB inhibits the growth of disease-causing bacteria such as *Salmonella* spp. or strains of *E. coli* (De Lange et al., 2010; Ross et al., 2010). In previous study it was shown that administration of probiotics (*Lactobacillus amylovorus* and *Enterococcus faecium*) positively influenced on gut microflora by reducing the number of enterobacteria and improved the nutrient utilization pigs (Ross et al., 2010). However, the effects of probiotics are highly species specific and affected by many factors.

Recently, essential oils (EO) have received more attention for use in swine feed as antibiotic alternatives. They are generally considered as safe, environment friendly and effective against pathogenic organisms. EO are natural, volatile compounds that are extracted from plant materials. The beneficial effects of essential oils include antibacterial, antifungal, antibiofilm, antiparasitic, antioxidant, antiviral, and anticancer (Adukwu et al., 2016). Owning to the antimicrobial or antibiotic effect, it has been recognized as a possible alternative to replace the AGP in swine feed. The EO which have potential to be used as antibiotic alternative in swine feed are carvacrol, thymol, cinnamaldehyde and citral while their effectiveness has been studies *in vivo* and in vitro. According to the previous studies, EO containing phenolic compounds (thymol, carvacrol and

eugenol) show strong antimicrobial activity than those containing other chemical structures such as aldehydes and terpenes (De Lange et al., 2010; Omonijo et al., 2018). Indeed, the combination of EO is more effective than individual EO due to their synergistic properties. Thymol and cinnamaldehyde are most commonly used as a combination. The results from *in vivo* study confirmed that blend of thymol and cinnamaldehyde had positive effects on the composition of gut microbiota, immunity status and growth performances in weaned piglets. The beneficial effect on gut microbiota was indicated by increased *Lactobacillus* counts and reduced *E. coli* counts in faces. Furthermore, increased level of phagocytosis rate, immunoglobulins and blood immune metabolites together with reduced diarrhoea index confirmed the favourable effect of combined EO in overall health in weaned piglets (Li et al., 2012).

1.4.3. Citral

Citral (3,7-dimethyl-2,6-octadienal) is an unsaturated aldehyde, mainly consists of a mixture of two monoterpene aldehydes namely neral (α-citral) and geranial (β-citral) (Subramaniyan and kumar Natarajan, 2017; Tamer et al., 2019). Unlike other EOs such as thymol and carvacrol, citral is a non-phenolic EO (Baschieri et al., 2017). It is a key compound of EO extracted from lemon scented herbal plants such as lemon myrtle, and lemongrass. This aromatic compound is widely used in food and cosmetic industries. The biological properties of citral include antibacterial, antifungal, antioxidant, insecticidal, anti-inflammatory and anticancer effects (Bouzenna et al., 2017; Subramaniyan and kumar Natarajan, 2017). The antibacterial property of citral has been confirmed *in vivo* and *in vitro* studies. Indeed, studies done on their potential applications in animal production is limited. To the best of our knowledge, no study was conducted to find the effect of citral in swine production. A previous research found that encapsulated citral alone or in combination with cinnamaldehyde significantly increased body weight of broiler chicken and reduced mortality. In the same study, increased *lactobacillus* and decreased *Enterococcus* and *Clostridium* were observed in ceca of citral supplemented chicken showing its potential benefits in improving gut health (Yang et al., 2020). In *in vitro* study, it has been reported that citral is effective against *E. coli* 0157:H7, *S. typhimurium*, *L. monocytogenes*, and *V. vulnificus* and the effect was dose dependent. The complete inhibition of *E. coli* 0157:H7 and *V. vulnificus* was observed at the concentrations of 500 µg/mL and 100 µg/mL respectively (Kim et al., 1995).

The antioxidant activity of citral also has been investigated in different experimental models including cell culture. The antioxidant property of citral arises from the presence of aldehydes. Citral protects oxidizable substrate from being oxidation by react with peroxyl radicals. In the oxidation process, the rate of self-termination and cross-termination of terpenoids are higher than oxidizable substrate. Therefore, chain termination process is increased and efficiency of autooxidation is reduced. However, the antioxidant activity of non-phenolic EO is different from chain breaking antioxidant and they act as terminating-enhancing antioxidant. The three major different aspects of these non-phenolic antioxidants are (i) antioxidant capacity is less than that of phenols and therefore requires higher concentrations (ii) effect has no linear relationship with concentrations, (iii) effectiveness depends on the chain termination rate of the oxidizable substrates, where being higher for substrates that have more modest chain -termination (Baschieri et al., 2017). Previously, it has been shown that antioxidant mechanism of citral is associated with the inhibition of lipid peroxidation and restoration of endogenous antioxidant defensive system. In intestinal epithelial cell line (ICE-6), citral significantly reduced aspirin induced oxidative stress by decreasing MDA level and increasing GSH level *in vitro* (Bouzenna et al., 2017). Furthermore, citral was found to protect human umbilical vein endothelial cells against oxidative damage induced by H_2O_2 by enhancing total antioxidant capacity and reducing hydroperoxides production (Sitral and Korur, 2010). Owning to strong antimicrobial and antioxidant effects is a good indicator which shows that citral has a potential to be used as antibiotic alternative in animal production, However, further experiments are needed to confirm the relevant properties.

1.4.4. Cinnamaldehyde

Cinnamaldehyde (CA) is an aromatic aldehyde and the major compound found in cinnamon EO which is obtained from the bark and leaves of trees from genus Cinnamomum (López-Mata et al., 2018). The cinnamon obtained from the bark is a spice and commonly used as aromatic and flavouring agent in the food and beverages. Interestingly, CA is listed as GRAS (generally recognized as safe) and approved by FDA to be used in foods (Food and Drug Administration). Moreover, CA isolated from cinnamon EO has a wide array of applications in medical products, cosmetics and perfumes (López-Mata et al., 2018; Doyle and Stephens, 2019).

In pigs, there is an interest in using CA as an antibiotic alternative in feed. The effects of CA on gut microbiota, growth performance, and health status of pigs have been shown in previous studies

(Michiels et al., 2007; Li et al., 2012; Blavi et al., 2016; Jiménez et al., 2020). CA has a greater antibacterial activity in the small intestine than in the stomach and caecum (Michiels et al., 2005). As shown in *in vitro* studies, the antibacterial activity of CA against porcine intestinal anerobic bacteria was more strong than other EOs namely carvacrol, thymol, eugenol, due to its strong inhibitory activity against coliform bacteria and *E coli* bacteria. Thus, CA is effective to be used to control pathogenic bacteria in pigs. Furthermore, CA has shown less or no inhibitory activity against lactobacilli and streptococcus (Michiels et al., 2005). Therefore, application of CA improves intestinal health through increasing beneficial bacteria and reducing the number of detrimental bacteria (Michiels et al., 2005; Michiels et al., 2007; Yan and Kim, 2012). The improvement of the immunity in pigs fed with CA supplemented diet is also associated with its beneficial effects on intestinal microbial composition (Yan and Kim, 2012).

Besides antimicrobial effect, CA exhibits various beneficial effects. Maternal exposure to flavours from plant extracts in sow diet affects the feed preference of weaned piglets. It was found that adding a blend of flavors from anethol, cinnamaldehyde, and eugenol in sow diet increased feed intake and daily body weight gain of piglets after weaning (Blavi et al., 2016). This result shows importance of CA as a sensory additive in conditioning pigs after weaning. Furthermore, CA has been demonstrated to have anti-tumor, anti-inflammatory and antioxidant activities. In cultured monocytes/macrophages, CA suppressed LPS-induced production of TNF, IL-6 and IL-1, indicating its anti-inflammatory activities(ref). Although CA could inhibit the production inflammatory mediators, it is not an LPS antagonist which could interfere LPS binding to cell surface. Moreover, CA has shown no effect on the expression of LPS recognising molecules namely TLR4 and CD14 (Chao et al., 2008).

Due to the synergistic activity, the antimicrobial effect of combination of EO or EO with organic acids are greater than their individual effects (Li et al., 2012; Jiménez et al., 2020). Li *et al.*, (2012) observed that feeding a blend of thymol and cinnamaldehyde improved feed intake, growth rate, feed conversion ratio, and immunity of weaner piglets. Moreover, it was found that EO containing thymol and cinnamaldehyde modulated gut microbiota and reduced the incidence of diarrhea by 50% in weaner pigs. The protective effect of the blend of CA and organic acid on intestinal health of *E.coli* challenged piglets was reported by Jiménez *et al.,* (2020) as demonstrated by reduced intestinal oxidative stress, higher ratio of villus height to crypt depth and increased intestinal cell

proliferation. In *in vitro* study, it was found that the combination of thymol and cinnamaldehyde improve tight junction integrity in Caco-2 cells (Jiménez et al., 2020). Importantly, CA did not show any toxic effect on gut epithelial cells up to the concentration of 50 mg/L (Putaala et al., 2017).

Antioxidant activity of CA is attributed to the phenolic and polyphenolic substances (Keshvari et al., 2013). As reported by Suryant *et al.*, (2018) CA possessed moderate antioxidant activity while its derivatives such as cinnamic acid, methyl cinnamate, and methyl-3-(2-hydroxy-5-nitrophenyl amino)-3-phenylpropanoate exerted higher antioxidant activities comparable to vitamin E. The study which was conducted to evaluate the antioxidant activity of EOs revealed that cinnamon EO exhibit strong DPPH radical scavenging activity, reducing capacity and superoxide anion scavenging activity which show its beneficial effect in neutralization of free radicals (Nanasombat and Wimuttigosol, 2011). As reported by Chao et al., (2008), CA reduced LPS-induced ROS production in monocytes/macrophages showing its antioxidant activity at cellular level.

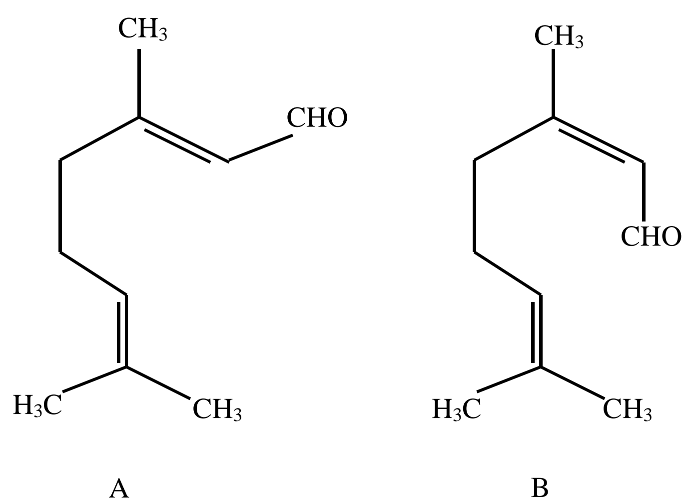


Figure 1.3. The chemical structures of citral: A.geranial and B. neral

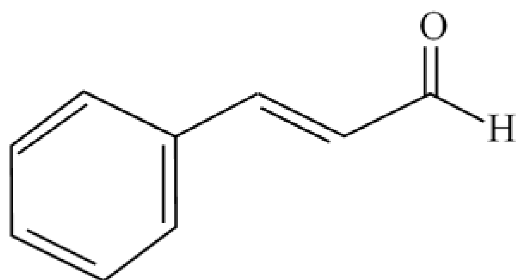


Figure 1.4. The chemical structure of cinnamaldehyde

CHAPTER II

HYPOTHESIS AND OBJECTIVES

2.1. Rationale

Intestinal infection of *E. coli* causes oxidative stress in swine. Oxidative stress is associated with the impairment of production and reproduction performances in animals. Traditionally, antibiotic growth promoters (AGP) are used in swine feed to control pathogenic infections and promote growth performances. Recently, usage of AGP in feed has been restricted in many countries including Canada due to safety concerns and as a precaution measurement. This restriction has put pressure on swine industry to look for alternatives. The potential usage of essential oils (EO) to act as antimicrobial compound has been investigated in many studies. However, the potential antioxidant property of those EO remains to be examined.

2.2. Hypothesis

- Lipopolysaccharide (LPS) could induce oxidative stress in intestinal epithelial cells.
- Essential oils: citral and cinnamaldehyde could attenuate LPS-induced oxidative stress in intestinal epithelial cells.

2.3. Objectives

The overall research objective was to investigate the protective effects of EO on endotoxin-induced oxidative stress in intestinal epithelial cells.

2.4. Specific aims

- To investigate the effect of LPS on oxidative stress in intestinal epithelial cells.
- To investigate the potential antioxidant effect of essential oils (citral and cinnamaldehyde) on LPS-induced oxidative stress in intestinal epithelial cells.

CHAPTER III

MATERIALS AND METHODS

3.1. Preparation of chemicals

Lipopolysaccharide (LPS) from *E.coli* O111:B4, citral and cinnamaldehyde were purchased from Sigma-Aldrich (Oakville, ON, Canada). LPS was dissolved in sterilized phosphate buffered saline (PBS) to prepare main stock (10 mg/mL) and stored in -20 °C until use. In the experiments, LPS was freshly prepared in cell culture medium. Both citral and cinnamaldehyde were dissolved in dimethyl sulfoxide (DMSO) and applied at the concentration of less than 0.1% (v/v) in cell culture medium. A combined application of citral and cinnamaldehyde was decided based on the initial experiments. Both citral and cinnamaldehyde were diluted up to 12.5 μM and blended at the ratio of 1:1.

3.2. Cell culture

The human intestinal epithelial cell line Caco-2 was purchased from the American Type Culture Collection (ATCC, Rockville, USA). Intestinal Caco-2 cells were cultured in dulbecco's modified eagle medium (DMEM), supplemented with 10% (*v/v*) fetal bovine serum at 37 °C and 5% CO_2. The basic culture was continued in 100-mm cell culture plates and split at the confluence of 90-95%, using 0.25% trypsin-EDTA (1×). Depending on the assay, cells were cultured in 6-well plates, 60 mm plates or 96-well plates. When cells reached 90-95% confluence, cells were washed with PBS and used for different treatments. To induce oxidative stress, Caco-2 cells were incubated with various concentrations (1, 5 and 10 μg/mL) of LPS for different time periods. To estimate the protective effect of essential oils on LPS-induced oxidative stress, cells were preincubated with citral, cinnamaldehyde or their combination for 30 minutes, followed by the LPS treatment for 4 hours. Oxidative stress related parameters were determined.

3.3. Cell viability assay

The effect of LPS on Caco-2 cell viability was determined using a colorimetric assay: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In brief, Caco-2 cells were plated in a 96-well plate at a concentration of 1×10^4 cells/well and incubated for 24 hours. The cells were then treated with different concentrations (1-100 μg/mL) of LPS for another 24 h. The yellow tetrazolium MTT was added to each well to yield a final concentration of 100 μM. After 4 h of incubation, the supernatant was aspirated and the MTT formazan was extracted in dimethyl sulfoxide (DMSO). The absorbance was measured at 540 nm using a SpectraMax M5 microplate reader.

3.4. Determination of lipid peroxidation

Malondialdehyde (MDA), a stable end-product of lipid peroxidation was measured using the thiobarbituric acid reactive substances (TBARS) assay. Following the treatments by LPS, citral and / or cinnamaldehyde, cells were washed with cold PBS and centrifuged at 5000g for 5 min. The cell pellets were then dissolved in cold PBS and sonicated. The cell lysates were suspended in 10%(w/v) phosphotungstic acid and incubated for 10 min at room temperature (RT) to precipitate the lipids. Following the centrifugation at 1000g for 10 min at RT, the cell pellets were collected. The cell pellets were then incubated for 1 hour at 90ºC with 0.67% (w/v) thiobarbituric acid. After the incubation, the samples were cooled, and color was extracted in n-butanol. The color extraction was facilitated by centrifugation at 1000g for 5 min and absorbance was measured at 532 nm wavelength. A concentration series of MDA was used as the standard. The protein content of the cell lysate was measured using Bradford method and used in normalization of TBARS value.

3.5. Determination of intercellular reduced glutathione

Intercellular reduced glutathione (GSH) level was measured by colorimetric method. Briefly, cells were washed with cold PBS followed by centrifugation at 5000g for 5 min. Cell pellets were suspended in cold PBS and sonicated. Sulphosalicyclic acid (10%) was added to cell homogenate at the ratio of 1:2 and centrifuged at 5000g for 10min at 4 °C. The supernatant was used in GSH measurement. In a 96 well plate, 40 µL of supernatant, 150 µL of 0.2 M sodium phosphate buffer and 10 µL of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were added and incubated for 10-15 min at RT. Absorbance was measured at 412 nm wavelength. A concentration series of GSH was used as the standard. The protein content of the cell lysate was measured using Bradford method and used in normalization of GSH value.

3.6. Determination of superoxide dismutase activity

Intercellular superoxide dismutase (SOD) activity was measured by a spectrophotometric detection method based on the inhibition rate of xanthine oxidase-mediated reduction of cytochrome c in cell lysate (Sarna et al., 2010). In brief, cell pellets collected after the treatments were homogenized in 0.05 M potassium phosphate buffer containing 100 mM EDTA (pH 7.8). Homogenate was centrifuged at 5000g for 5 min and supernatant was used for the assay. Prior to the enzymatic reactions of the samples, calibration was performed to determine the volume of

cytochrome c and xanthine oxidase. A mixture of 0.25 mL of 0.15 mM cytochrome c, 0.15 mL of 2 mM xanthine, and 0.5 mL of homogenization buffer were added into a cuvette and placed in the spectrophotometer. A 5-8 μL of diluted xanthine oxidase was added to the cuvette and the rate of increase in absorbance was recorded at 550 nm wavelength at every 15 sec for 3 min. The volume of cytochrome c and xanthine oxidase was adjusted until the rate of change in absorbance reached to 0.05 per minute. These calibrated volumes of cytochrome c and xanthine oxidase were used in enzymatic reactions.

The calibrated volume of cytochrome c, 0.15 mL xanthine, 40 μL supernatant and reaction buffer as required to make up the total volume up to 0.9 mL were added into a cuvette at RT. The cuvette was then placed in spectrophotometer and calibrated amount of xanthine oxidase was added to initiate the reaction. The rate of change in absorbance was measured at 550 nm wavelength and recorded at every 15 sec for 3 min. Two concentrations of SOD as to yield 50% decrease in the rate of absorbance (IU activity) was used as the standard. Calculations were performed according to a previous study (Amarakoon, 2017) and protein concentrations of the supernatants measured by Bradford method were used to normalize SOD activities in the samples.

3.7. Statistical analysis

Results were analyzed using two-tailed Student's t-test. *P* values less than 0.05 were considered statistically significant. Results were expressed as mean ± standard error (SE). The number of replicates per treatment varies in between 3-26.

CHAPTER IV

RESULTS

4.1. Effect of lipopolysaccharide on viability of Caco-2 cells

The effect of LPS on cell viability was examined by MTT assay. LPS treatment (1–100 µg/mL) did not affect the viability of Caco-2 cells (Fig. 4.1).

4.2. Induction of oxidative stress by lipopolysaccharide in intestinal epithelial cells

Effect of lipopolysaccharide on lipid peroxidation

To evaluate the effect of LPS on oxidative stress, intercellular MDA level was measured using TBARS assay. Caco-2 cells were incubated with LPS for 4, 24 and 48 hours. According to the data, MDA level in the cells increased with an elevation of the LPS concentration for 4 hours (Fig. 4.2A). Similarly, MDA levels increased in LPS-treated cells, dose dependently for 24 and 48 hours (Fig. 4.2B, and Fig. 4.2C). The lowest concentration of LPS which could induce lipid peroxidation in Caco-2 cells is 1µg/mL. This concentration was selected for further experiments. As shown in Fig. 4.1D, significant effect of LPS (1µg/mL) on MDA level started at 2 hours and peaked at 12 hours. Then it decreased and remained until 48 hours (Fig. 4.2D).

MDA is used as a biomarker for oxidative stress. Therefore, these results suggest that LPS induce oxidative stress in Caco-2 cells. Such an effect was dose and time dependent.

Effect of lipopolysaccharide on reduced glutathione (GSH)

To investigate the effect of LPS on non-enzymatic antioxidant in Caco-2 cells, intercellular GSH level was measured. As shown in Fig. 4.3A, GSH level in the cells decreased with increasing LPS concentration for 4 hours incubation time. Further, the lowest concentration of LPS which could significantly reduce GSH level in Caco-2 cells is 1µg/mL.

These results suggest that LPS induce oxidative stress in intestinal epithelial cells might be mediated through the depletion of GSH.

Effect of lipopolysaccharide on superoxide dismutase (SOD) activity

To determine the effect of LPS on enzymatic antioxidant in Caco-2 cells, intercellular SOD activity was measured. As shown in Fig. 4.3B, SOD activity in the cells decreased with an elevation of LPS concentration for 4 hours incubation time. Unlike for MDA and GSH level, significant effect

of LPS on SOD activity was not observed at the concentration of 1µg/mL. The lowest concentration of LPS which could significantly reduce SOD activity in Caco-2 cells was 5 µg/mL.

These results suggest that LPS induce oxidative stress in intestinal epithelial cells through the depletion of antioxidant enzyme SOD.

4.3. Effect of citral on lipopolysaccharide-induced oxidative stress in intestinal epithelial cells

Caco-2 cells were incubated with LPS to induce oxidative stress. The MDA value was significantly increased in the LPS (1µg/mL) treated cells. Pretreatment with citral at the concentrations of 12.5 and 25 µM significantly lowered the MDA level in LPS-treated cells. Interestingly, citral reduced MDA level to the base level at the concentration of 25 µM (Fig. 4. 4). As shown in Fig. 4.5, the GSH level was significantly decreased in LPS (1µg/mL) treated cells. Such inhibitory effect was reversed by citral at the concentration of 25 µM. As to SOD activity in the cells, the inhibitory effect was observed at the concentration of 5µg/mL of LPS. However, preincubation with citral (25 µM) restored SOD activity in LPS-treated cells (Fig. 4.6).

These results suggested that inhibitory effect of citral on LPS-induced oxidative stress in intestinal epithelial cells might be mediated via inhibition of lipid peroxidation and restoration of endogenous antioxidants namely GSH and SOD.

4.4. Effect of cinnamaldehyde on lipopolysaccharide-induced oxidative stress in intestinal epithelial cells

Caco-2 cells were incubated with LPS to induce oxidative stress. As shown in Fig. 4.7, MDA level in Caco-2 cells significantly increased with LPS (1µg/mL) treatment and all concentrations (5, 12.5, and 25 µM) of cinnamaldehyde (CA) tested reduced intercellular MDA level. Importantly, CA at the concentration of 12.5 µM completely abolished LPS-induced MDA production. Furthermore, cells incubated in the presence of LPS (1µg/mL) showed significant reduction in GSH level. Pretreatment with CA (12.5 µM) significantly restored the GSH level in LPS-induced cells (Fig. 4.8).

These results indicate that protective effect of CA on LPS-induced oxidative stress in gut epithelial cells might be mediated through the inhibition of lipid peroxidation and restoration of GSH.

4.5. Effect of combination of citral and cinnamaldehyde on lipopolysaccharide-induced oxidative stress in intestinal epithelial cells

Caco-2 cells were incubated with LPS for 4 hours to induce oxidative stress. According to the data reported on effect of citral and cinnamaldehyde (CA) alone on LPS-induced MDA production in Caco-2 cells, both essential oils showed a significant inhibitory effect at the concentration of 12.5 µM. Therefore, it was decided to use 12.5 µM concentration of both essential oils at 1:1 ratio to determine the combined effect. According to the results, cells incubated with LPS at the concentration of 1µg/mL significantly increased MDA level and decreased GSH level. Pretreatment with the combination of citral and CA effectively reduced the MDA level and restored GSH level in Caco-2 cells (Fig.4.9A and Fig. 4.9B).

Results suggest that combination of essential oils (citral and CA) protect intestinal epithelial cells against LPS-induced oxidative stress through the inhibition of lipid peroxidation and restoration of GSH.

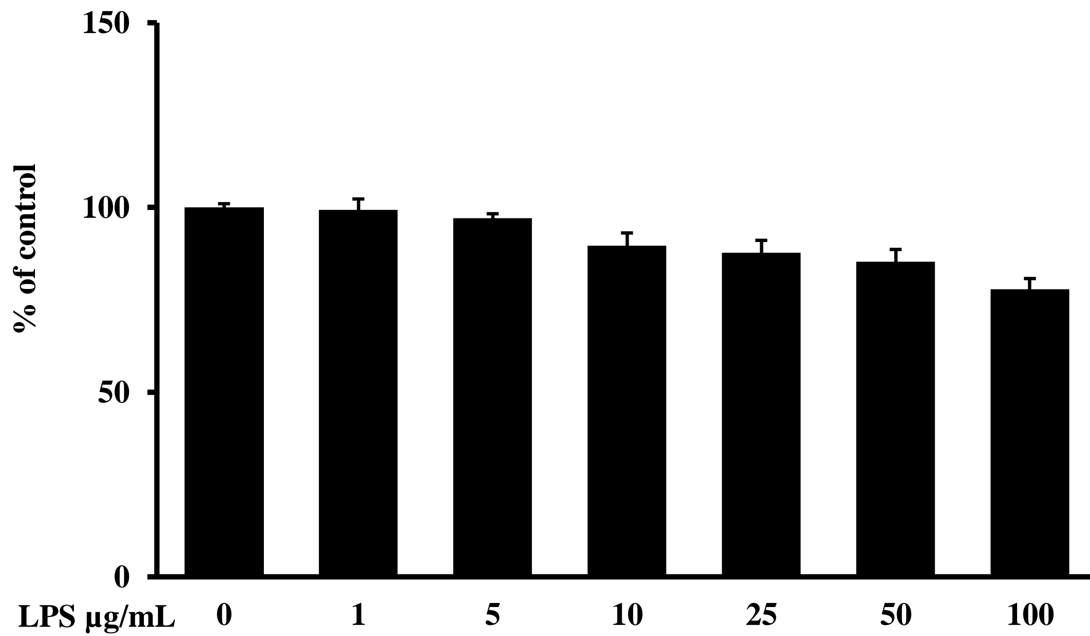
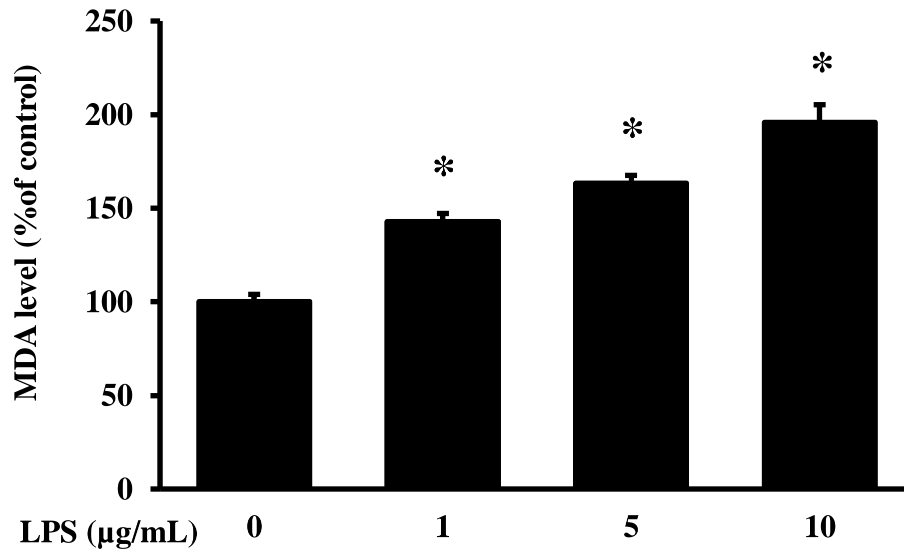


Figure 4.1. Effect of lipopolysaccharide on cell viability of Caco-2 cells

Caco-2 cells were incubated with lipopolysaccharide (LPS) (1-100 µg/mL) for 24 h. Cell viability was assessed using the MTT assay. Cells incubated without LPS was considered as control. Cell viability for control cells was arbitrarily expressed as 100. Results are expressed as means ± SE. *, Significant at $P < 0.05$ compared with control.

A



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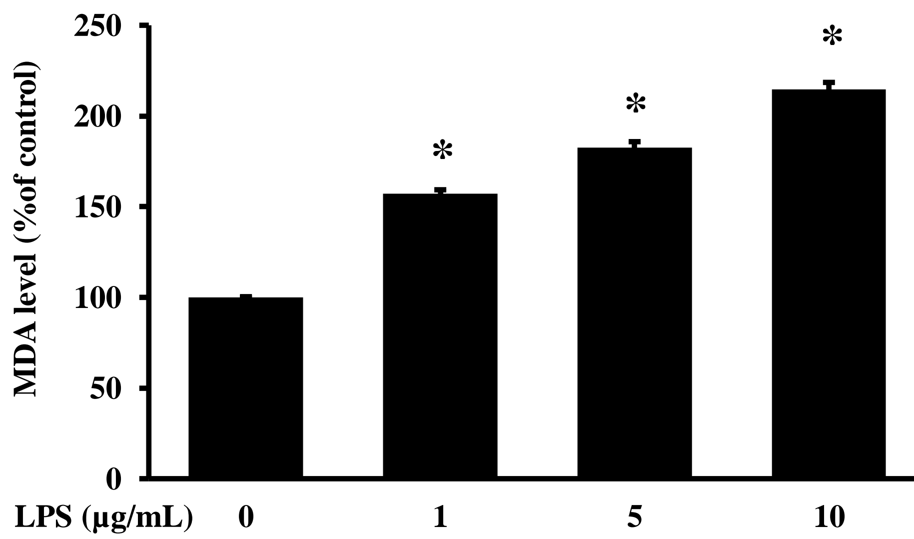

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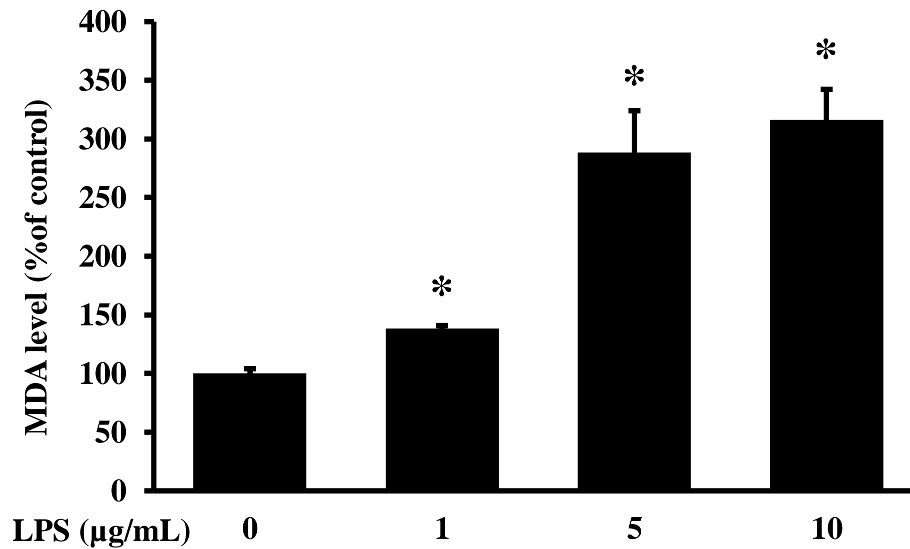

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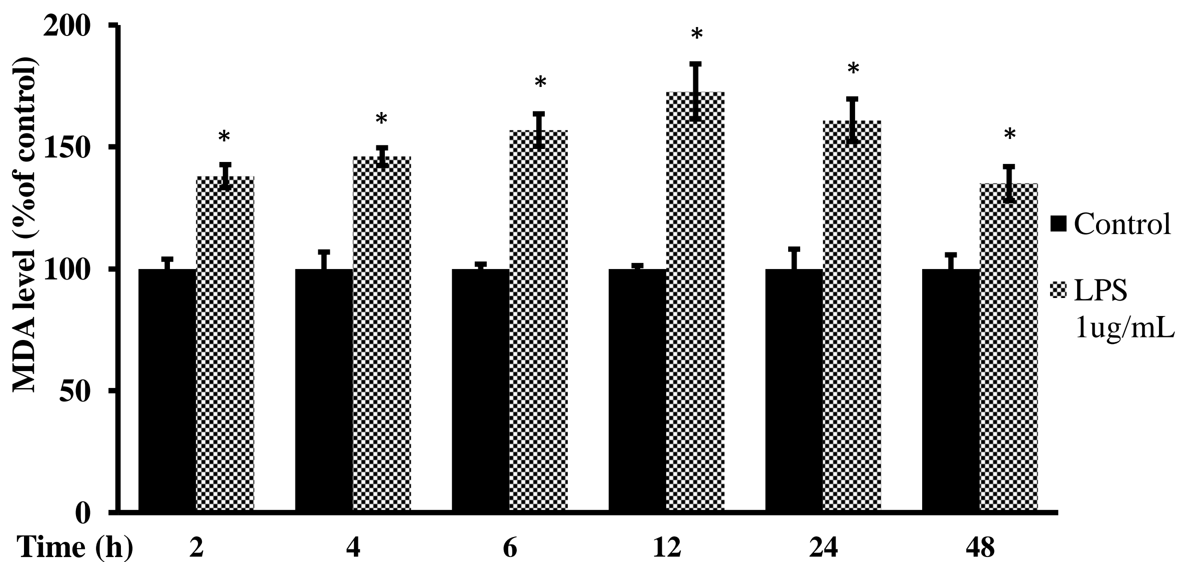
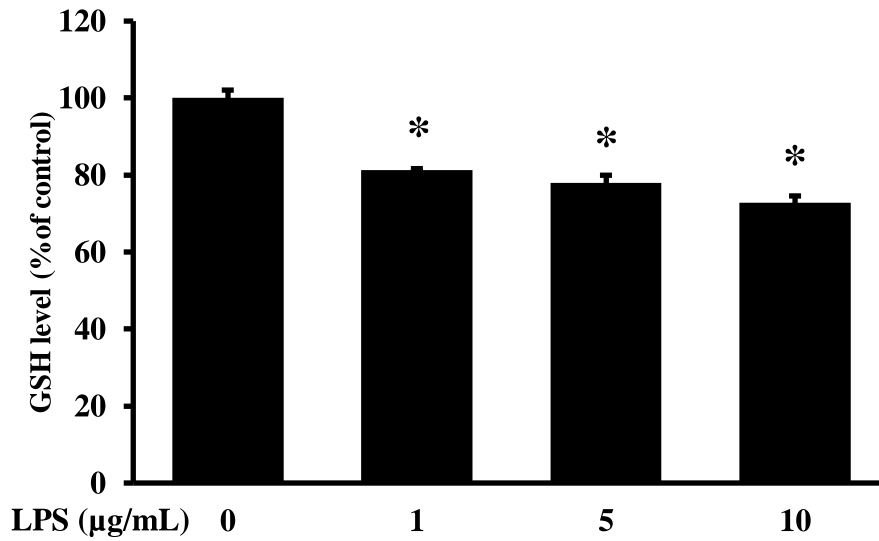


Figure 4.2. Effect of lipopolysaccharide on the malondialdehyde production in Caco-2 cells. Caco-2 cells were incubated in the absence (control) or presence of lipopolysaccharide (LPS) (1, 5, and 10 μg/mL) for **(A)** 4h, **(B)** 24h and **(C)** 48h. **(D)** cells were incubated with LPS at the concentration of 1μg/mL for various time points. Intercellular malondialdehyde (MDA) level was measured by thiobarbituric acid reactive substance (TBARS) assay. The MDA level in control cells was arbitrarily expressed as 100. Results are expressed as means ± SE. *P<0.05 compared with the control cells.

A



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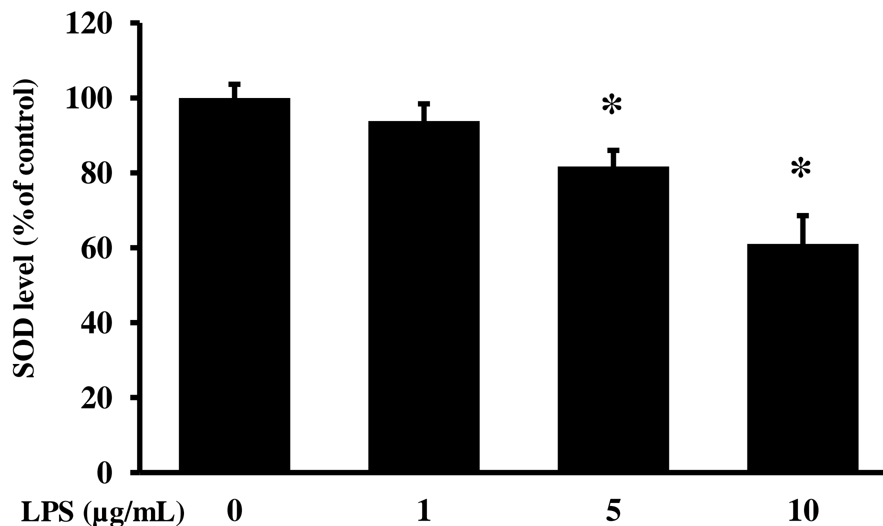


Figure 4.3. Effect of lipopolysaccharide on reduced glutathione and superoxide dismutase activity in Caco-2 cells.

Caco-2 cells were incubated in the absence (control) or presence of lipopolysaccharide (LPS) (1, 5, and 10 µg/mL) for 4 hours. (A) intercellular reduced glutathione (GSH) level and (B) superoxide dismutase (SOD) activity were measured in each group. The GSH level and SOD activity in control cells was arbitrarily expressed as 100. Results are expressed as means ± SE. *P<0.05 compared with the control cells.

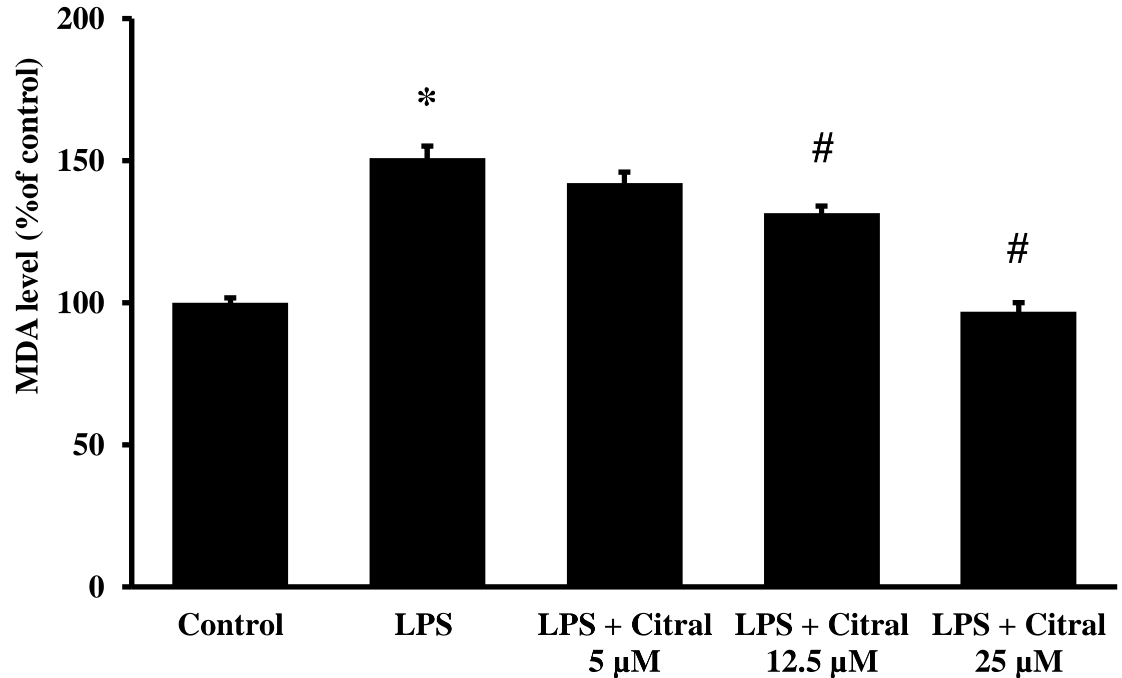


Figure 4.4. Effect of citral on lipopolysaccharide-induced malondialdehyde production in Caco-2 cells.

Caco-2 cells were preincubated with citral at the concentrations of 5, 12.5 and 25 µM for 30 min followed by 4 h incubation in the presence of lipopolysaccharide (LPS) (1 µg/mL). Cells incubated without citral or LPS was considered as control. Intercellular malondialdehyde (MDA) levels was measured by thiobarbituric acid reactive substance (TBARS) assay. The MDA level in control cells was arbitrarily expressed as 100. Results are expressed as means ± SE. *$P<0.05$ compared with the control cells, #, $P < 0.05$ compared with LPS-treated cells.

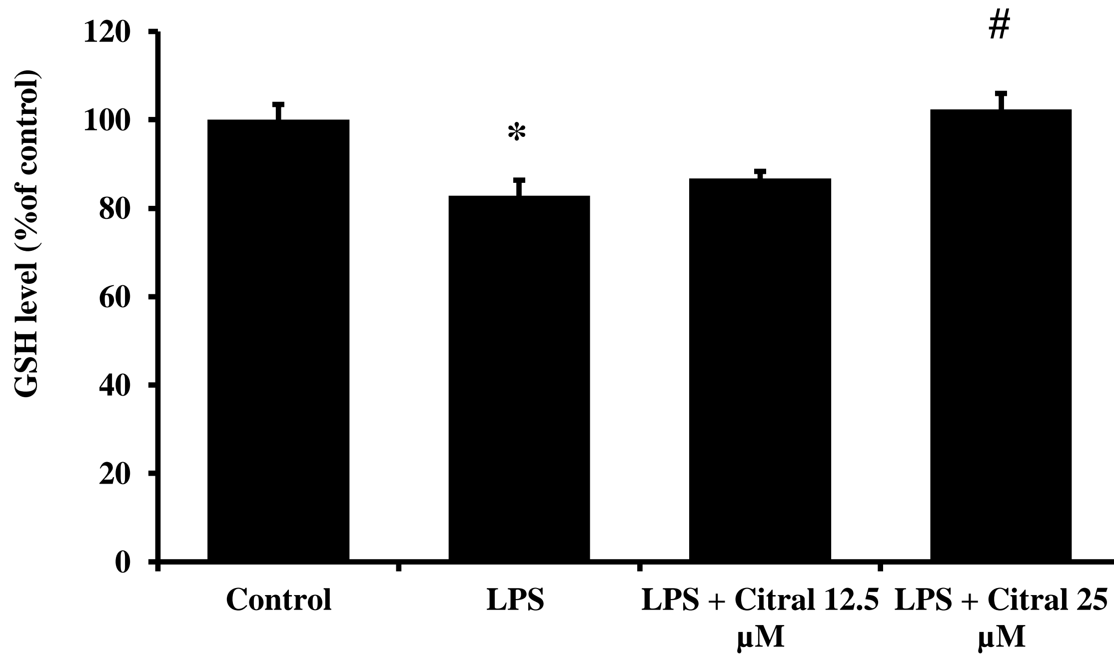


Figure 4.5. Effect of citral on lipopolysaccharide-induced reduced glutathione in Caco-2 cells

Caco-2 cells were preincubated with citral at the concentrations of 12.5 and 25 µM for 30 min followed by 4 h incubation in the presence of lipopolysaccharide (LPS) (1 µg/mL). Cells incubated without citral or LPS was considered as control. Intercellular reduced glutathione (GSH) level was measured in each group. The GSH level in control cells was arbitrarily expressed as 100. Results are expressed as means ± SE. *P<0.05 compared with the control cells, #P< 0.05 compared with LPS-treated cells.

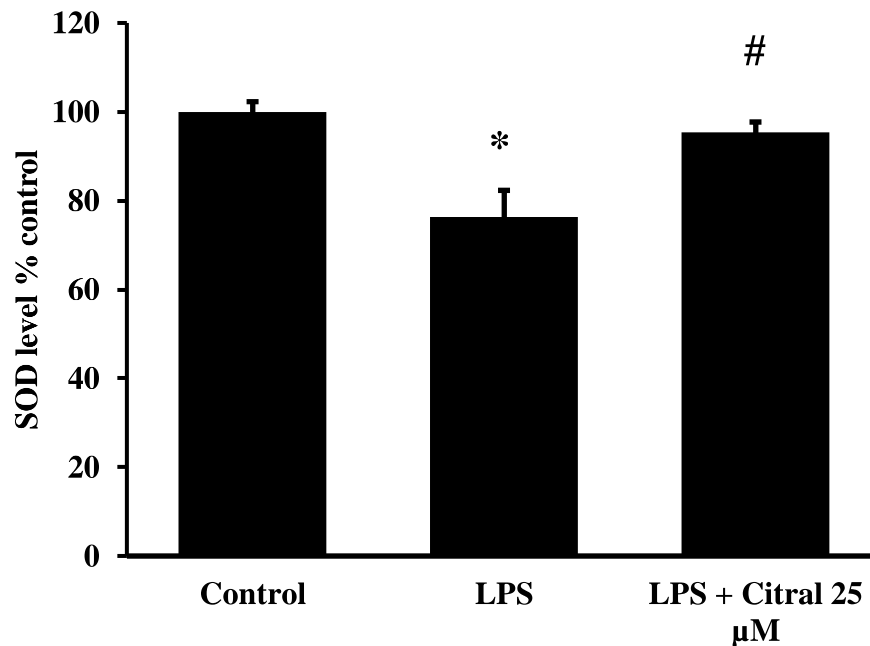


Figure 4.6. Effect of citral on lipopolysaccharide-induced superoxide dismutase activity in Caco-2 cells

Caco-2 cells were preincubated with citral (25 µM) for 30 min followed by 4 h incubation in the presence of lipopolysaccharide (LPS) (5 µg/mL). Cells incubated without citral or LPS was considered as control. Intercellular superoxide dismutase (SOD) activity was measured in each group. The SOD activity in control cells was arbitrarily expressed as 100. Results are expressed as means ± SE. *P<0.05 compared with the control cells, #P< 0.05 compared with LPS-treated cells.

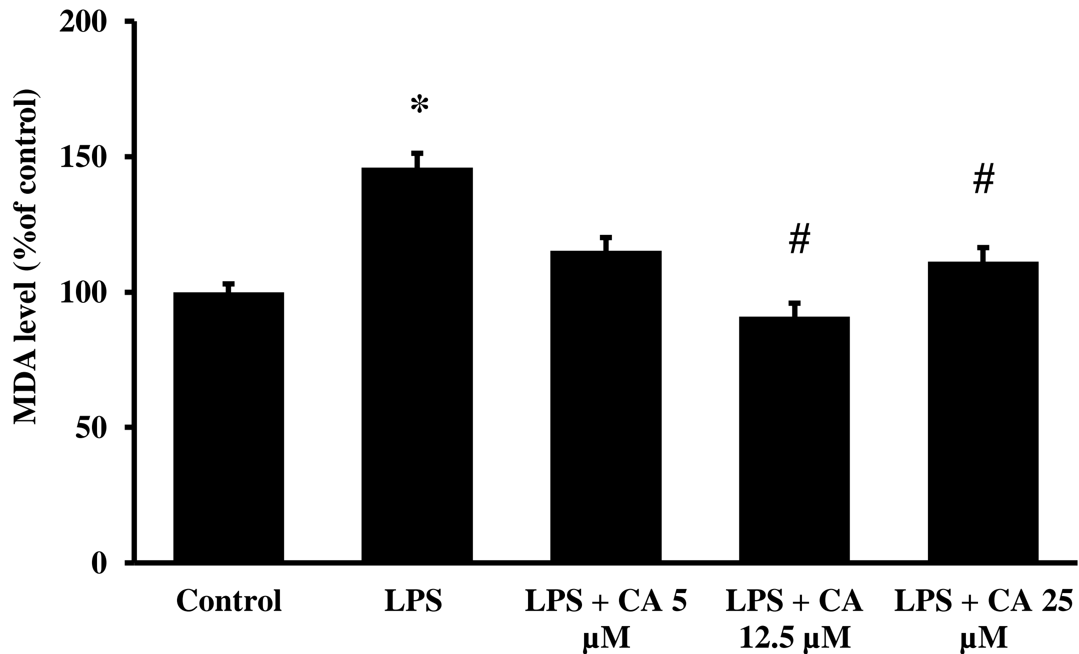


Figure 4.7. Effect of cinnamaldehyde on lipopolysaccharide-induced malondialdehyde production in Caco-2 cells.

Caco-2 cells were preincubated with cinnamaldehyde (CA) at the concentrations of 5, 12.5 and 25 µM for 30 min followed by 4 h incubation in the presence of lipopolysaccharide (LPS) (1 µg/mL). Cells incubated without CA or LPS was considered as control. Intercellular malondialdehyde (MDA) level was measured by thiobarbituric acid reactive substance (TBARS) assay. The MDA level in control cells was arbitrarily expressed as 100. Results are expressed as means ± SE. *P<0.05 compared with the control cells, #P< 0.05 compared with LPS-treated cells.

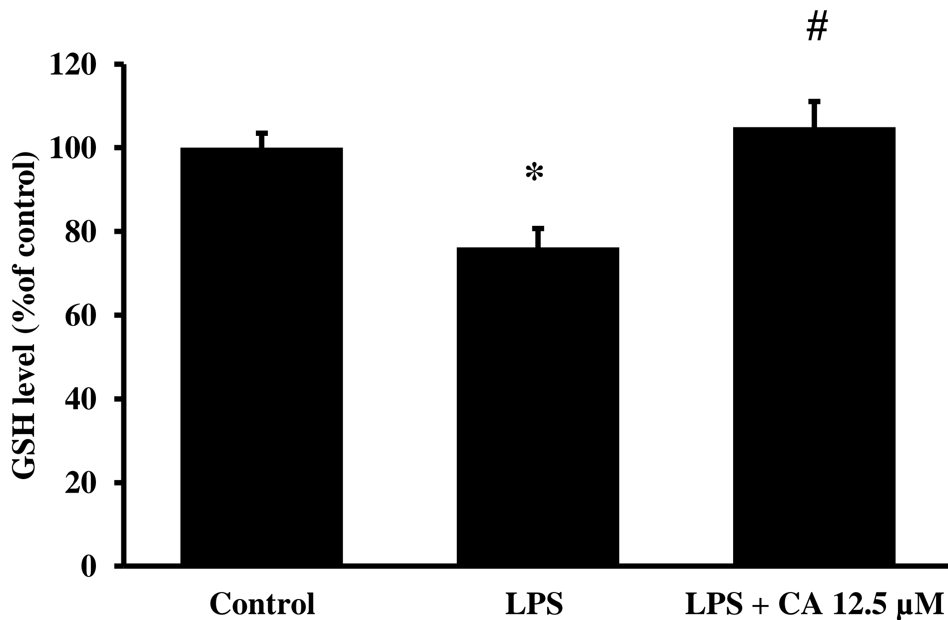
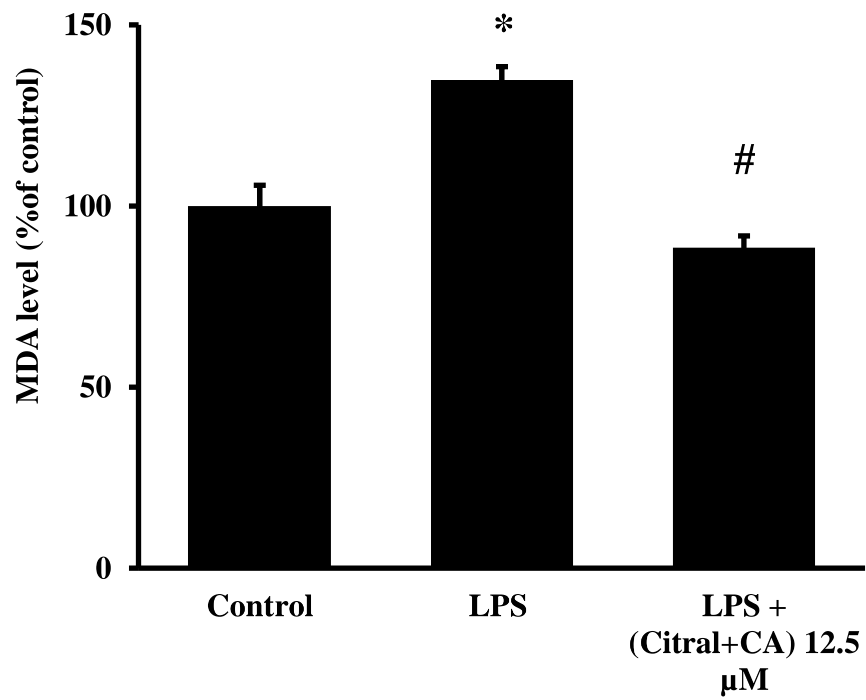


Figure 4.8. Effect of cinnamaldehyde on lipopolysaccharide-induced reduced glutathione in Caco-2 cells.

Caco-2 cells were preincubated with cinnamaldehyde (CA) at the concentration of 12.5 µM for 30 min followed by 4 h incubation in the presence of lipopolysaccharide (LPS) (1 µg/mL). Cells incubated without CA or LPS was considered as control. Intercellular reduced glutathione (GSH) level was measured in each group. The GSH level in control cells was arbitrarily expressed as 100. Results are expressed as means ± SE. *P<0.05 compared with the control cells, #P< 0.05 compared with LPS-treated cells.

A



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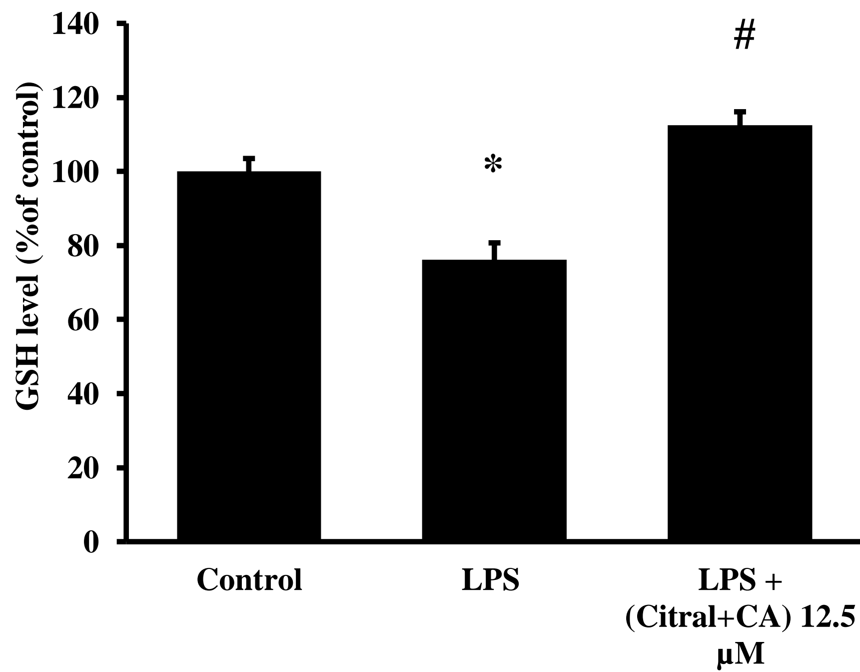

Figure 4.9. Effect of combination of citral and cinnamaldehyde on lipopolysaccharide-induced malondialdehyde production and reduced glutathione in Caco-2 cells

Caco-2 cells were preincubated with the combination of citral and cinnamaldehyde (CA) at the concentrations of 12.5 µM (1:1 ratio) for 30 min followed by 4 h incubation in the presence of lipopolysaccharide (LPS) (1 µg/mL). Cells incubated without citral+CA or LPS was considered as control. **(A)** intercellular malondialdehyde (MDA) level was measured by thiobarbituric acid reactive substance (TBARS) assay. **(B)** Intercellular reduced glutathione (GSH) level was measured in each group. Values in control cells were arbitrarily expressed as 100. Results are expressed as means ± SE. *$P<0.05$ compared with the control cells, #$P< 0.05$ compared with LPS-treated cells.

CHAPTER V

DISCUSSION

5.1. Induction of oxidative stress in intestinal epithelial cells by lipopolysaccharide

Intestinal epithelial cells play an important role in innate immune responses against pathogenic bacteria. In fact, these cells are continuously exposed to microbial derived compounds such as LPS, flagellin A and lipoteichoic acids from intestinal microbial community (Lotz et al., 2007). LPS, also known as an endotoxin, is one of the virulent factors employed by gram-negative bacteria to cause infections. Being a component of the outer membrane of cell wall of gram-negative bacteria, LPS acts as an immunoreactive surface antigen. Therefore, its involvement in host-pathogen interaction leads to initiate innate immune responses (Rietschel et al., 1993). Briefly, the pathogen recognition receptors (PRRs) present in the host cells detect the pathogen-associated molecular patterns (PAMPs). TLR4 is the key PPR which recognizes lipid A portion of LPS. The binding of LPS to TLR4 triggers the immune system (Ramachandran, 2014). LPS is released from the cell wall when bacteria are multiplying or disintegrating. Released LPS exhibits biological activities and shows similar symptoms like gram-negative bacteraemia. Furthermore, LPS in the lysate of dead bacteria also exerts pathophysiological effects including fever and hypertension (Rietschel et al., 1993). Therefore, LPS is considered as a well documented endotoxin to study bacterial infectious diseases (Adewole et al., 2016).

Endotoxin is widely applied in pigs as an experimental model to induce bacterial infections (Wyns et al., 2015). However, *in-vitro* studies to determine the effects of pathogens or infections on intestinal epithelial cells of pigs are restricted due to limited availabilities of porcine intestinal epithelial cell lines (Oswald, 2006). Therefore, human and murine intestinal cell lines are used instead. The Caco-2 cell line has been widely employed as a cell model for studying the small intestinal epithelium. These cells show morphological and functional characteristics that are similar to small intestinal enterocytes (Sambuy et al., 2005). Interestingly, they grow in monolayer, form brush border membrane with microvilli and show tight junctions between adjacent cells. Importantly, this cell line has been effectively used in oxidative stress related studies as they express different endogenous antioxidants and oxidative stress related signalling pathways (Wijeratne et al., 2005). Oxidative stress is the shift in the balance between production of free radicals (ROS and RNS) and ability of antioxidant system to detoxify them. In swine production, oxidative stress has negative impacts on animal performances, health status and well being. Therefore, it has been recently received much attention in studies to determine the factors that

affect oxidative stress and different interventions to overcome it. There is an array of nutritional and environmental factors affect the development of oxidative stress in the pigs. The major risk factors for oxidative stress include weaning, pathogenic infections, mycotoxins and high oxidant containing diet (Amarakoon, 2017).

In the present study, the impact of LPS on oxidative stress in intestinal epithelial cells (Caco-2 cells) was examined. Malondialdehyde (MDA), a stable product of lipid peroxidation was measured using TBARS assay and used as a biomarker of oxidative stress. The findings indicated that LPS derived from *E. coli* caused oxidative stress in Caco-2 cells, characterised by elevated level of intercellular MDA production. Such an effect was dose and time dependent. At the lowest concentration of 1µg/mL LPS, MDA concentration was about 1.5-fold higher compared with control cells at all the tested time points. This effect was an earlier response to endotoxins as increased lipid peroxidation started after 2 hours of the treatment and continued for 48 hours. LPS induce overproduction of both mitochondrial and intercellular ROS in gut epithelial cells. Excessive ROS damages cell components and negatively affects cellular biochemical process. Membrane lipids are highly susceptible to oxidation due to high content of polyunsaturated fatty acids. MDA are produced during lipid peroxidation caused by ROS (Phaniendra et al., 2015). A previous study also showed that MDA level in Caco-2 cell lysate increased with an increase in the LPS dosage (Wu et al., 2018). Another study showed that *E. coli* infection caused intestinal oxidative stress and thereby increased MDA level in the small intestine and serum in weaned piglets (Amarakoon, 2017). In the present study we used LPS derived from *E. coli* as virulent factor to induce the infection. These results demonstrate that LPS is one of the virulent factors causing oxidative stress during *E. coli* infection.

Endogenous antioxidant system includes enzymatic and non-enzymatic antioxidants which protect body against oxidative stress. The major enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) while non-enzymatic antioxidants include glutathione. In the present study, LPS significantly depleted reduced glutathione (GSH) level and SOD activity in Caco-2 cells. Such inhibitory effect showed a dose dependent correlation. The lowest concentration of LPS which exerted a significant inhibitory effect on GSH, and SOD were 1µg/mL and 5µg/mL respectively. These results suggested that the inhibitory effect of LPS on GSH was greater than that of SOD. Similar results were reported by Russo *et al.* (2012)

showing that LPS from *E. coli* serotype O127:B8 decreased GSH level in Caco-2 cells at the concentration of 1µg/mL.

Glutathione is the most abundant cellular thiol antioxidant, present in millimolar concentrations in the intestinal epithelial cells. In healthy cells, majority of them present in reduced from and it is oxidized to GSSH (oxidized glutathione) under the oxidative stress condition. Therefore, the ratio of GSH/GSSG is an useful indicator of oxidative stress where the ratio decreases with increased level of oxidative stress (Birben et al., 2012; Circu and Aw, 2012). LPS cause oxidative stress through the overproduction of ROS (Circu and Aw, 2012). Therefore, endogenous antioxidant system counteracts the oxidant effects through the participation in detoxification of ROS and ROS derived toxic molecules. GSH scavenges ROS through direct and indirect mechanisms (Espinosa-Diez et al., 2015). There are other redox related enzymes that are also involved in these mechanisms. In the process of H_2O_2 detoxification, GSH donates electrons to H_2O_2 and reduces to H_2O and O_2. This reaction is catalyzed by glutathione peroxidase (GSH-Px). Glutathione reductase (GR) reduces GSSG back to GSH by utilizing NADPH as an electron donor (Birben et al., 2012). In the current study, cellular GSH level decreased as they were involved in neutralizing ROS and oxidized to GSSG. Depletion of GSH level decreases GSH/GSSG ratio and causes the activation of inflammatory signalling pathways (Birben et al., 2012). Proinflammatory cytokines, mainly TNF-α, IL-1, and IL-6 exert negative effects on metabolic pathways which lead to reduce the feed intake and growth parameters of pigs (Webel et al., 1997).

SOD also plays an important role in oxidative stress as it detoxifies the most toxic ROS: superoxide anions. SOD catalyses the dismutation of O_2^- into O_2 and H_2O_2. The formed H_2O_2 is then reduced to H_2O by catalase and GSH-Px (Birben et al., 2012). In the present study, LPS caused a depletion of SOD activity in intestinal epithelial cells. Our results agreed with the previous studies (Wu et al., 2018; Xiong et al., 2019), which reported decreased SOD enzyme activity in LPS treated Caco-2 cells. This change might be a result of increased SOD utilization to neutralize excessive ROS. The depletion in both GSH and SOD can lead to an increase in lipid peroxidation in intestinal epithelial cells.

5.2. Effect of citral on lipopolysaccharide-induced oxidative stress in intestinal epithelial cells

Production of pro-inflammatory cytokines and ROS is the early response of host innate immune system to microbial infections. Increased production of ROS often occurs at the initial stage of the inflammation. Excessive ROS production not only causes oxidative stress but also stimulates the expression of pro-inflammatory cytokines, which lead to progression of inflammation. Therefore, inhibition of oxidative stress at the initial stage during infections may be an important mechanism to reduce the progression of inflammation and inflammation related diseases (Liang and Kitts, 2018). Oxidative stress in the gastrointestinal tract is associated with the pathogenesis of many gastrointestinal diseases such as IBD, celiac, intestinal ischemic injury and colon cancer in human (Pérez et al., 2017). In swine, oxidative stress cause poor growth performances, sepsis, endotoxemia, mulberry heart disease, porcine stress syndrome and poor reproductive performances (Amarakoon, 2017). In recent years, many countries including Canada have restricted use of antimicrobial growth promoters (AGP) to control pathogenic infections in animal production. Therefore, the use of plant-derived compounds to control infections in monogastric animals has received increasing attention. Essential oils (EO) are volatile compounds extracted from plants. They have been recognized as an alternative to AGP in swine feed. In the present study, we examined the effect of citral on LPS-induced oxidative stress in intestinal epithelial cells.

Our results indicated that citral dose dependently lowered lipid peroxidation in LPS-treated Caco-2 cells, as indicated by reduced MDA level. In the previous study, the protective effect of citral against lipid peroxidation was observed in the rat small intestine epithelial cells (Bouzenna et al., 2017). The ability of citral to attenuate lipid peroxidation is linked to its antioxidant capacity. Considering its chemical composition, aldehyde present in citral is associated with antioxidant property and shows direct as well as indirect antioxidant activities against ROS (Yang et al., 2013; Baschieri et al., 2017). In contrast to the chain breaking antioxidants, citral neutralizes ROS such as peroxyl radical through the participation in oxidation reactions as terminating enhancing antioxidant (Baschieri et al., 2017). The greater reducing power of citral allows it to donate hydrogen atoms to break free radical chain reaction (Bouzenna et al., 2017). Furthermore, antioxidative property of citral is associated with the inhibition of ROS and NO generation and depletion of $p47^{phox}$ protein level. The cytosolic subunit $p47^{phox}$ is required for the activation of superoxide producing enzyme, NADPH oxidase. Therefore, the inhibitory effect of citral on

p47^{phox} subunit may be responsible for its inhibitory effect on NADPH oxidase-mediated superoxide production (Yang et al., 2013).

Non-enzymatic antioxidant: GSH acts as a first line of defence against oxidative stress. Our results demonstrated that citral (25 uM) increased intracellular GSH level in the cells exposed to LPS. This finding agreed with the previous study that citral (0.5 µg/mL) increased GSH level in rat small intestine epithelial cells, compared to the cells exposed to aspirin (Bouzenna et al., 2017). Under the oxidative stress, GSH involves in removing ROS, through the reactions catalysed by GSH-Px and oxidized to GSSG. Glutathione reductase (GR) catalyzes the reduction of GSSG to GSH. Citral has been shown to restore antioxidant enzymes (SOD, CAT, GSH-Px and GR) under oxidative stress condition (Subramaniyan and kumar Natarajan, 2017). Therefore, the increased GSH might be due to efficient conversion of GSSG to GSH by increased enzymatic activities.

Nrf2 (nuclear factor erythroid 2-related factor 2) signaling pathway is activated under oxidative stress to enhance endogenous antioxidant defensive system. Nrf2 regulates the expression of an array of antioxidant enzymes including heme oxygenase-1 (HO-1), NAD(P)H dehydrogenase quinone 1 (NQO1), and SOD (Sun et al., 2020). In the present study, we demonstrated that citral (25uM) upregulated SOD activity in LPS-treated Caco-2 cells. The beneficial effect of citral on SOD activity might be linked to its ability to activate Nrf2 signalling pathway.

5.3 Effect of cinnamaldehyde (CA) on lipopolysaccharide-induced oxidative stress in intestinal epithelial cells

Gastrointestinal infections after weaning cause large economic losses in the pig industry. Therefore, maintaining gut health is important to improve production performances and overall health of animals as well as to reduce financial loss (Lalles et al., 2007). Dietary and management strategies are implied to improve gut health in swine production. *E. coli* , the most common pathogen involved in gastrointestinal infections elicits intestinal and systemic oxidative stress in piglets (Fouhse et al., 2016; Amarakoon, 2017). Oxidative stress is one of the critical factors associated with pathological conditions, reduced production and reproduction parameters in pigs. AGP are applied in swine feed to control bacterial infections, and improve growth performance (Omonijo et al., 2018). Recently, there is a growing interest to replace the use of antibiotics by

natural alternatives. Cinnamaldehyde (CA) has gained an interest as an antibiotic alternative because of its antimicrobial activities (Doyle and Stephens, 2019). However, its protective effect on intestinal epithelial cells has not been documented. Our results indicated that CA at the concentration of 12.5 uM attenuated lipid peroxidation in LPS-treated Caco-2 cells. Moreover, CA (12.5 μM) restored GSH level in LPS-treated cells. The inhibition of lipid peroxidation and altered endogenous antioxidant status by CA showed its antioxidant activity against LPS-induced oxidative stress in intestinal epithelial cells.

Cinnamon essential oil consists of variety of chemical compounds: CA (96.8%), alpha-capaene (0.2%), alpha-murolene (0.11%), para-methoxy cinnamaldehyde (0.6%), and delta-cadinen (0.4%) (Keshvari et al., 2013). CA is the major active compound which exhibits several pharmaceutical properties such as antibacterial, antioxidant, antifungal and antidiabetic (Doyle and Stephens, 2019). Antioxidant activity of CA is attributed to its phenolic and polyphenolic components [25]. CA has strong free radical scavenging activity in quenching DPPH (α, α-diphenyl-β-picrylhydrazyl), superoxide radical and NO (Subash-Babu et al., 2014). DPPH is a stable free-radical molecule used in *in vitro* assay to evaluate antioxidant activity of compounds. Decreased MDA level in CA treated cell in the present study might be due to removal of ROS by CA. Several studies have been published on the use of CA as an antioxidant in food production. CA has significant antioxidant effect in ground meat (Naveena et al., 2014) and vegetable oils (Keshvari et al., 2013). However, antioxidant activity of CA at cellular level has not been investigated. In our study, CA at the concentration of 25 μM increased MDA level, compared to the cells treated at 12.5 μM concentration. Similarly, Naveena et al., (2014) reported that CA tended to increase peroxidation above certain concentration and induced oxidative stress at higher concentrations. Therefore, it is important to determine the optimal CA concentration to be added into animal feed in *in vivo* studies.

The beneficial effect of CA on endogenous antioxidant system was demonstrated in previous studies (Huang et al., 2011; Subash-Babu et al., 2014). In the present study, CA restored GSH level in LPS-treated intestinal epithelial cells. GSH is the most powerful non-enzymatic antioxidant which shows higher electron donation and detoxification capacity of ROS and peroxides (Huang et al., 2011; Birben et al., 2012). CA enhances GSH synthesis and activates the phase II detoxifying enzyme expression. CA also increases the cellular protein level of GCLC (γ-

Glutamylcysteine ligase catalytic subunit), a catalytic subunit of γ-glutamylcysteine ligase (GCL) enzyme. GCL is the rate-limiting enzyme in GSH biosynthetic pathway. Furthermore, CA regulates phase II detoxifying enzymes expression, glutathione-S-transferases (GST) and heme oxygenase-1 (HO-1) through the modulation of Nrf2 signalling pathway (Huang et al., 2011).

Combination of EO exerts strong antibacterial effect than individual EOs alone, due to a synergistic effect. In the present study, the antioxidant activity of the combination of citral and CA was examined. We found that the blend of citral (12.5 μM) and CA (12.5 μM) decreased MDA level and increased GSH level in Caco-2 cells. These results suggested that the blend of citral and CA showed a protective effect against LPS-induced oxidative stress.

CHAPTER VI

CONCLUSION AND FUTURE PERSPECTIVES

Conclusion

In conclusion, LPS caused a significant increased in oxidative stress biomarker lipid peroxidation (MDA) and a reduction of endogenous antioxidants (SOD and GSH) in intestinal epithelial cells. Such a detrimental effect was time and dose dependent. Preincubation of cells with essential oils (citral, cinnamaldehyde) effectively attenuated LPS-induced lipid peroxidation as well as restored the SOD and GSH levels. Our results suggest that citral and cinnamaldehyde have a protective effect against LPS-mediated oxidative stress through down-regulation of lipid peroxidation and improving the endogenous antioxidant system. Supplementation of essential oils in feed may exert a beneficial effect on oxidative stress-induced gut injury in animals during infection.

Future directions

Future studies are warranted to investigate (1) the molecular mechanisms of oxidative stress and the protective mechanism of selected essential oils in LPS-treated intestinal epithelial cells; (2) the effect of LPS and selected essential oils on other oxidative stress related signalling pathways such as inflammation in intestinal epithelial cells; (3) the effect of LPS on oxidative stress in the intestine and the underlying mechanisms in animals and (4) the effect of citral and/or cinnamaldehyde supplementation on LPS-induced intestinal oxidative stress, gut health and nutrient absorption in animal models.

CHAPTER VII

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