REGULATION OF OXIDATIVE STRESS IN WEANED PIGLETS CHALLENGED WITH *ESCHERICHIA COLI*

By

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A Thesis submitted to Faculty of Graduate studies of

The University of Manitoba

in fulfillment of the requirements of the degree of

Master of Science

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ABSTRACT

Oxidative stress is a deleterious process that causes various pathological processes in humans and animals. The objectives of this study were to investigate the changes in oxidative status in weaned piglets after an *E. coli* K88+ challenge and to determine the effects of dietary supplementation of red osier dogwoods plant products and epidermal growth factor (EGF) on oxidative stress in these piglets. TBARS and superoxide dismutase enzyme activity were measured as oxidative stress biomarkers. *E. coli* challenge caused oxidative stress in weanling piglets. Dietary supplementation of red osier dogwoods plant products attenuated oxidative stress induced by *E. coli* challenge in weanling piglets. Further, dietary supplementation of epidermal growth factor also attenuated the generation of free radicals in *E. coli* challenged weanling piglets. This study concluded that red osier dogwoods as well as EGF can protect weanling piglets from oxidative stress induced by infection.

ACKNOWLEDGEMENT

First and foremost, I would like to express my deepest gratitude to my supervisor Dr. Karmin O for giving me this invaluable opportunity to pursue graduate studies in Canada. I am forever grateful for her immense guidance, encouragement, support and for the great mentorship always received through out this period.

Secondly, I would like to thank the members of my committee, Dr. Martin Nyachoti and Dr. Rotimi Aluko who shared their expertise with me and provided me with their invaluable suggestions and comments throughout my research work.

Further, I would like to thank our collaborators, Dr. Nyachoti's group for their immense contribution on behalf of my project. Next, I would like to acknowledge my lab members Victoria Sid and Yvette Shang for their continuous assistance, encouragement and friendship. I highly value their assistance and time dedicated in my early technical training.

More importantly, I would like to acknowledge my family for the support and care given to me in succeeding my studies. Special thanks to my loving husband Vidura Senaratne for providing me with all the moral support and care during all the easy and hard times in my life. Heartiest thanks to my beloved late parents who taught me the importance of education & nourished me with all the courage and strength I bear in me. I am also grateful to my brother for being a huge strength in my life.

Lastly, I would like to acknowledge the funding agencies that supported financially for the research work of my master's program.

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LIST OF ABBREVIATIONS

(ACN) Anthocyanins

(ADG) Average daily gain

(ADFI) Average daily feed intake

(AF) Aflatoxin

(ALT) Alanine aminotransferase

(AOX) Antioxidant

(AST) Aspartate aminotransferase

(ATP) Adenosine triphosphate

(BHT) Butylated hydroxytyl.

(CAT) Catalase

(DNA) Deoxyribonucleic acid

(DON) Deoxyvalenol

(DTNB) 5,5'-dithio-bis (2-nitrobenzoic acid)

(ETEC) Enterotoxigenic Escherichia coli

(ETHX) Ethoxiquine

(F) Fumonisin

(GIT) Gastrointestinal tract

(GPx) Glutathione peroxidase

(GSH) Reduced glutathione

(GSSG) Oxidized glutathione

(HIV) Human Immuno deficiency virus

(HNO_2) Nitrous acid

($H2O_2$) Hydrogen peroxide

(HOCl) Hypochlorous acid

(HOO.) Hydroperoxyl radical

(LOOH) Lipid peroxide

(MDA) Malondialdehyde

(MHD) Mulberry heart disease

(NADPH oxidase) Nicotinamide adenine dinucleotide phosphate oxidase

(N_2O_3) Dinitrogen trioxide

($NO^{\cdot}$) Nitric oxide

(NOS) Nitric oxide synthases

($OH^{\cdot}$) Hydroxyl radical

(ONOO-) Peroxynitrite

($O_2^{\cdot -}$) Superoxide anion

(OT) Octratoxin

(PUFA) Polyunsaturated fatty acid

(PWD) Post weaning diarrhea

(PSS) Porcine stress syndrome

(RNA) Ribonucleic acid

($ROO^{\cdot}$) Peroxyl radicals

(ROS) Reactive oxygen species

(SCFA) Short chain fatty acid

(SDS) Sodium dodecyl sulphate

(SOD) Superoxide dismutase

(TBARS) Thiobarbeturic acid reactive substance

(TBHQ) Tetrabutyl hydroquinone

(ZEN) Zeralenone

(EGF) Epidermal growth factor

CHAPTER I

GENERAL INTRODUCTION

Oxidative stress is a deleterious process that causes tissue injury by damaging cell structures and functions, resulting from the accumulation of excessive free radicals. It is considered as a major risk factor that is associated with various pathological processes in humans as well as in animals. Oxidative stress occurs due to an imbalance between the generation of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and their removal by antioxidants. ROS and RNS can be either free radicals that contain an unpaired electron in the outer orbit or non-radical reactive species which does not have an unpaired electron in the outer orbit but are highly unstable and reactive. These reactive species are produced during normal cellular ATP production in the mitochondria or by immune cell activation. Even though at low concentrations these can be beneficial to the cell functioning, when there are excessive amounts they can cause adverse effects to the cells and organs. Oxidative stress can affect livestock by causing severe disease conditions to animals and impairing the performance and reproduction. Swine is one of the most susceptible species to oxidative stress. Even though swine producers have not paid much attention towards the effects of oxidative stress in swine, they may have noticed them as effects of vitamin E deficiency. However, those effects might be due to the oxidative stress. In swine, prolonged oxidative stress is associated with conditions such as reduced performance, decreased immunity, muscle degeneration, stroke, mulberry heart disease, diarrhoea, destruction of liver tissue and reproductive disorders (Rice and Kennedy, 1989; Stern et al., 1995). Diets with a high concentration of polyunsaturated fatty acids, ingestion of mycotoxins through contaminated feed, weaning stress and pathogenic infection are reported as risk factors for oxidative stress in swine (Nagata et al., 1998; Wang et al., 2008; Lu et al., 2014; Wu et al., 2014).

Weaning, which is the removal of the suckling piglets from their mother and introducing them to a solid diet is the most difficult period in the life time of pigs. Sudden environmental, social and

nutritional changes caused by weaning can develop a stressful situation in those piglets, which cause feed refusal. Lower nutritional status due to poor feed intake can make them highly susceptible to infections. One such very common infection in weaned piglets is *E. coli* infection. *E. coli* infection, an important cause of death in weaned piglets worldwide, causes severe diarrhea and sudden death, mainly by the pathotype enterotoxigenic *E. coli* (ETEC) K88+ (Fairbrother et al., 2005). Effect of *E. coli* infection on oxidative status of piglets has not been investigated previously. We hypothesized that *E. coli* infection during weaning could cause oxidative stress in piglets.

The most widely used preventive measure of the *E. coli* infection in piglets is the application of sub-therapeutic levels of antibiotics in the feed. However, there are concerns of antibiotic usage in animal feed due to the risk of development of antibiotic resistance. Some countries have already banned the usage of antibiotics in animal feed as it has become a major concern among consumers of the animal products. Therefore, animal scientists worldwide are paying more attention in finding an effective alternative antimicrobial agent to replace antibiotics in animal feed. In that context, natural products or plant extracts have drawn much attention.

In this study, we studied one such potential plant named Red osier dogwoods (*Cornus stolonifera*) which is a native North American plant rich in several antimicrobial and antioxidative phenolic compounds. This has been used previously by native North American people as a traditional medicine to treat human illness such as diarrhoea, fever and skin diseases. It has been suggested that supplementing piglet diets with phenolic compounds can prevent diarrhea disease caused by enterotoxigenic *E. coli* (Verhelst et al., 2010; Verhelst et al., 2014). However, the potential of Red osier dogwoods supplementation to prevent *E. coli* infection or its antioxidative effects have not been reported.

CHAPTER II

LITERATURE REVIEW

2.1. Oxidative stress

Oxidative stress is a pathophysiological condition that arises from an imbalance between generation of free radicals and neutralization of those free radicals by the activity of antioxidants in the body. The discovery of free radicals in the biological materials owns a history of about sixty years (Commoner and Ternberg, 1961). Within last few decades there has been a rapid growth in research on the importance of free radicals in biological systems, its favorable and unfavorable effects on biomolecules and free radical neutralising activity of antioxidants. These molecules can either be reactive oxygen species (ROS) or reactive nitrogen species (RNS). These can be either from endogenous or exogenous sources. They show harmful effects as well as beneficial effects depending on their molecular concentration in the biological systems. At low or moderate concentrations, these are beneficial in regulating several physiological processes including cell proliferation, migration, hypertrophy, differentiation, an metabolism, whereas higher concentrations lead to harmful effects (Dröge, 2002). At higher concentrations these reactive species can attack biomolecules of the body such as proteins, lipids and DNA altering their structure and functions to cause tissue and organ injury (Dhalla et al., 2000). Hydroxyl radical and peroxynitrite can damage membrane lipids and lipoproteins by a process called lipid peroxidation. This reaction leads to the formation of malondialdehyde (MDA) and conjugated diene compounds, which are cytotoxic and mutagenic. Lipid peroxidation starts a radical chain reaction, spreads rapidly and affects a great number of lipid molecules destroying the membrane lipid (Pham-Huy et al., 2008). Damage to cell membranes can increase membrane permeability and destroy the integrity of tissues. Proteins may also be damaged by ROS/RNS, causing structural changes which leads to disturbed protein activity due to altered or mutated expression. Oxidative damage to DNA leads to the formation of different oxidative DNA lesions which can

cause mutations leading to various diseases in humans and animals (Dröge, 2002). The body has a defense mechanism to neutralize these highly reactive molecules. It comprises of either endogenous or exogenous antioxidant compounds which are capable of converting reactive species to non-reactive, harmless compounds (Finkel and Holbrook, 2000). Any imbalance of reactive species and their neutralization by antioxidants of the body which is called oxidative stress, has been considered as a major risk factor associated with various pathological processes in humans and animals (Valko et al., 2007; Pham-Huy et al., 2008). Researchers therefore, are focusing on various disciplines for suppressing oxidative stress in order to prevent its deleterious effects.

2.1.1. Reactive species

Reactive species includes free radicals as well as non-radical reactive species. Free radicals are those molecules with one or more unpaired electrons in its outer orbit. Due to these unpaired electrons, these molecules become very unstable and thus are highly reactive. Due to this high reactivity, they tend to react with various organic substrates in the body such as lipids, proteins, DNA and alter their structure and function (Finkel and Holbrook, 2000). The non-radical reactive species do not contain un- paired electrons in the outer shell but are highly reactive. They also contribute to the oxidative stress. All these reactive species can be either from endogenous or exogenous sources. As endogenous synthesis, they can be produced by electron leakage from the electron transport chain during the mitochondrial energy production (Dröge, 2002). Apart from that, free radicals can be generated endogenously by immune cell activation, mental stress, ischemia, cancer and aging whereas exogenous free radicals are from sources such

as environmental pollution, smoking, alcohol consumption, heavy metals, certain drugs, industrial solvents, certain foods and radiation (Pham-Huy et al., 2008).

2.1.1.1. Reactive oxygen species (ROS)

ROS are the radicals and non-radical reactive species derived from oxygen (Dröge, 2002). They represent the most important class of radical species generated in the living system. Molecular oxygen, which is a radical itself, has a unique electronic configuration. Addition of one electron to dioxygen results in formation of the most important radical which is the superoxide anion radical ($O_2^{\bullet -}$), which is considered the primary source of ROS in the living systems. The production of superoxide occurs mostly within the mitochondria of a cell through the metabolic processes or following oxygen activation by physical irradiation (Pham-Huy et al., 2008). This radical can react with other molecules to generate secondary ROS. The mitochondrial electron transport chain is the main source of ATP generation in the mammalian cell. During this process, a small number of electrons can be leaked to oxygen, forming the superoxide radical. It has been estimated that 1 to 3% of all electrons in the transport chain leak to generate super oxide radical instead of reducing oxygen to water (Finkel and Holbrook, 2000; Cohen and Nyska, 2002; Valko et al., 2007).

Hydroxyl radical, •OH is another important ROS which has a high reactivity, making it a very dangerous radical with a very short *in vivo* half-life of 10^{-9} seconds (Sies, 1993). Extra superoxide anion radicals ($O_2^{\bullet -}$) in the body facilitate •OH radical production from H_2O_2 by releasing "free iron" from iron-containing molecules which contributes to the generation of highly reactive hydroxyl radicals through a reaction called "Fenton" reaction. (Fe^{2+} + H_2O_2 →

Fe^{3+} + OH^- + $^{\bullet}OH$) (Cohen and Nyska, 2002). Thus, under stress conditions, superoxide acts as an oxidant facilitating •OH production from H_2O_2 by making Fe^{2+} available to Fenton reaction.

Other reactive species derived from oxygen are the peroxyl radicals (ROO•). The simplest peroxyl radical is HOO• which is the protonated form of the superoxide radical and it is termed as either hydroperoxyl radical or perhydroxyl radical (Cohen and Nyska, 2002; Valko et al., 2007). It has been demonstrated that this radical is responsible for fatty acid peroxidation in living organisms (Aikens and Dix, 1991).

2.1.1.2. Reactive nitrogen species

Reactive nitrogen species includes nitric oxide ($NO^{\cdot}$) which is an abundant reactive radical. $NO^{\cdot}$ is generated in biological tissues by specific nitric oxide synthases (NOSs). $NO^{\cdot}$ has a very short half-life of only a few seconds in an aqueous environment (Valko et al., 2007). It is soluble in both aqueous and lipid media; therefore, can easily diffuse through cytoplasm and plasma membranes. In the extracellular environment, $NO^{\cdot}$ reacts with oxygen and water to form nitrate and nitrite anions. Oxidative burst occurs during the inflammatory processes where immune cells produce both the superoxide anion and nitric oxide. Under these conditions, nitric oxide and superoxide anion react together to produce another active molecule, peroxynitrite anion ($ONOO^-$), which is a potent oxidising molecule that can cause DNA fragmentation and lipid oxidation (Finkel and Holbrook, 2000).

2.1.1.3. Non-radical reactive species

Non-radical reactive species can be either ROS or RNS but they do not contain un-paired electrons in the outer orbit but are highly reactive. Hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite ($ONOO^-$), dinitrogen trioxide (N_2O_3), lipid peroxide (LOOH), are a few non radical reactive species which can easily lead to free radical reactions in living organisms (Pham-Huy et al., 2008). H_2O_2 (a non-radical) is produced by the action of several oxidase enzymes, including amino acid oxidase and xanthine oxidase. H_2O_2 is also produced in the peroxisomes of the cells during oxygen consumption which then use to oxidize other molecules. Peroxisome also contains catalase enzyme that break down H_2O_2 to water and thereby maintain lower levels of this toxic molecule. When peroxisomes are damaged, these molecules can be released to the cytosol. Hypochlorous acid (HOCl), another non-radical reactive species is produced by the neutrophil-derived enzyme, myeloperoxidase, which oxidizes chloride ions in the presence of H_2O_2. $ONOO^-$ is produced by the reaction of nitric oxide and the superoxide anion (Cohen and Nyska, 2002).

2.2. Effect of oxidative stress in livestock

Oxidative stress can affect the livestock industry by causing severe diseases to animals and thereby attenuating their performance and production. It is highly detrimental to meat animal production and is considered to be a major risk factor associated with various pathological processes (Lykkesfeldt and Svendsen, 2007; Kataria and Kataria, 2010). Excess free radicals damage animal cells and tissues and may ultimately impair health, growth and performance while negatively affecting meat flavor, color, texture and nutritive value. A fall in performance

caused by oxidative stress might be regarded as a vitamin E deficiency by animal producers, but it can be actually due to a lack of antioxidants. Although vitamin E is an antioxidant, there are alternatives that help prevent oxidative stress. Oxidative stress effects in animals are mostly studied in pigs, cattle, poultry and horses.

In ruminants, apart from symptoms like drop in performance and decreased immunity, mastitis, pneumonia are some diseases associated with oxidative stress. Levels of lipid hydro- peroxides were increased in erythrocytes isolated from dairy cows with acute mastitis and levels of ascorbate were decreased in dairy cattle with subclinical mastitis caused by *Staphylococcus aureus*, *Streptococcus agalactiae* or *E. coli* (Kleczkowski et al., 2004; Ranjan et al., 2005). The detection of increased lipid peroxidation and lowered ascorbate levels suggest that the disease caused udder related oxidative stress (Lykkesfeldt and Svendsen, 2007). In bronchopneumonic calves, it was found that isolated granulocytes produced ten times as much superoxide radicals and have lower plasma superoxide dismutase compared with healthy calves (Ledwozyw and Stolarczyk, 1991). Another study found that isolated neutrophils released from diseased animals produced large amounts of NO. and myeloperoxidase, which in combination might result in nitrotyrosine formation (protein damage) (Wessely-Szponder et al., 2003).

Table 1. Major reactive oxygen and nitrogen species

Formula	Name
Reactive oxygen species	
$^1O_2$	Singlet oxygen
H_2O_2	Hydrogen peroxide
HOO·	Hydroperoxyl radical
HO·	Hydroxy radical
$O_2^{\cdot-}$	Superoxide anion
O_3	Ozone
OCl−	Hypochloride anion
ROOH	Hydroperoxide
ROO·	Peroxyl radical
RO·	Alkoxyl radical
Reactive nitrogen species	
·NO	Nitric oxide
N_2O	Nitrous oxide
ONOO−	Peroxynitrite
$\cdot NO_2$	Nitrogen dioxide
N_2O_3	Dinitrogen trioxide
ONOOH	Peroxynitrous acid
NO−	Nitroxyl anion
NO+	Nitrosyl cation
HNO_2	Nitrous acid
NO_2Cl	Nitrosyl chloride
NO_2^-	Nitrite
NO^{2+}	Nitronium ion
RSNOs	Nitrosothiols

In poultry, in extensive production systems, birds are exposed to several factors that can induce oxidative stress. This includes dietary factors, environmental factors and management related factors. During stress, the hypothalamus-pituitary adrenal axis is activated and glucocorticoid hormone is released from the adrenal cortex. Glucocorticoid is essential for maintaining life and normal growth. However, excessive glucorticoid causes growth inhibition, immune system suppression and induction of oxidative stress in chicken (Taniguchi et al., 2001). Such uncontrolled oxidative reactions can lead to damage of cellular tissues and can lead to cell death, severe oxidative stress and metabolic diseases in poultry (Surai, 2000).

In pigs, symptoms such as drop in performance, decreased immunity, muscle degeneration, increased risk of stroke in fast growing pigs, mulberry heart disease have been observed due to oxidative stress (Rice and Kennedy, 1989). Further, porcine stress syndrome which determined by an abnormal accumulation of lactic acid in the cell compartments, osteochondrosis, due to an altered metabolism of bone growth, loss of appetite, diarrhoea and destruction of liver tissue might be seen during oxidative stress which will be discussed in detail further in the next chapter. ((Christian and Lundstrom, 1992; Stern et al., 1995; Lu et al., 2014)

2.3. Swine health

Health is a critical aspect of all animal production systems. Proper health management practise to prevent diseases and to maintain a good health of the animals is a major concern in every animal production system. In swine production, as in any other animal production system, ensuring good reproductive performance for consistent pig numbers throughout each phase of production cycle with less mortality numbers is vital. Rapid growth of pigs without any disease outbreak may also

contribute to high productivity or the profit of the swine industry. Like other animal species, pigs are susceptible to many infectious and non-infectious diseases. Infectious diseases include bacterial, fungal or viral infections. Viral infections such as porcine adenovirus, African swine fever virus, parvovirus, porcine arterivirus, classical swine fever virus, influenza virus are common in pigs that cause severe losses to the swine industry. Many diseases can be caused by bacterial infections such as brucellosis, clostridiosis, colibacillosis, salmonellosis, tuberculosis and streptococcosis and many more. Non-infectious diseases can be caused by nutrient deficiencies or excesses, mycotoxins, toxic minerals, gases as well as chemicals (Straw et al., 2006). Therefore, in order to obtain a sustainable productivity in the swine industry, prevention and control of infectious and non-infectious diseases and maintenance of good health of animals is of greater importance. Several factors can affect the health status of pigs in a farm such as the management condition, environmental factors and host factors. Therefore, health management program of a swine industry should consider all these important aspects in maintaining the health of animals.

2.3.1. Dietary impact on swine health

Diet provides an important tool for management manipulations in maintaining animal health and the control of disease. The type of feed ingredients, quality, methods of presentation and the amounts of feed provided as well as dietary additives are part of the disease prevention process. Dietary manipulations focus on efficient gain by maximising digestibility and minimising adverse health complications. Ensuring adequate consumption of all essential nutrients in required amounts is central to maintain a good health of pigs. Animals that gain a proper nutrition through their diet are more resistant to infections due to better body tissue integrity and

improved immunity to pathogens. Also, proper nutrition is essential for rapid recovery from diseases. Therefore, diet and health are interrelated and depend upon each other. Nutritional deficiencies and feed contamination, which make the quality of the diet poorer, could cause negative impacts on the health status of pigs. Nutritional deficiencies are rare in today's pig production systems because there is a better understanding of the nutritional requirement during each phase of life cycle under different production conditions. There is also an increased ability of estimating nutrient contents, digestibility and bioavailability of swine diets ingredients. However, there can be rare occasions of nutritional deficiency due to errors in feed manufacturing or formulation. Feed contamination with pathogens or toxins could also play a major role in swine health.

2.4. *E. coli* infection in piglets

When considering the infectious diseases in swine, post weaning piglets are most susceptible to infections. Weaning is the most critical period in the life time of piglets as it exposes piglets to completely different nutritional, environmental, and social conditions. Sudden withdrawal of sow milk and exposure to less digestible plant based dry diets cause a remarkable reduction of feed intake in piglets. This reduced feed intake leads to physiological, microbiological, and immunological changes in the gut as well as lower growth performance in piglets. Weaned piglets also have a higher gastric pH which might be caused by the lower acid secretion capacity of stomach due to lowered lactic acid production from lactose (Efird et al., 1982). The high gastric pH value after weaning is believed to contribute partially to the susceptibility to enteric infections (Heo et al., 2013). *E. coli* infection is regarded as a major cause of post-weaning diarrhea in piglets, which is also called post-weaning enteric colibacillosis (Nagy and Fekete,

1998; Fairbrother et al., 2005; Nagy and Fekete, 2005). It is characterized by frequent excretion of watery feces during early weeks of post weaning and is one of the major problems in swine industry. This is mainly caused by enterotoxigenic *E. coli* (ETEC), a pathotype that produces adhesins which make bacteria adhere to the intestinal wall and release enterotoxins, the causative agent of severe diarrhea (Cutler and Gardner, 1988; Heo et al., 2013). The adhesins aid bacteria colonization in the intestine by keeping them adhered to the wall. The enterotoxins produced by *E. coli* can be either heat-labile or heat-stable toxins. Heat-labile toxins may increase the secretion of sodium, chloride and hydrogen carbonate ions to the intestinal lumen while heat-stable toxins may inhibit the absorption of water and salt from the intestinal walls (Pluske et al., 2002). The actions of these toxins result in accumulation of water and electrolytes in the intestinal lumen, which exceed the absorptive capacity of the large intestine (Nagy and Fekete, 1998) leading to diarrhea, dehydration, poor appetite, supressed growth and even death of the piglets. Therefore, it is vital to make dietary interventions to minimize number of *E. coli* pathogens in the gut as well as to inhibit their adherence to the gut wall and minimize post-weaning diarrhea in piglets.

2.5. Management of swine infection

2.5.1. Natural microbiota in the pig intestine

Naturally there is a large population of microbes in the large intestine in piglets due to slow digesta turn over, low redox potential and higher short chain fatty acid contents (SCFA) (Gaskins et al., 2002). Microbial composition in the large intestine is highly diverse and beneficial to the host. It has been reported that microbial fermentation in the hindgut provides 5-20% of the total

energy production in pigs (Friend et al., 1963). However, in the small intestine, microbes compete with the host for nutrients. In the upper small intestine, the rate of digesta flow is higher so that the bacteria are easily washed out. Therefore, bacteria colonize and adhere only to the mucus layer or epithelial layer. In the distal small intestine, there is a larger number and diversity of bacterial population that compete for nutrients in the host. It was reported that 6% of the dietary energy can be lost in pigs due to nutrient consumption by gut bacteria (Vervaeke et al., 1979). Also, these bacteria make amino acids unavailable for the pigs and produce toxic products such as amines, ammonia, phenols and indoles (Macfarlane and Macfarlane, 1995).

In the small intestine, gram positive bacteria is predominant (Stewart, 1997), which can produce more than one toxic metabolite (Gaskins, 2001). It is believed that even certain types of inhabiting non-pathogenic microbes in the swine small intestine can depress growth by competing for the nutrients available in the gut. Therefore, it is necessary to modulate the intestinal microbiota as well as to prevent the pathogenic infection in order to maintain healthy farm pigs.

2.5.2. Use of antimicrobial compounds

2.5.2.1. Antibiotics

Antibiotics are antimicrobial drugs that can either kill or inhibit the growth of bacteria, therefore, they can be used to prevent and treat bacterial infections in humans and animals. Penicillin was the first chemical compound discovered in 1928 by Alexander Fleming to have antibacterial properties. Some antibiotics may show antiprotozoal properties but they are not effective for viruses. When an illness is detected as a result of a bacterial infection, an antibiotic

administration is practiced as a means of treatment for the particular illness. Sometimes they could be used as a preventive measure in patients but limited only to at- risk individuals such as those taking immunosuppressive drugs, having weakened immune systems, cancer patients and those who undergo surgery.

2.5.2.2. Antibiotic usage in livestock industry

Antibiotics have been widely used in the livestock industry for years for efficient production of pork, beef, poultry meat and other animal products. They can be used at therapeutic levels to treat the diseases of animals while sub therapeutic levels can be used to prevent the diseases (Viola and DeVincent, 2006). Sulfonamides were one of the first antibiotics to be tested on animals in the late 1930's. Initially, they found that they are detrimental because it caused reduced growth and agranulocytosis in rats, but later researchers found out that it reduced mortality and increased growth when supplementing rats with sulfonamides and essential vitamins (Visek, 1978). Livestock producers added antibiotics to their feeding programs after their growth enhancing capabilities were reported in the early 1950's.

When antibiotics were included in animal diets at lower levels it can improve growth rate and efficiency of feed utilization, reduce mortality and morbidity, and improve reproductive performance (Cromwell, 2002). Therefore, livestock producers, mostly swine and poultry producers tend to include antibiotics in the diets of animals as a regular ingredient. There are several modes of action that have been proposed for growth enhancing ability of antibiotics. Inhibition of sub-clinical infections, reduction of growth-depressing microbial metabolites, reduction of microbial nutrient use, and enhanced uptake and use of nutrients through the thinner

intestinal wall associated with antibiotic-fed animals are the known modes of action of antibiotics (Francois, 1962; Visek, 1978; Anderson et al., 2000).

2.5.2.3. Antibiotic usage in swine

In swine production, a large proportion of antibiotics have been used. Over the past few decades, it was estimated that 70 to 80% of pig starters, 70 to 80% of grower feeds, 50 to 60% of finisher feeds, and 40 to 50% of sow feeds contained antimicrobial agents (Cromwell, 2002). The FDA has approved 17 antibiotics to be used in the swine industry and 14 of them are used as growth promotants. Research shows that a wide variety of antibiotics have growth-promoting ability. Zinc-bacitracin, chlortetracycline, oleanomycin, oxytetracycline, tylosin, penicillin, neomycin, tilmicosin, sulfamethazine, and sulfathiozole have all been shown to improve growth performance of pigs (Cromwell, 2002). Injectable antibiotics typically stimulate a growth response only if they are absorbed into the gastrointestinal (GI) tract, while antibiotics not absorbed into the GI tract only stimulate growth responses when included in the diet (Visek, 1978).

Many studies have shown that dietary supplementation of antibiotics improves feed intake and daily weight gain in young pigs (Bunch et al., 1963; Roof and Mahan, 1982) but less effective for finishing pigs (Clawson and Alsmeyer, 1973; Moser et al., 1985). This improved response of piglets is thought to be due to prevention of bacterial infection by antibiotics in piglets. Young pigs are highly susceptible to infection because sow's milk contain lower amounts of immunoglobulin at 3 weeks after farrowing (Miller et al., 1961) and also piglets are weak at producing their own antibodies until around 6 weeks after weaning (Cromwell, 2001). Therefore,

in-feed antibiotics help protect against pathogenic infection and promote their growth. Continuous feeding of antibiotics over a long time period may lower the bacterial population and lower the disease potential in pigs (Jukes, 1955). However, once pigs are at the finishing stage, their immune system is well developed and addition of antibiotics in diet is less effective. Response to antibiotics is also related to environmental factors such as cleanliness of the facility. A study reported that antibiotic feeding is more effective in piglets reared in facilities that were not thoroughly cleaned compared to the piglets reared in cleaner facilities (Hays and Speer, 1960).

Antibiotics are believed to alter the requirement of energy, protein, fat, vitamins and minerals in animals (Visek, 1978). Dry matter and energy digestibility were increased when pigs were fed with antibiotics (Ravindran et al., 1984). Contrarily, some studies found that the dry matter, organic matter and carbohydrate digestibility were decreased when piglets were fed with antibiotics but no growth reduction was observed indicating that there might be a higher utilization of absorbed energy in these pigs fed with antibiotics (Beames and Lloyd, 1965; Eggum et al., 1982). Feeding antibiotics can also increase the digestibility of crude fat (Eggum et al., 1982) and increase lipid absorption by depressing the bile acid transforming bacteria such as *Lactobacillus and Enterococcus* (Gaskins et al., 2002). This also might lead to the growth promotion in pigs fed antibiotics. Antibiotic feeding has beneficial effects on protein metabolism as well. A study reported that pigs fed a low protein diet supplemented with the antibiotic aureomycin showed a similar growth rate as the pigs fed a high protein diet (Catron et al., 1952). Other studies also reported that feeding antibiotics could increase protein digestibility and N retention (Beames and Lloyd, 1965; Eggum et al., 1982; Gaskins et al., 2002) whereas some

studies demonstrated that antibiotic feeding also increased the synthesis of certain vitamins and the absorption of minerals as well (Visek, 1978).

The in-feed antibiotic usage has many beneficial effects in increasing production efficiency in swine. Use of antibiotics in piglet feeds has been reported to reduce the piglet mortality by 50%. Also antibiotic feeding had shown beneficial effects on reproductive performance when antibiotics were fed before breeding (Cromwell, 1991). Increased number of piglets born alive, higher birth weights, increased litter sizes and increased farrowing rates were observed when fed antibiotics. These benefits of antibiotics on reproduction are believed to be due to the prevention of uterine infection and stimulation of uterine tissue cell metabolism. Feeding antibiotics during farrowing and lactating stages also showed beneficial effects on piglet survival and weaning weights (Cromwell, 2002).

2.5.2.4. Recent concerns for antibiotic usage in livestock production

In recent years, there have been concerns regarding the use of antibiotics in animal feeds due to the risk of development of antibiotic resistance. Even though some researchers believe that the growth promoting mechanisms of antibiotics in animals are independent from the mechanisms that lead to bacterial resistance (Visek, 1978), there is evidence suggesting that supplementing continuously low dosages of antibiotics leads to acceleration of the development of resistant bacteria (McDermott et al., 2002). In certain countries, it has become a major health issue so that they have set up restrictions for using antibiotics in animal feed. Even though people still prefer getting antibiotics for illnesses from their doctors, they refuse to consume products from antibiotic-fed livestock animals. As a result of this concern, the EU has already banned the use of

antibiotics in animal feed and other countries are also looking at alternatives for antibiotics as growth promotants in animal feed.

Government of Canada also released a federal framework for action in October, 2014, towards antimicrobial resistance and use in Canada. It is a framework that maps out a coordinated, collaborative federal approach in responding to the threat of antimicrobial resistance (AMR). According to that, both health Canada and Canadian Food Inspection Agency work in collaboration with provincial partners and animal health stake holders to strengthen veterinary oversight of medically-important antimicrobials for food-producing animals. Both Health Canada and the Canadian Food Inspection Agency believe that professional oversight is necessary on the use of existing antimicrobial drugs in food animal production in order to attain the goal of facilitating the prudent use of antimicrobials and conserving antimicrobial effectiveness. Agriculture and Agri-Food Canada will work with the livestock and poultry sectors to encourage the increased adoption of animal health practices that ultimately reduce the use of antimicrobials in animal production (Federal Action Plan on Anti-microbial Resistance and Use in Canada, 2015).

2.6. Oxidative stress in swine

2.6.1. Risk factors of oxidative stress in swine

2.6.1.1. High oxidant containing diet

Diets with a high concentration of poly unsaturated fatty acids (PUFA) have increased potential for nutrient oxidation (Chahboun et al., 1990). Specifically, use of by-products with greater concentrations of PUFA, enhances the potential for nutrient oxidation and oxidative stress in

meat animals. Moreover, free radicals which are induced by increased amounts of oxidized lipids, can attack macromolecules in the body such as lipids, protein, and nucleic acids (Valko et al., 2006) and can impair animal health and growth. According to a study (Lu et al., 2014), pigs fed a diet high in oxidants i.e. a diet enriched with 5% oxidized soy bean oil, without any antioxidant supplementation, displayed poor health and performance. They observed, during the grower phase, the growth rate and the gain to feed ratio were poor in animals fed a high oxidant diet compared to the animals fed a conventional diet. But, in the same study, when the diets were switched to a conventional corn-soy diet in the finisher phase, after 83 days on trial, their growth rate and the gain to feed ratio were improved. The same study showed that the diet high in oxidants also had negative effects on liver function (Lu et al., 2014). There is evidence that enlarged liver in pigs subjected to oxidative stress was due to the cytotoxic effects of oxidation products accumulated in the body system (Esterbauer, 1993). The elevated concentrations of hepatic enzymes such as alanine transaminase (ALT), and aspartate transaminase (AST) are commonly used as diagnostic indicators of liver injury and are considered as biomarkers for liver function. The above mentioned study (Lu et al., 2014) revealed that levels of ALT in blood were elevated after supplying high oxidant diet to pigs, indicating liver injury. They also observed elevation in plasma bilirubin concentrations in high oxidant diet, which might be due to erythrocyte hemolysis. The erythrocyte membrane is also susceptible to oxidative stress due to its high content of polyunsaturated fatty acids. High doses of free radicals or oxidation products from the oxidative stress diet probably caused a destruction of the erythrocyte membrane structure (Nakazawa and Nagatsuka, 1980). In another study, malondialdehyde (MDA), a biomarker for oxidative stress was found in higher amounts in finishing barrows after oxidized

oil was applied in the diet, whereas antioxidant supplementation in the diet reduced plasma and muscle TBARS(Boler et al., 2012).

2.6.1.2. Pathogenic infection

It is well known that massive production of ROS and RNS by activated macrophages in the inflammatory environment provides a first line of defense against pathogens. ROS and RNS are the most important micromolecules involved in destroying and eliminating invading pathogens. A study showed that hydrogen peroxide (H_2O_2) could kill *E. coli* in a dose-dependent manner (Imlay and Linn, 1986). H_2O_2 at low concentrations oxidized DNA of *E. coli*, whereas at higher concentrations it simultaneously oxidized several biomolecules of *E. coli*. On the other hand, microbial pathogens can induce oxidative stress in infected host cells by generating more free radicals as the first line of defense (Schweizer and Peterhans, 1999). Ding et al. (2007) reported that *H. pylori* infection played an important role in epithelial injury by inducing oxidative stress. Oxidative stress associated with *H. pylori* infection can alter epithelial cell proliferation, apoptosis and oxidative DNA damage (Baik et al., 1996; Clément et al., 1998). Apart from inducing production of free radicals in the host, infected pathogens produce free radicals inside themselves. It was reported that, *H. pylori* itself also generates ROS (Nagata et al., 1998) and that ROS accumulate in gastric epithelial cells (Bagchi et al., 1996) to cause oxidative stress. Supplementation of antioxidants to *H. pylori* infected cells inhibited the apoptosis induced by infection in gastric epithelial cells in vitro (Ding et al., 2007).

Another important free radical, nitric oxide is also known to inhibit bacterial respiration and DNA replication (Fang, 2004). Therefore, pathogen-induced inflammation is associated with an

increase in ROS and RNS production. But, these excessive production of ROS can eventually lead to an imbalance of oxidants and antioxidants and therefore cause oxidative stress in the host (Dröge, 2002). Moreover, it has been observed that these reactive molecules cannot distinguish between the structure of host molecules, cells and tissues and infectious agents (Sorci and Faivre, 2009) so that they can harm not only the infecting pathogen but the host cells and tissues of the host as well. For instance, humans infected with HIV suffer from an increased amount of serum hydroperoxides and malondialdehyde and reduced availability of antioxidants (both enzymatic and non-enzymatic) (Pace and Leaf, 1995). Therefore, it is clear that activation of the inflammatory response depletes antioxidants and exposes the host to increased risk of oxidative stress (Sorci and Faivre, 2009). More specifically in swine, a study reported that *E. coli* infection postweaning attenuated the antioxidant defense system in weanling piglets (Jiang et al., 2014). They observed lower plasma GSH-Px activity and total antioxidant capacity (T-AOC) in the *E. coli* infected group without any antioxidant supplementation than the groups supplemented with antioxidants.

2.6.1.3. Mycotoxins

Another risk factor for oxidative stress in swine is mycotoxins. Mycotoxins are a larger group of naturally occurring fungal secondary metabolites that are not utilized by fungal organisms as meant by "secondary", but instead are excreted into the medium where they grow. These mycotoxins are of greater importance because of their ability to cause unfavourable or toxic effects in humans and animals when ingested. There is evidence that ingestion of mycotoxins through contaminated feed can cause oxidative stress in pigs. Swine are fed largely a grain based diet so that exposure to mycotoxin is unavoidable. The most often reported mycotoxin types of

swine feeds are aflatoxin (AF), zearalenon (ZEN) and trycothecsenes i.e. deoxyvalenol(DON) and fumonisins (F). It is evident that, fumonisin B1 (FB1) increases the oxidative DNA damage. A study reported increased DNA strand breaks and malondialdehyde adducts in rat liver and kidney *in vivo* when fed FB1 contaminated feed (Abel and Gelderblom, 1998). FB1 is involved in the disruption of sphingolipids biosynthesis pathway and thereby affects numerous cell functions and signaling pathways such as apoptosis and mitosis and thus contributes to carcinogenesis (Merrill Jr et al., 2001; Stockmann-Juvala and Savolainen, 2008).

Furthermore, it was reported that one of the underlying mechanisms for FB1-induced cytotoxicity and carcinogenicity is oxidative damage. An increased production of ROS was detected in cultured rat hepatocytes following the exposure to aflatoxin B1 (AFB1) (Shen et al., 1995). Increased levels of oxidative stress markers i.e. catalase, total anti-oxidant capacity, hydrogen peroxide, nitric oxide were observed in blood of piglets fed a DON-contaminated diet (Wu et al., 2014). Furthermore, increased levels of oxidative stress markers (heme oxygenase 1) were observed in growing piglets even when the diet contained acceptable levels DON-contamination declared by European Commission Recommendation (2006/576/EC) i.e 0.9 mg/kg of diet (Alizadeh et al., 2015). Several studies reported that ZEN causes oxidative damage and cytotoxicity and inhibit DNA and protein synthesis in swine epithelial cells (Marin and Taranu, 2012; Wan et al., 2013). Therefore, it is clear that mycotoxins play an important role in oxidative stress in swine.

2.6.1.4. Weaning of piglets

Weaning is the most difficult period for piglets as they are exposed to stress by separating from their mothers, by mixing with other piglets and changing the diet from milk to solid feed. Due to the changes in feed, the favourable microbial composition in their gut changes and piglets become highly susceptible to harmful bacteria during this period (Fairbrother et al., 2005; Heo et al., 2013). Gut inflammation can occur in piglets due to immaturity of the gut and poor immunity. This can lead to digestive disorders which ultimately result in severe diarrhoea and may even lead to the death of the piglets (Heo et al., 2013). Induced inflammation under these conditions is known to be associated with oxidative stress which severely affects piglet health at this period. It has been reported that oxidative status is exacerbated during the first week after weaning compared to suckling time without any visible clinical signs on the piglets (Wang et al., 2008; Zhu et al., 2012). It has been suggested that this condition appears to be due to low plasma vitamin E and vitamin C conditions. Furthermore, it has been reported that newborn piglets exhibit a low vitamin E level and glutathione peroxidase (GSH-Px) activity at birth. Another study revealed that early weaning resulted in increased expression of genes that promoted oxidative stress (Wang et al., 2008). They observed decreased food intake, body weight, small intestinal weight, increased levels of oxidized glutathione (GSSG) to reduced glutathione (GSH) ratio of weaned piglets compared to suckling piglets of the same age. Sulfur amino acids, cysteine and methionine are vital reductants that are essential to maintain glutathione concentration through the trans-sulfuration pathway. However, during early stages of post weaning, deficiency of these amino acids can occur due to poor feed intake (Li et al., 2001) and this can result in the imbalance of pro and antioxidants leading to oxidative stress in piglets.

2.6.2. Effects of oxidative stress on swine health

2.6.2.1. Poor performance

Poor growth and performance were observed in swine due to oxidative stress. A fall in performance caused by oxidative stress might typically be regarded as a vitamin E deficiency, but it can be lack of other antioxidants. A study reported that pigs fed a diet high in oxidants, without any antioxidant supplementation showed poor growth and performance. They observed poor weight gain and feed intake in pigs fed a high oxidant diet compared to pigs fed a conventional diet (Lu et al., 2014). Another study conducted by Shi-bin et al. (2007) demonstrated that oxidative stress in weanling piglets induced by feeding diets containing oxidized fish oil or injecting with diquat, a moderately toxic herbicide, resulted depressed growth performance in piglets. They observed lower average daily gain, average daily feed intake and increased gain: feed ratio in piglets due to oxidative stress. Arsenic (As) is an element that cause cytotoxicity by inducing oxidative damage (Lee and Ho, 1995). Depressed average daily gain and increased gain: feed ratio was also observed in growing piglets due to oxidative stress when they were fed a diet supplemented with As (Wang et al., 2006).

2.6.2.2. Sepsis and endotoxemia

Sepsis is the harmful inflammatory response from the host to an infection and is characterized by multiple organ failure (Cohen, 2002). Similarly, endotoxemia is the presence of toxins in blood, which occurs in response to severe bacterial infections that can result in high morbidity and mortality and requires intensive care and treatment. Sepsis and endotoxemia syndrome in piglets are a relatively frequent event, which is characterised by altered vascular tone and organ

perfusion. Porcine endotoxemic shock seems to be associated with increased oxidative stress and damage. Previous studies reported that free radical and the cyclooxygenase enzyme catalyzed oxidation of arachidonic acid occurred during endotoxemia in pigs (Basu and Eriksson, 1998, 2000). They observed decreased antioxidant levels and increased levels of oxidative stress parameters in pigs induced with sepsis. This increased levels of free radicals and arachidonic acid metabolites can cause pulmonary and renal vasoconstriction and increase vascular permeability. This seems to be involved in tissue injury associated with sepsis and endotoxemia in pigs (Basu and Eriksson, 1998).

2.6.2.3. Mulberry heart disease

Mulberry heart disease (MHD) is characterized by brief period of respiratory distress and sudden death due to heart muscle failure in fast growing pigs. MHD in pigs is a condition known to be associated with oxidative stress (Rice and Kennedy, 1989) and is believed to be related to vitamin E deficiency and diets containing unsaturated fat (Moir and Masters, 1979). Vitamin E is a well-known biological antioxidant and due to its ability to dissolve in lipids, it protects the cell membranes by scavenging free radicals. Deficiency of vitamin E in pig diets and excess amounts of unsaturated fats, therefore, can cause increased levels of free radicals and cause lipid peroxidation leading to various pathological conditions particularly MHD in growing pigs (Rice and Kennedy, 1989). This disease is founded most commonly in 2 - 4 months old piglets, with affected animals showing severe dyspnoea, cyanosis, recumbancy and forced walking all leading to immediate death.

2.6.2.4. Porcine stress syndrome

Porcine stress syndrome (PSS) is a disease condition that causes a significant problem in swine industry and it is characterized by heavily muscled animals and sudden death of pigs. Animals having PSS may also show muscle tremors and nervousness. This syndrome, determined by an abnormal accumulation of lactic acid in the cell compartments is believed to be related with increased levels of reactive oxygen species in the cell compartments (Christian and Lundstrom, 1992).

2.6.2.5. Poor reproductive performance

Oxidative stress is believed to cause many reproductive disorders in pigs. The adverse effects of oxidative stress to reproduction involves damage to sperm and oocyte DNA, disrupted testicular functions, dysfunction of the ovary and endometrium and decreased fertility rate of animals. Elevated ROS concentrations are known to induce sperm immobilization via depletion of intracellular ATP and lipid peroxidation, which causes sperm cell death. Spermatogenesis in testes is a very active and replicative process to generate sperm at a higher rate. This high rate of cell division is accompanied with high production of free radicals due to high amounts of mitochondrial oxygen consumption by germinal epithelium (Aikens and Dix, 1991). An imbalance of free radical generation and detoxification can cause oxidative stress and damage to cellular lipids, proteins, amino acids, sugars, nucleic acids, and mid pieces in sperm and testicular tissues. This may lead to subsequent poor semen qualities and ultimately lead to infertility of male animals (Zhong and Zhou, 2013). Oxidative stress has been implicated in the pathogenesis of lead and cadmium-induced reproductive diseases in animals (Patra et al., 2011).

Similar to male animals, free radicals exert functions in female reproductive system with involvement in the pathophysiology of preeclampsia (Buhimschi et al., 1998), endometriosis (Uchiide et al., 2002); birth defects and infertility (Celi, 2011). The risk of abortions in gestating sows can also be caused by oxidative stress (Zhong and Zhou, 2013).

2.7. Antioxidants

In order to prevent oxidative stress and the associated tissue damage, the balance between oxidants and antioxidants of the body is vital. Antioxidants may donate electrons to oxidants and neutralize the unstable status of oxidants, preventing tissue damage by rendering them inert to cellular macromolecules. Major enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase, catalase (CAT) while non-enzymatic antioxidants in the body include ascorbic acid (Vitamin C), α - tocopherol (Vitamin E), glutathione (GSH), carotenoids, flavonoids etc. (Valko et al., 2006). Glutathione is a major antioxidant in the body due to several functions it performs to counteract and eliminate oxidants. According to Masella et al. (2005), glutathione is also a cofactor of several detoxifying enzymes against oxidative stress, e.g. glutathione peroxidase (GPx), glutathione transferase. GSH also scavenges hydroxyl radical and singlet oxygen directly, detoxifies hydrogen peroxide and lipid peroxides by the catalytic action of glutathione peroxidase and furthermore it is able to regenerate other important antioxidants, Vitamins C and E, back to their active forms. The enzyme SOD, catalyses the dismutation of superoxide anion into dioxygen and H_2O_2 while catalase removes H_2O_2 leaking from the electron transport chain and GSH reductase facilitates the recycling of GSH. These antioxidants are vital in maintaining the redox status of the cells by counteracting oxidants and thereby preventing the harm caused by oxidants (Cohen, 2002).

2.7.1. Use of antioxidants in swine

Although a lot of endogenous antioxidants are present in the animal body, it is important to provide exogenous antioxidants to animals to maintain their health and performance at times of oxidative stress. Exogenous antioxidants can be either natural or synthetic. Natural antioxidants act either as free radical scavengers or structural components of enzymes that react and neutralize free radicals (Ullrey, 1981). Synthetic antioxidants such as ethoxyquin (ETHX), tert-butylhidroquinone (TBHQ), butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) are added to ingredients in the diet that are susceptible to oxidation such as cereal grains, animal fats, vegetable oils, fish oil and meal, and vitamin premixes (Thorisson et al., 1992).

Vitamin E and selenium (Se) are the most frequently added antioxidants in animal feed. Vitamin E is believed to react with lipid peroxide and attenuate glutathione peroxidase enzyme activity (Ullrey, 1981). Lauridsen et al. (1999) found that rapeseed oil added at 6% rate of the diet (as fed basis) supplemented with vitamin E for finishing pigs increased α-tocopherol concentration in plasma and skeletal muscle while TBARS production was decreased in liver and skeletal muscle. Also increased levels of α-tocopherol were observed in piglet serum and liver and skeletal muscle in finishing pigs when diets were supplemented with α-tocopherol acetate (Mason et al., 2005).

Selenium dependant AOX activity also has been widely evaluated in swine nutrition. Increased GSH-Px activity in plasma (Mahan et al., 1999), liver and muscle (Zhan et al., 2007) has been reported when growing-finishing pigs were fed either with an organic or inorganic Se source. Zhan et al. (2007) also reported that pigs supplemented with an organic Se source (selenomethionine) in the diet showed decreased TBARSlevels in skeletal muscle.

Feeding black raspberry to swine provided the antioxidative protection in the gut of the weanling piglets due to the high content of anthocyanin in the black raspberry (Wu et al., 2006). They found that the antioxidant capacity and concentration of anthocyanins within the GI contents positively correlated with each other throughout the GI tract of the weanling pigs.

A combination of carvacrol, cinnamaldehyde and capsicum oleoresin beneficially affected gastrointestinal ecosystem and gastric emptying of weaned pigs (Manzanilla et al., 2004). The mixture effectively protected pig's blood lymphocytes against oxidative DNA damage at 271.2 mg/kg feed concentration. Its effect was reported as comparable to that of 90.4 mg/kg of vitamin E. Antioxidant capacity of propylene glycol extracts of *Calendula officinalis* (*Calendula off*. 1 – extract from petals, 3 ml/day; *Calendula off*. 2 – extract from whole flowers tops, 3 ml/day) and vitamin E (38.4 mg/day) were evaluated against oxidative stress induced by high PUFA intake in pigs. The extracts effectively prevented oxidative DNA damage in peripheral lymphocytes (measured as % DNA in the tail of the comet), but did not prevent lipid peroxidation.

Moreover, providing pigs with an antioxidant blend and vitamin E attenuated the adverse effects caused in pig growth parameters and carcass quality by high oxidant diets (Lu et al., 2014). When pigs were fed high oxidant diet rich in oxidized lipids (5% of oxidized soy bean oil in the diet), they observed poor ADG and ADFI compared to the animals fed a conventional soy-corn diet, as mentioned in the previous sections. However, when they fed animals a high oxidant diet comprised of added vitamin E at 11 IU/kg or antioxidant blend including a blend of ethoxyquin and propyl gallate at 135 mg/kg, they observed better growth performance during the grower phase and carcass characteristics such as dressing percentage, moisture percentage, extractable fat content of meat, drip loss in loin muscle similar to the animals fed a normal conventional diet. Furthermore, when weanling piglets subjected to *E. coli* challenge were fed with a mixture of

polyphenols extracted from apples, grape seeds, green teas and olive leaves, increased plasma GSH-Px and T-AOC activity were observed, indicating that these polyphenols enhanced the in vivo antioxidative defense system (Jiang et al., 2014). Therefore, it is evident that supplying antioxidant as a feed additive in swine diets can eliminate harmful effects caused by oxidative stress.

2.8. Red osier dogwoods

Red osier dogwoods (*Cornus stolonifera*/ *Cornus sericea*) is a native North American plant that grows naturally in every Canadian province and also in the northern parts of the United States. This plant is an ornamental shrub which is widely used in landscaping purposes. It has a small shrub like appearance and contain small white flowers in summer or early fall while containing white berries in the fall. This plant has been used as a traditional medicine by native Americans to treat diarrhea, fever and skin diseases (Ritch-Krc et al., 1996; Obomsawin, 2007; Tushingham and Eerkens, 2016).

This plant material (barks and leaves) has been demonstrated to contain a total phenolic concentration ranging from 40 to 220 mg/g depending on the season, highest in the month of August, and these include anthocyanins, gallic acid, ellagic acid, quercetin, kaempferol and cyanine (Isaak et al., 2013). Gallic acid has been shown to have antioxidative capacity in mice (Rasool et al., 2010) whereas ellagic acid induced apoptosis in cancer cells (Losso et al., 2004). Moreover, quercetin has been reported to reduce inflammation and oxidation damage caused by *Helicobacter pylori* in the mucosa of guinea pigs (González-Segovia et al., 2008) whereas anthocyanins showed antioxidative properties in piglets (Mason et al., 2005).

2.9. Epidermal growth factor

Apart from investigating the natural plant extracts as alternatives to antibiotics, the stimulatory effects of milk born growth factors on developmental and protective effects on intestine has been a subject for considerable investigation. These growth factors include a variety of proteins and peptides capable of stimulating cell growth and facilitate different cell functions.

Epidermal growth factor (EGF) is a single chain polypeptide comprising 53 amino acids with a molecular weight of 6kDa. EGF has been found in milk of several species including humans and swine but the concentration in milk is highly variable among species (Zijlstra et al., 1994). In humans and swine, average concentrations are 50-150 ng/mL and 200ng/mL respectively. It has been reported that in swine colostrum, levels as high as 1500ng/ml were detected (Jaeger et al., 1987). Milk EGF could be derived from either maternal circulation or from synthesis within the mammary gland itself. In most species, concentrations of EGF are highest in colostrum and decline rapidly thereafter. EGF was found to have many effects in mammalian tissue such as enhanced proliferation and differentiation of intestinal epithelial cells, initiation and stimulation of enzyme production in the intestine (Jaeger et al., 1987). For milk-borne growth factors to elicit effects on the intestine of the suckling neonate, they must survive the digestive process and arrive in sufficient concentration to bind to their intestinal receptors.

In order to act on target cells, it has to bind a 170kDa membrane bound glycoprotein receptor (Carpenter, 1984). The EGF receptor has been identified in piglets from esophagus to ileum. In adult pigs, EGF receptor has been identified in gastric parietal cells. It has been reported that the expression of EGF receptor is higher in weaned piglets than new born pigs. But suckling piglets have less EGF receptor expression than new born piglets. It has been identified that EGF receptor is predominantly located in the basal regions of the villi and in the crypts (Kelly et al.,

1993). Kelly et al. (1992) observed EGF binding to both apical and basolateral membranes of jejunal enterocytes from newborn and weaned pigs, suggesting that EGF may act at either luminal or serosal surfaces. Interestingly, EGF binding was not detected in suckled pigs, suggesting that receptors were either occupied, blocked, and (or) down-regulated by factor(s) present in milk.

It has been shown in many studies that EGF affects the GI epithelial maturation and function. In 21-day-old newly weaned piglets, oral administration of EGF at 372 mg/day increased jejunal lactase and sucrase specific activities by 77 and 97%, respectively, after 3 days of treatment (Jaeger et al., 1990). Subcutaneous administration of EGF at 60 mg/kg of BW per day to 3-day-old suckling piglets for 3 days increased the specific activities of sucrase and maltase in the middle and distal small intestine and reduced the specific activity of lactase in the distal small intestine (James et al., 1987). Addition of EGF (0.5 mg/ l) to the culture media increased protein synthesis rate by 2-fold in jejunal explants from neonatal pigs (Black and Ellinas, 1992). Supplementation of EGF at 0.5 or 1.0 mg/ l to milk replacer increased villus height and lactase specific activity in a dose response fashion when fed to rotavirus-infected newborn pigs for 8 days (Zijlstra et al., 1994). Kingsnorth et al. (1990) reported increased tensile strength of gastric wounds in young pigs after 5 days of intraperitoneal infusion of EGF at 0.5 mg/kg per day.

In 4-week old rabbits, orogastric EGF treatment daily, starting 3 days prior to infection with enteropathogenic *E. coli* prevented the occurrence of diarrhoea and reduction of body weight gain, inhibited *E. coli* colonization in the small and large intestine, improved jejunal maltase and sucrase activities, and reduced microvillus injury (Buret et al., 1998). It has been reported that orally administered EGF is not subjected to degradation in the GI lumen and can pass through the intestinal cell walls intact. In neonatal animals, after trans epithelial transport to the

circulation, intact EGF might reach proliferative crypt cells, because gastric secretion is attenuated and proteolytic digestion is incomplete in the newborn (Hartma et al., 1961).

However, direct administration of recombinant EGF is very costly for routine application in swine production. Taking this matter into account, Cheung et al. (2009) generated a strain of *Lactococcus lactis* (*L. lactis*) capable of expressing porcine EGF (EGF-LL). *L. lactis* is a non-pathogenic, non-invasive, non-colonizing gram positive lactic acid bacterium. Lactic acid producing bacteria are generally regarded as safe and widely used in the production and preservation of fermented food products. *L. lactis* is found to be metabolically active in all compartments of intestine (Drouault et al., 1999). It makes this bacterium capable of delivering recombinant protein to the intestine. Many studies demonstrated the ability of this bacteria to express and secrete biologically active cytokines (Steidler et al., 2000; Steidler et al., 2003; Frossard et al., 2007). The local delivery of strain of *L. lactis* developed by Cheung et al. (2009) which is capable of expressing porcine EGF demonstrated beneficial effects on intestinal growth of early weaned mice. Same research team demonstrated that the supplementation of this EGF-LL to early weaned pigs enhanced the proliferation and maturation of intestinal cells, increased the intestinal length and improved growth performance (Kang et al., 2010; Bedford et al., 2012). Also, addition of *L. lactis*-free fermentation supernatant to the feed of weaned piglets showed increased intestinal sucrose and alkaline phosphatase activity as well as enhanced growth performance indicated by increased daily body weight gain, final body weight and gain : feed (Bedford et al., 2014).

Furthermore, EGF was also shown to reduce the colonization of pathogens in the intestinal epithelium. Supplementation of EGF facilitated the recovery of intestine in piglets from a rotavirus infection (Zijlstra et al., 1994). Also, Oral administration of EGF inhibited

enteropathogenic *Escherichia coli* induced diarrhea and prevented a reduction in weight gain in rabbits (Buret et al., 1998). Therefore, we hypothesized that EGF supplementation may have beneficial effects on preventing *E. coli* infection and associated complications such as diarrea in weaned piglets while improving growth performance. In this study, we investigated the effects of EGF on antioxidative defense system and oxidative stress in weaned piglets induced by *E. coli* infection post weaning.

CHAPTER III

HYPOTHESIS AND OBJECTIVES

3.1. Research objectives

The overall research objective is to find alternatives to in-feed antibiotics to be included in weanling piglet diets to protect them from infection and promote growth and to evaluate their effects on oxidative stress of piglets

3.2. Hypothesis

- *E. coli* infection post-weaning causes oxidative stress in weanling piglets
- Dietary supplementation of red osier dogwoods plant products has inhibitory effects against oxidative stress in piglets challenged with *E. coli*
- Dietary supplementation of epidermal growth factor (EGF) has inhibitory effects against oxidative stress in piglets challenged with *E. coli*

3.3. Specific objectives

- To measure the oxidative stress parameters in weanling piglets after *E. coli* challenge
- To determine the antioxidative effects of dietary supplementation of Red osier dogwoods plant material (ground leaves and barks) at two levels (2% and 4% of the total diet) in piglets challenged with *E. coli*
- To determine the antioxidative effects of dietary supplementation of EGF expressing *Lactococcus lactis* inpiglets challenged with *E. coli*

CHAPTER IV

MATERIALS AND METHODS

4.1. Animals Care

All the piglets were reared according to the guidelines of Canadian Council on Animal Care (CCAC, 2009). The experimental protocol (F13-002/2) was reviewed and approved by the University of Manitoba Animal Care Committee.

4.2. Animal and housing

Twenty-eight individually housed male and female piglets (Durocn × [Yorkshire×Landrace]) weaned at 21 d with an initial average bodyweight of 7 kg (Table 2) were obtained from Glenlea Swine Research Unit, University of Manitoba. They were divided into four groups to comprise seven piglets in each, and assigned to four dietary treatments including a corn-soy bean meal wheat based control diet for a 14-day study. Piglets were kept in individual pens in a temperature controlled room where feed intake was monitored and pigs were weighed on d 0, 7 and 14 to determine body weight gain. Another group of 4 piglets fed the control diet was kept in a separate room and served as an unchallenged control (UC). All experiments were conducted in the T. K. Cheung Centre for Animal Science Research at the University of Manitoba (Winnipeg, MB).

4.3. Experimental diet preparation

4.3.1. Experiment 1 – diets supplemented with red osier dogwoods or antibiotics

The red osier dogwoods plants grown in Swan River, Manitoba, were harvested in September 2015 and air dried at room temperature. The leaves (60%) and barks (40%) of red osier dogwoods were finely ground together to pass a 1mm sieve.

Four iso- nitrogenous and iso-caloric diets were formulated to meet the nutrient requirement of 7 to 11kg pigs. The dietary treatments were as follows;

a) A corn-wheat and soybean meal-based diet (negative control, **NC**),

b) NC plus 2% dogwoods in the diet (**ROD2**)

c) NC plus 4% dogwoodsin the diet (**ROD4**)

d) NC plus antibiotics (55mg/100g of Aueromycin 220; Positive control, **PC**)

Table 2. The composition of experimental diets of experiment 1 (as fed basis)

Item	NC	ROD2	ROD4	PC
Corn	46.5	45.5	45.5	46.5
Wheat	15.0	14.0	13.0	15.0
Soybean meal	31.0	31.0	30.0	31.0
Red osier dogwood	0	2.0	4.0	0
Vegetable oil	3.0	3.0	3.0	3.0
Limestone	1.0	1.0	1.0	1.0
Monocalcium phosphate	1.4	1.4	1.4	1.4
Iodized salt	0.33	0.33	0.33	0.33
Vitamin-Mineral premix[1]	1.00	1.00	1.00	1.00
Lysine	0.470	0.470	0.470	0.470
DL-Methionine	0.140	0.140	0.140	0.140
Threonine	0.130	0.130	0.130	0.130
Tryptophan	0.03	0.03	0.03	0.03
Aureomycin 220 G	0	0	0	0.055
Calculated nutrient content of experimental diets (% DM)				
Crude protein	20.98	20.93	20.49	20.98
Ether extract	2.2	2.2	2.3	2.2
NDF	9.3	9.3	9.3	9.3
ADF	3.5	3.7	3.8	3.5
Calcium	0.8	0.83	0.82	0.80
Available phosphorus	0.4	0.4	0.4	0.4
Lysine	1.49	1.48	1.44	1.49
Methionine+Cysteine	0.8	0.8	0.8	0.8
Threonine	0.9	0.9	0.9	0.9
Tryptophan	0.3	0.3	0.3	0.3
Valine	1.0	0.9	0.9	1.0

[1]Supplied per kilogram of diet: vitamin A, 8,250 IU; vitamin D3, 825 IU; vitamin E, 40 IU; vitamin K, 4 mg; thiamine, 1 mg; riboflavin, 5 mg; niacin, 35 mg; pantothenic acid, 15 mg; vitamin B12, 25 µg; biotin, 200 µg; folic acid, 2 mg; Cu, 15 mg (copper sulfate); I, 0.21 mg as Ca(IO3)2; Fe, 100 mg (ferrous sulfate); Mn, 20 mg (manganese oxide); Se, 0.15 mg (sodium selenite); and Zn, 100 mg (zinc oxide); NC, corn-soybean meal-wheat based control diet, ROD2, 2% dogwoods in control diet, ROD4, 4% dogwoods in control diet, PC, antibiotic supplemented diet.

4.3.2. Experiment 2 – diets supplemented with EGF or antibiotics

EGF solution (1.89 mg/ml) and supernatant without EGF was received from Department of Animal Biosciences, University of Guelph.

A corn-soybean meal wheat based basal diet was prepared per the NRC (2012) recommendations for 7-11 kg pigs. Composition and the calculated nutrient content of the control diet is indicated in Table 3.

From the basal diet, four dietary treatments were prepared as follows;

Treatment	Description
NC	Corn soy bean meal wheat based control diet + supernatant without EGF
EGF1	NC + EGF solution @ 120 μg/kgBW/day
EGF2	NC + EGF solution @ 180 μg/kgBW/day
PC	NC + Antibiotic (Aureo S-P 250,0.25% feed + supernatant without EGF)

For the control and antibiotic mixed groups, supernatant without EGF were added similar to the volume added to EGF groups for the consistency of all the diets. EGF and supernatant without EGF were mixed with the morning diets each morning just before feeding. The procedure of mixing EGF and supernatant without EGF with diets to obtain the required concentration is amended at the end.

Table 3: Composition of the basal diet of experiment 2 (as-fed basis)

Item	Control diet
Corn	46.5
Wheat	15.0
Soybean meal	31.0
Vegetable oil	3.0
Limestone	1.0
Monocalcium phosphate	1.4
Iodized salt	0.33
Vitamin-mineral premix [a]	1.00
Lysine	0.470
DL-Methionine	0.140
Threonine	0.130
Tryptophan	0.03
Calculated nutrient content of experimental diets (% DM)	
Crude protein	20.98
Ether extract	2.2
NDF	9.3
ADF	3.5
Calcium	0.8
Available phosphorus	0.4
Lysine	1.49
Methionine+cysteine	0.8
Threonine	0.9
Tryptophan	0.3
Valine	1.0

[a] Supplied the following per kilogram of diet:8250 IU of vitamin A, 835 IU of vitamin D3, 40 IU of vitamin E, 25 µg of vitamin B12, 4 mg of vitamin K, 25 µg of niacin, 600 mg of choline, 12 mg of riboflavin, 200 µg of biotin, 4.5 mg of pyridoxine, 4 mg of folic acid, 2 mg of thiamin, 50 mg of Mn, 150 mg of Zn, 120 mg of Fe, 25 mg of Cu, 0.35 mg of Se, 0.4 mg of I.

4.4. Bacterial challenge

Piglets were allowed a 7-day adaptation period to their respective diets and then orally challenged with 6ml of enterotoxigenic *E. coli* (ETEC) K88+ on day 8 (except UC group) as previously reported (Opapeju et al., 2009). This was same for both experiment 1 and 2. The concentration of *E. coli* used for experiment 1 is 5×10^{9} cfu/ml and 2×10^{13} cfu/ml for experiment 2.

4.5. Sample Collection

Blood samples (2 ml) were collected via jugular venipuncture into serum tubes at 0, 6, 24, 48 hours and 6 days after inoculation with *E. coli* K88+ (Fig. 4.1). The serum tubes containing blood were left to stand at room temperature for 3 hours and then centrifuged at 1500 × g for 15 minutes at 4ºC and were stored at -80ºC until required for analysis. Piglets were euthanized to collect tissues on day 14. Intestinal samples were collected from each piglet, washed with saline and immediately frozen in liquid nitrogen until brought into the laboratory and stored in -80 ºC until required for analysis. Sample collection procedure was same for both experiment 1 and 2.

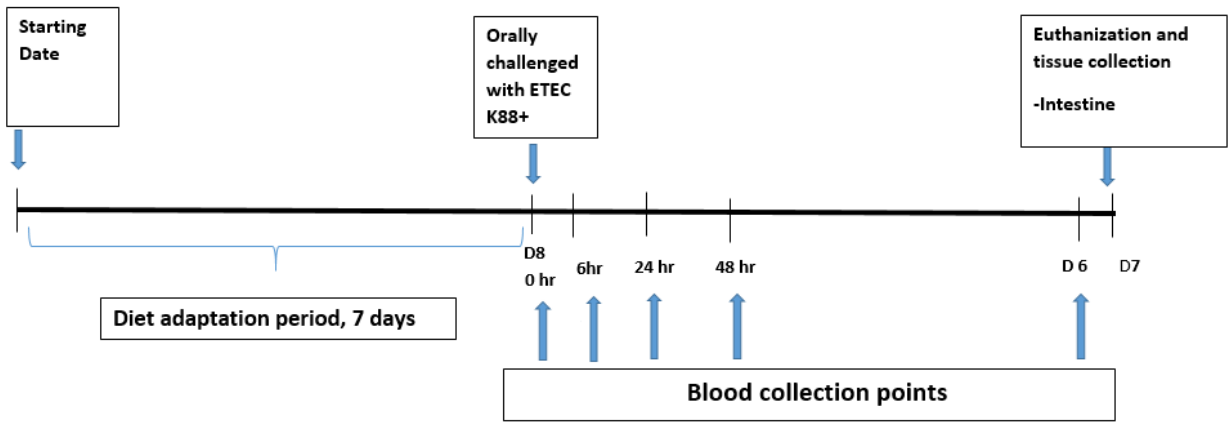


Figure 4.1. Sample collection at different time points

Piglets were allowed for a diet adaptation period of 7 days and challenged with *E. coli* on day 8. Blood samples were collected from each piglet at 0, 6, 24, 48 hours and 6 days after *E. coli* challenge and intestine samples were collected on day 7, upon euthanization.

4.6. Determination of lipid peroxidation

Lipid peroxidation in serum and intestinal tissue was determined via measurement of amounts of thiobarbituric acid reactive substances (TBARS) as previously reported (Yagi, 1976; Sarna et al., 2012) with minor modifications. Briefly, enterocytes were scraped from the ileum and the homogenate was prepared using 0.1 M potassium phosphate buffer (pH = 7.8). The suspension was centrifuged at 3000 ×g for 10 min in 4ºC and an aliquot of supernatant was incubated for 1 hour at 90ºC with 3ml of reaction mixture consisting of 0.8% of thiobarbituric acid, 20% acetic acid, 8% sodium dodecyl sulphate (SDS) and distilled water. The color was extracted using n-butanol and the absorbance of the butanol extract was measured at 532 nm. A series of known concentrations were prepared and measured the absorbance at 532nm as the standard. Total proteins were quantified through the Bradford method and TBA were normalized to protein. TBARS were done for serum and ileum samples from experiment 1 and 2.

Calculation 1

$$\text{TBARS} = \frac{(\text{Absorbance reading} / \text{slope of the standard curve}) \times \text{volume of butanol}}{\text{Amount of protein in sample}}$$

To determine TBARS in serum, an aliquot of serum was mixed with 10% phosphotungstic acid, incubated for 10 min at room temperature and centrifuged at 3000 × g for 10 min in room temperature. Supernatant was removed and pellet was incubated for 1 hour at 90ºC with 500µl of 0.67% TBA. The color was extracted using n-butanol and the absorbance of the butanol extract

was measured at 535 nm. A series of known concentrations were prepared and absorbance was measured the at 535nm. TBARS were expressed as nmol/µl of serum.

Calculation 2

Standard curve was drawn against the known TBARS concentrations of the standards and their respective absorbance.

$$\text{TBARS} = \frac{(\text{Absorbance reading} / \text{slope of the standard curve}) \times \text{volume of butanol}}{\text{Volume of serum used}}$$

4.7. Determination of Superoxide Dismutase (SOD) Activity Levels

SOD activity in ileum and serum collected at the end of the experimental duration in the experiment 1 was determined by measuring the inhibition rate of xanthine oxidase mediated reduction of cytochrome c as previously reported (Hwang et al., 2011; Sarna et al., 2012). Briefly, the scraped enterocytes from the ileum were homogenized in homogenization buffer containing 0.05M potassium phosphate buffer and 0.1M EDTA. The suspension was centrifuged at 15000g for 10 min. Total proteins were quantified by the Bradford method and protein content of each sample was equalized to 5µg/µL.

In a 1mL cuvette, 0.25mL of 0.15mM cytochrome c, 0.15mL of 2mM xanthine, 0.5 mL of homogenization buffer were added and placed in the spectrophotometer. A 5-8 µL aliquot of diluted xanthine oxidase was added to the cuvette and the rate of increase in absorbance was measured and recorded every 15 sec up to 4 min at 550 nm. The amount of cytochrome c and

xanthine oxidase was adjusted to yield the rate of increased absorbance as 0.05 per minute. The amount of cytochrome c and xanthine oxidase to yield such a rate was used as the calibrated amounts for the rest of the procedure and for the calculation.

Calibrated amounts of cytochrome c, 0.15mL xanthine, 20µL of sample, reaction buffer to make the total up to 0.9mL were added in a cuvette at 25ºC. The reaction was initiated by adding calibrated amounts of xanthine oxidase and the rate of change in absorbance at 550nm was measured by measuring the absorbance every 15 seconds for 4 min. Standard was prepared by replacing sample with SOD. SOD was added (10-25U) to yield 50% decrease in the rate of absorbance which was defined as 1U of activity.

Calculation

From the calibration of xanthine oxidase, the ΔAbs per min was determined (ΔXO). From the standards, the ΔAbs per min was determined for different amounts of SOD (i.e., ΔSOD1, ΔSOD2). Percentage of maximum rate of changes in absorbance was calculated for SOD as follows;

$$\% \text{ of maximum rate of change in Abs for SOD1} = (\Delta SOD1/ \Delta XO) \times 100$$

$$\% \text{ of maximum rate of change in Abs for SOD2} = (\Delta SOD2/ \Delta XO) \times 100$$

These % of maximum rate of changes in absorbance were plotted against the units of the SOD in each volume used. Using the slope (M) and the intercept (C) of the standard curve, the units of SOD required for 50% decrease in the rate of change in absorbance was calculated (This amount

was defined as 1 U of SOD activity). Using this value, SOD activity of the samples was calculated using the following formula;

$$100 - \frac{(\Delta Abs / min)\ sample \times 100}{\Delta XO}$$

SOD units required for 1U of activity

4.8. Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA) followed by the Newman Keuls post hos test. Data were presented as means ± SEM. P values less than 0.05 were considered statistically significant.

CHAPTER V

RESULTS

5.1. Experiment 1 - Effect of dietary supplementation of Red osier dogwoods plant products on oxidative stress in *E. coli* challenged weanling piglets

5.1.1. Body weights of the animals

Initial body weights of the piglets were similar when the experiment started. After the diet adaptation period, they had relatively similar body weights. At the end of the experimental duration, the body weights of piglets were also relatively similar among the groups (Table 4)

5.1.2. Lipid peroxidation

As shown in Fig. 5.1.1, serum TBARS levels in the *E. coli* challenged (NC) group and unchallenged control group (UC) at each sample collecting time point were compared. Before challenge and 6 hours after challenge, serum TBARS levels were similar in both groups. At 24 hours after *E. coli* challenge, the serum TBARS levels were significantly increased ($p < 0.05$) in the NC group compared to the UC group. TBARS levels remained elevated until 48 hours and even up to 6 days after *E. coli* challenge ($p < 0.05$). Before and 6 hours after *E. coli* challenge (Fig. 5.1.2(A) & (B), the TBARS levels appeared to be similar in all the groups of piglets, regardless of any dietary treatment. At 24 hours after *E. coli* challenge, serum TBARS levels were increased significantly ($p< 0.05$) not only in the NC group but also in the ROD2 group compared to the UC group. There was no significant difference in serum TBARS levels among the ROD4, PC groups and the UC (Fig. 5.1.2 (C)). Similarly, at 48 hours and 7 days after challenge, respectively, the serum TBARS levels remained elevated ($p < 0.05$) in the NC group and the ROD2 as compared to UC group, whereas the ROD4 group as well as the PC group had similar TBARS levels as the UC (Fig. 5.1.2(D), (E)). Ileum TBARS levels were also

significantly increased ($p < 0.05$) in the NC group and the ROD2 group as compared to the UC group whereas the ROD4 group and the PC group had similar TBARS levels as that in the UC group (Fig. 5.1.3).

5.1.3. SOD activity levels

The antioxidant enzyme SOD activity in the serum and ileum was significantly decreased ($p < 0.05$) in the NC group and the ROD2 group compared to the control group (UC) (Fig. 5.1.4 (A) & (B)). Groups supplemented with ROD4 and PC showed higher SOD activity in the serum and ileum similar to the UC group.

Table 4. Average body weight gain (Kg) of piglets at day 1, 7 and 14 of the experiment 1

Group	Initial BW	BW at day 7	BW at day 14	BW gain
NC	6.91 ± 0.25	7.66 ± 0.25	10.30 ± 0.34	3.39 ± 0.34
ROD2	6.87 ± 0.19	7.27 ± 0.30	9.14 ± 0.71	2.26 ± 0.38
ROD4	6.96 ± 0.17	7.31 ± 0.15	9.83 ± 0.27	2.87 ± 0.30
PC	6.95 ± 0.15	7.66 ± 0.24	9.71 ± 0.45	2.76 ± 0.37
UC	6.98 ± 0.46	7.95 ± 0.74	10.05 ± 0.49	3.07 ± 0.49

NC (corn-wheat soybean meal based control), **ROD2** (NC plus 2% Dogwoods), **ROD4** (NC plus 4% Dogwoods), **PC** (NC plus antibiotics), **UC** (unchallenged control fed NC); **BW**, body weight (expressed in kg ± SEM). The difference of body weight gain within each group was not statistically significant.

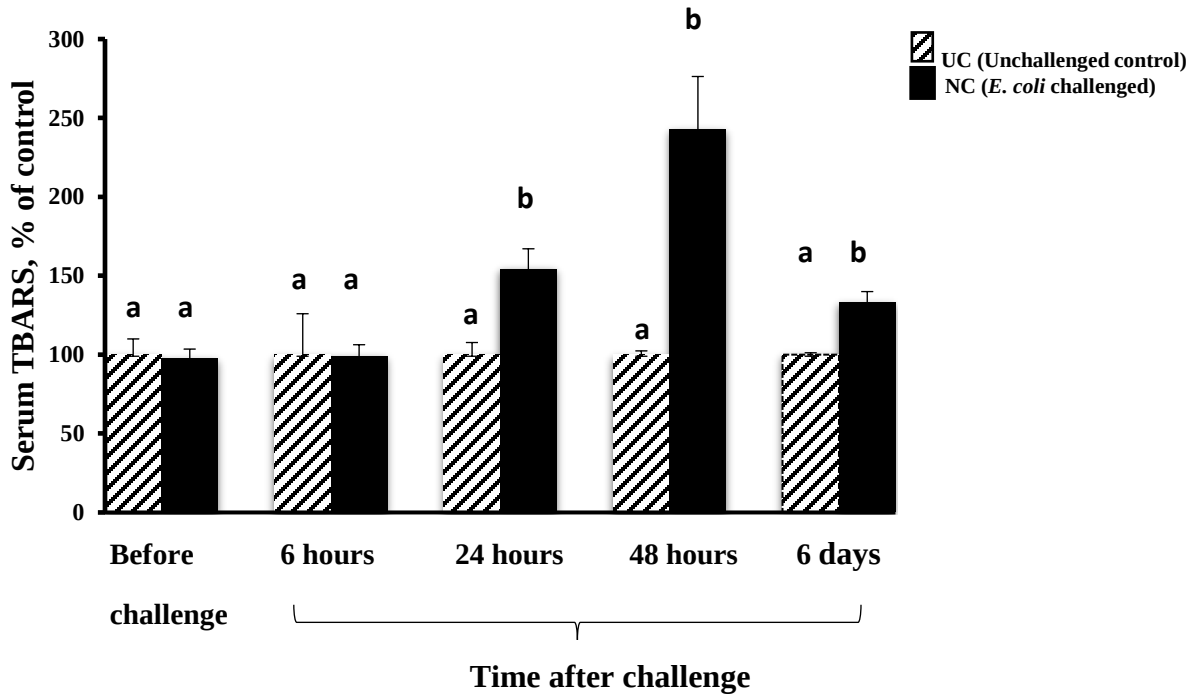
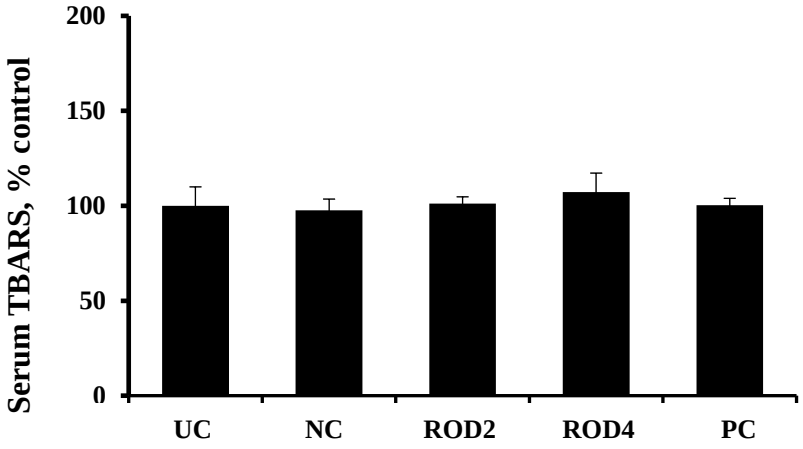


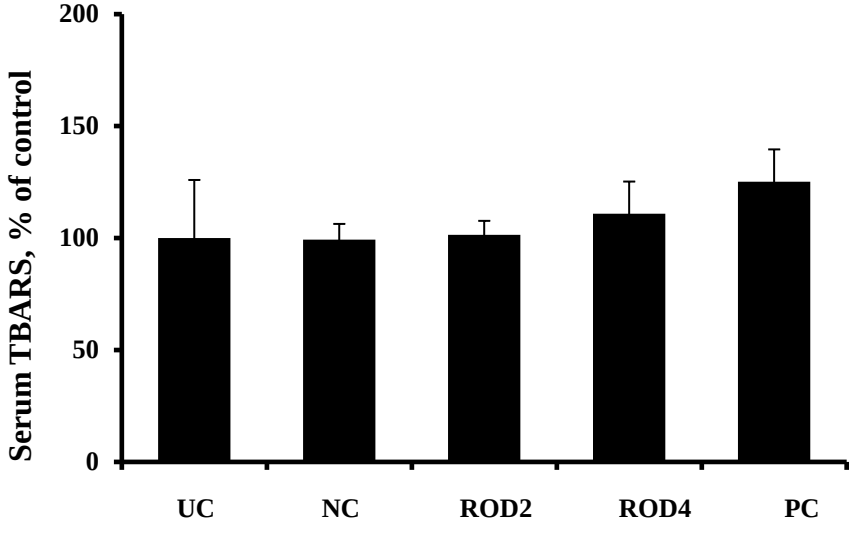
Figure 5.1.1. Comparison of serum TBARS levels in *E. coli* challenged group and unchallenged group at different time points

Serum TBARS levels of NC and UC groups at 0, 6, 24, 48 hours and 6 days after *E. coli* challenge, were determined and compared; NC, corn-wheat soy bean meal based control diet (n = 7); UC, unchallenged control fed NC diet (n = 4); a, b means within a raw with no common subscripts differ ($p < 0.05$). TBARS= thiobarbeturic acid reactive substances

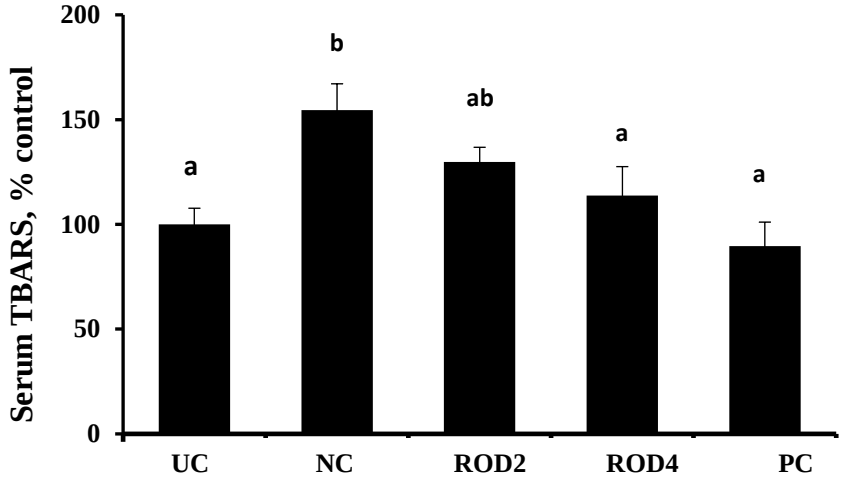
A.


Serum TBARS, % control
200
150
100
50
0
UC
NC
ROD2
ROD4
PC

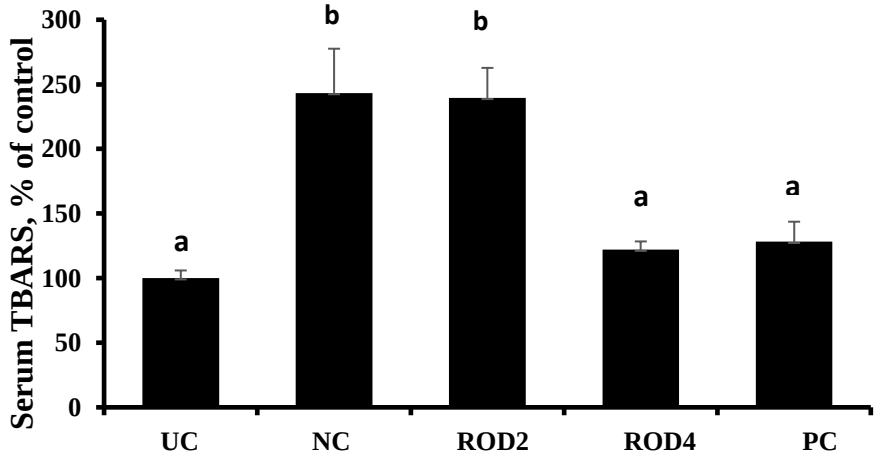
B.


Serum TBARS, % of control
200
150
100
50
0
UC
NC
ROD2
ROD4
PC

C.


Serum TBARS, % control
200
150
100
50
0
a
b
ab
a
a
UC
NC
ROD2
ROD4
PC

D.


Serum TBARS, % of control
300
250
200
150
100
50
0
a
b
b
a
a
UC
NC
ROD2
ROD4
PC

E.

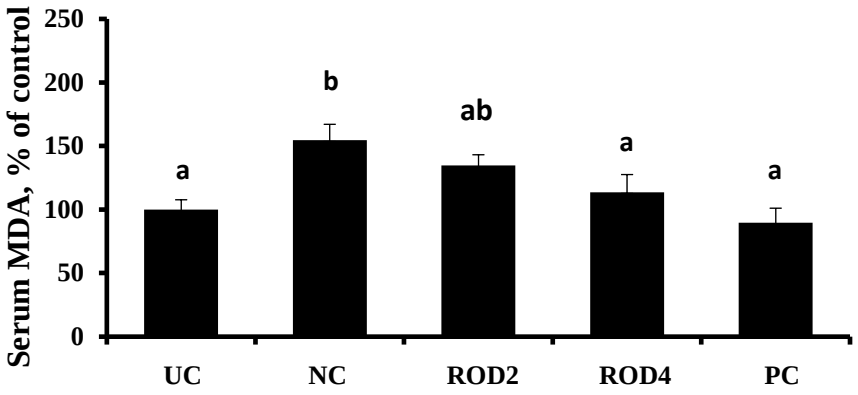


Figure 5.1.2: Serum TBARS levels at different time points.

Serum TBARS levels in all the experimental groups were determined for each time point. NC, corn-wheat-soybean meal based control diet (n = 7); ROD2, NC plus 2% dogwoods (n = 7); ROD4, NC plus 4% dogwoods (n = 7); PC, NC plus antibiotics (n = 7); UC, unchallenged control fed NC (n = 4). (A) Serum TBARS levels before *E. coli* challenge, (B) Serum TBARS levels at 6 hours after *E. coli* challenge, (C) Serum TBARS levels at 24 hours after *E. coli* challenge, (D) Serum TBARS levels at 48 hours after *E. coli* challenge, (E) Serum TBARS levels at 6 days after *E. coli* challenge; a, b means within a raw with no common subscripts differ ($p < 0.05$). TBARS= thiobarbeturic acid reactive substances

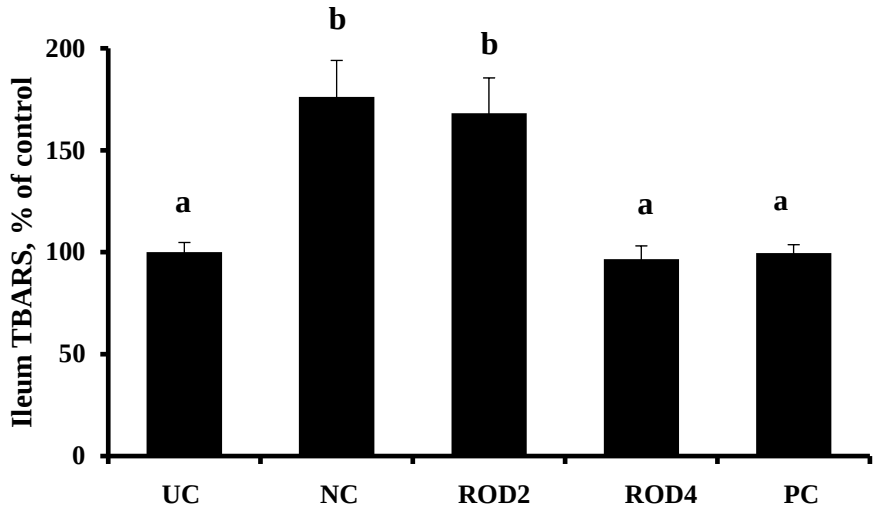
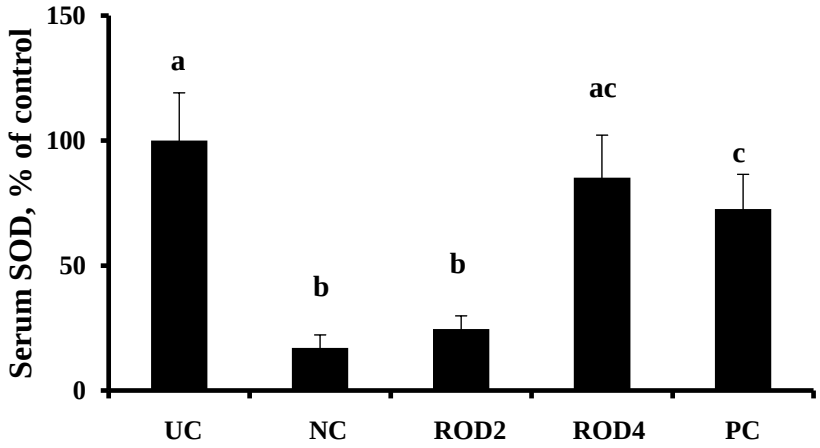


Figure 5.1.3. Ileum TBARS levels at 7 days after *E. coli* challenge.

Ileum TBARS levels at 7 days after *E. coli* challenges were determined. NC, corn-wheat & soybean meal based control diet (n = 7); ROD2, NC plus 2% dogwoods (n = 7); ROD4, NC plus 4% dogwoods (n = 7); PC, NC plus antibiotics (n = 7); UC, unchallenged control fed NC (n = 4); a, b means within a raw with no common subscripts differ ($p < 0.05$). TBARS= thiobarbeturic acid reactive substances

A.



B.

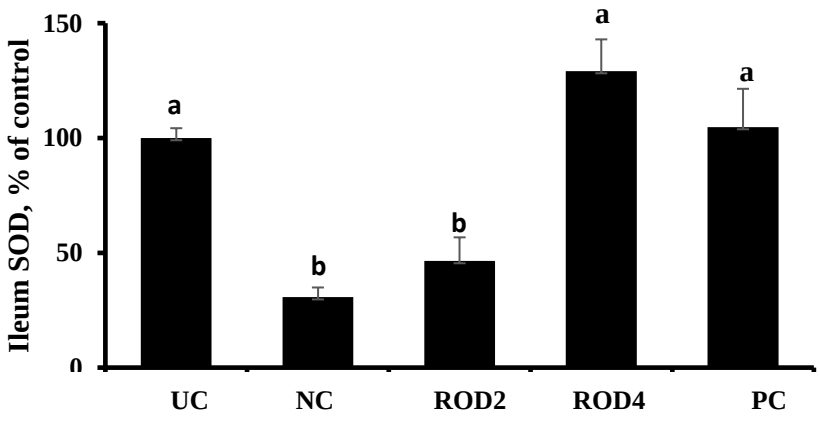


Figure 5.1.4. SOD activity levels in serum and ileum

Serum and ileum SOD levels at 6 days and 7 days after *E. coli* challenge respectively were determined. NC (corn-wheat soybean meal based control diet, n = 7); ROD2 (NC plus 2% dogwoods, n = 7); ROD4 (NC plus 4% dogwoods, n = 7); PC (NC plus antibiotics, n = 7); UC (unchallenged control fed NC, n = 4). (A) Serum SOD levels at 6 days after *E. coli* challenge, (B) Ileum SOD levels at 7 days after *E. coli* challenge; a, b, c means within a raw with no common subscripts differ ($p < 0.05$). SOD = superoxide dismutase.

5.2. Experiment 2 - Effect of dietary supplementation of epidermal growth factor on oxidative stress in *E. coli* challenged weanling piglets.

5.2.1. Body weight gain

Initial body weights of the piglets were similar when the experiment was started. After the diet adaptation period, PC as well as EGF1, EGF2 groups had relatively higher bodyweights compared to NC. At the end of the experimental duration also, the body weights of piglets in EGF supplemented groups i.e. EGF1 and EGF2 as well as antibiotic supplemented group (PC) were higher compared to NC group. Overall bodyweight gain was significantly increased in EGF2 and PC groups compared to other groups (Table 5).

5.2.2. Lipid peroxidation

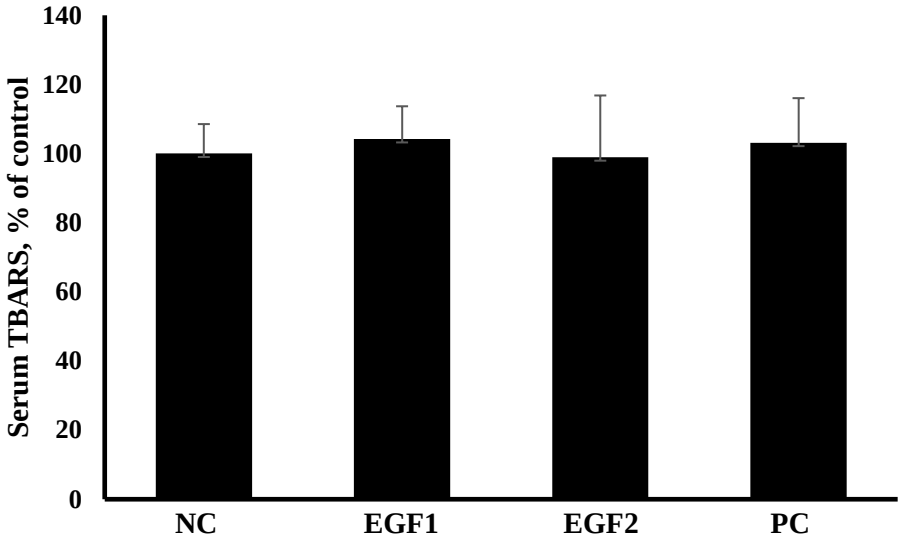
TBARS levels in the serum of piglets were compared at different time points of the experiment i.e. 24 hours, 48 hours and 6 days after *E. coli* challenge. As indicated in Fig. 5.2.1(A), there is no change in TBARS levels between treatment groups at 24 hours after *E. coli* challenge. At 48 hours after *E. coli* challenge, NC and EGF2 groups had elevated levels of TBARS compared to the EGF2 and PC groups even though the data is not statistically significant (Fig. 5.2.1(B)). However, at 6 days after *E. coli* challenge (Fig. 5.2.1(C)), EGF1, EGF2 and PC groups showed significantly lower levels of TBARS than the NC group. Similar trend was observed in the ileum TBARS levels i.e. EGF1, EGF2 and PC groups had significantly lower levels of TBARS than NC group as showed in Fig. 5.2.2.

Table 5. Average bodyweight gain of piglets at day 1, 7 and 14 of experiment 2

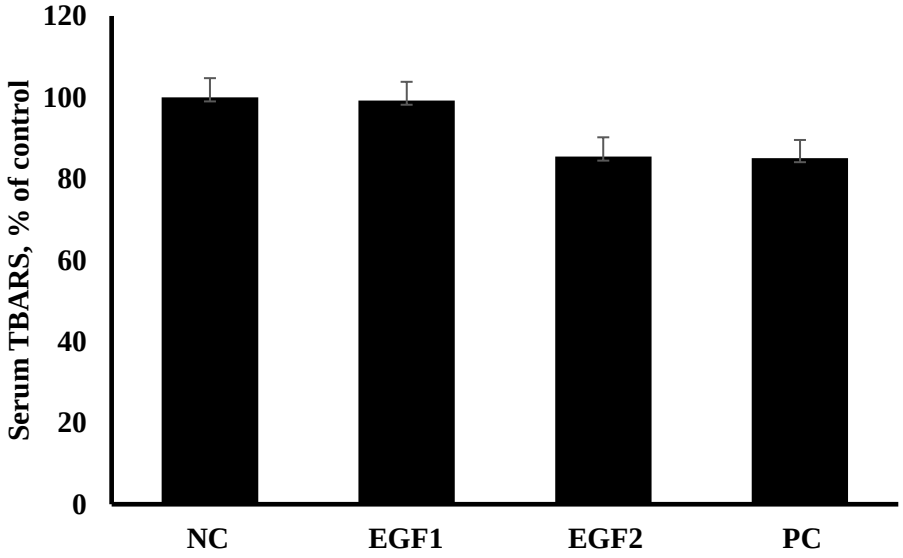
Group	Initial BW	BW at day 7	BW at day 14	BW gain
NC	6.39 ± 0.20	7.01 ± 0.10	8.44 ± 0.16	2.05 ± 0.14[a]
EGF1	6.39 ± 0.13	7.23 ± 0.22	8.79 ± 0.21	2.40± 0.21[a]
EGF2	6.39 ± 0.17	7.29 ± 0.24	8.89 ± 0.24	2.51± 0.11[b]
PC	6.40 ± 0.18	7.38 ± 0.20	8.97 ± 0.22	2.57 ± 0.16[b]
UC	6.41 ± 0.13	7.17 ± 0.33	8.82 ± 0.29	3.41± 0.17[b]

NC, *E. coli* challenged group fed control diet; **EGF1**, *E. coli* challenged group fed NC + EGF solution @120 µg/kgBW/day; **EGF2**, *E. coli* challenged group fed NC + EGF solution @180 µg/kgBW/day fed; **PC**, *E. coli* challenged group fed NC + Antibiotic (Aureo S-P 250,0.25% feed; **UC**, unchallenged group fed control diet. a, b means within a raw with no common subscripts differ significantly ($p < 0.05$); BW, body weight (expressed in kg ± SEM)

A)


Serum TBARS, % of control
140
120
100
80
60
40
20
0
NC
EGF1
EGF2
PC

B)


Serum TBARS, % of control
120
100
80
60
40
20
0
NC
EGF1
EGF2
PC

C)

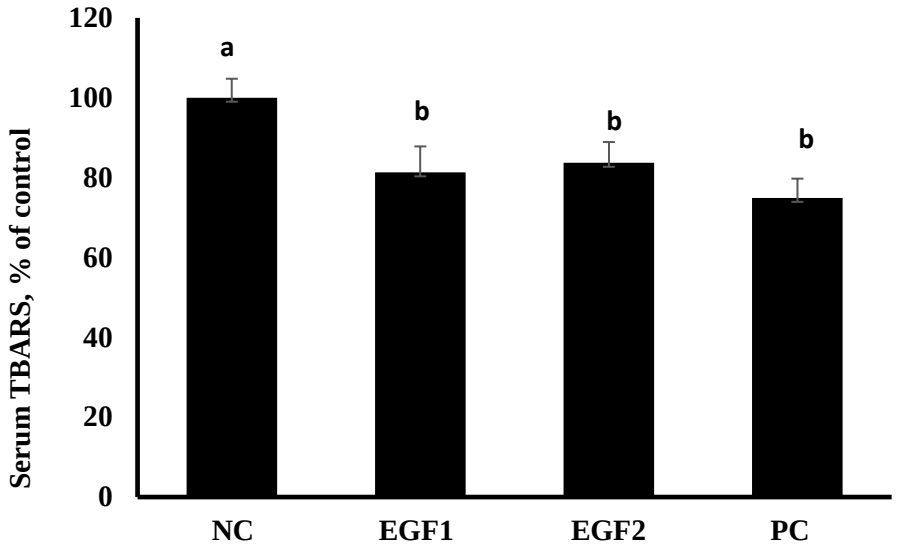


Figure 5.2.1. Serum TBARS levels at different time points

Serum TBARS levels in all the experimental groups were determined for 3 time points. NC, negative control, *E. coli* challenged group fed corn-wheat soybean meal based control diet (n = 7); EGF1, NC plus EGF solution @120 µg/kgBW/day (n = 7); EGF2, NC plus EGF solution @180 µg/kgBW/day (n = 7); PC, positive control, NC plus antibiotics (n = 7). (A) Serum TBARSlevels at 24 hours after *E. coli* challenge, (B) Serum TBARSlevels at 48 hours after *E. coli* challenge, (C) Serum TBARS levels on day 6 after *E. coli* challenge; a, b means within a raw with no common subscripts differ ($p < 0.05$). TBARS= thiobarbeturic acid reactive substances

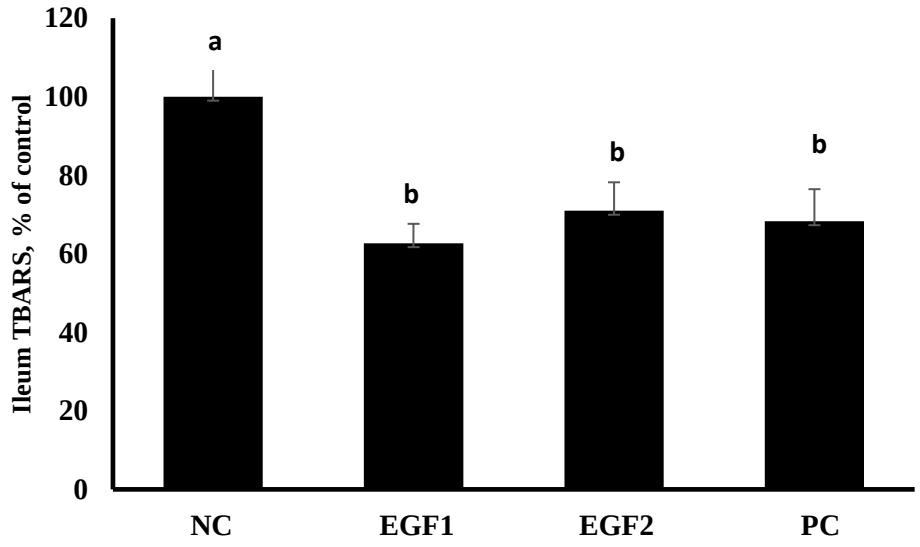


Figure 5.2.2. Ileum TBARS levels at 7 days after *E. coli* challenge.

Ileum TBARS levels on day 7 after *E. coli* challenge were determined. NC, negative control, *E. coli* challenged group fed corn-wheat & soybean meal based control diet (n = 7); EGF1, NC plus EGF solution @120 μg/kgBW/day (n = 7); EGF2, NC plus EGF solution @180 μg/kgBW/day (n = 7); PC, positive control, NC plus antibiotics (n = 7); a, b means within a raw with no common subscripts differ ($p < 0.05$). TBARS= thiobarbeturic acid reactive substances

CHAPTER VI

DISCUSSION

6.1. Study 1 – Effect of dietary supplementation of red osier dogwoods plant products on oxidative stress in *E. coli* challenged weaned piglets

6.1.1. *E. coli* challenge caused oxidative stress in piglets

Weaning is a critical period in life time of pigs due to poor intestinal barrier functions, compromised immune functions and increased disease susceptibility arising from sudden separation of piglets and mothers which expose the piglets to completely different environmental, social, and nutritional conditions (Heo et al., 2013). During this critical period, post weaning diarrhea is a major risk factor for piglet death. Among several other factors, *E. coli* infection is considered as a major cause for post weaning diarrhea in piglets (Fairbrother et al., 2005; Heo et al., 2013). This diarrhea is responsible for economic losses due to mortality, morbidity, decreased growth rate, and cost of medication. Post weaning diarrhea due to *E. coli* is caused primarily by ETEC, a pathotype that is characterized by production of adhesins. *E. coli* adheres to the intestine of piglets using these adhesins, which facilitates the colonization and enterotoxin production. These enterotoxins can induce ion imbalance across the intestinal wall and lead to severe fluid loss leading to severe diarrhea (Fairbrother et al., 2005). Several physiological, immunological, morphological and nutritional aspects have been studied regarding *E. coli* infection and piglet diarrhea. However, to our knowledge, studies are rarely done to examine the changes in oxidative status of piglets due to *E. coli* infection.

Oxidative stress, which is the imbalance of oxidants and antioxidants in the body is a critical factor associated with many pathological conditions in humans and animals. Changes in oxidative status and antioxidant defense mechanism have been previously studied in swine in correlation with many aspects such as diet, weaning, reproduction etc. (Zhu et al., 2012; Zhong and Zhou, 2013; Lu et al., 2014). In a previous study, compromised antioxidant defense system

was also observed in weanling piglets that were subjected to *E. coli* challenge (Jiang et al., 2014). In the present study, we examined the effect of *E. coli* infection on oxidative status of weaned piglets. As an oxidative stress biomarker, TBARS levels in serum and small intestine were measured to determine the oxidative stress. TBARS, which is a measure of lipid peroxidation is widely used as a biomarker for oxidative stress in many studies (Jiang et al., 2011; Sarna et al., 2012; Alizadeh et al., 2015). TBARS levels are elevated during oxidative stress due to lipid peroxidation that takes place in cells when free radical levels are increased. In the present study, when the piglets were challenged with *E. coli*, we observed increased TBARS levels in the serum and the small intestine in piglets compared to the unchallenged piglets. These results suggest that there were elevated levels of reactive oxygen species in the intestine and blood circulation in piglets after *E. coli* challenge which resulted in lipid oxidation and elevated TBARS levels. Some previous studies also reported that pathogenic infection induced production of free radicals in the host. *Helicobacter pylori* infection induced the production of reactive oxygen species including superoxide ion and hydroxyl radical, in human gastric mucosal cells (Bagchi et al., 1996; Ding et al., 2007)

ROS production in the GI tract is a key factor in the development of inflammatory disorders. The GI tract acts as a physical barrier preventing pathogens from entering the body and confining them in the lumen. Intestinal mucosa can provide inflammatory signals to infectious pathogens while producing tolerance signals to commensal harmless bacteria. When there is an inflammatory signal due to an invading pathogen, the general defense mechanism is activated. This includes secretion of polysaccharides by goblet cells, release of antimicrobial peptides by Paneth cells and secretion of antibodies by circulating blood cells (Heo et al., 2013). But when integrity of the intestinal barrier is destroyed it would lead to initiation of inflammatory response.

Altered intestinal barrier function activates phagocytes and ROS production for antimicrobial defense. Meantime, intestinal epithelial cells also produce ROS which regulate intracellular signals for barrier repair and healing. Therefore, the initial response of ROS production might be beneficial to counteract the invading pathogen. However, uncontrolled generation of ROS and inadequate removal of ROS by antioxidants will alter the redox balance leading to the trigger of pathological processes. Prolonged over-production and accumulation of these reactive species in the cells lead to oxidative stress and eventually can modify cellular biomolecules including protein, lipids and DNA to cause tissue damage.

In the present study, the elevated levels of reactive species detected by TBARS method in intestine and serum, might be due to the activation of immune cells against the invading *E. coli* pathogen. Attachment of *E. coli* pathogen to the intestinal wall and their multiplication in the intestine might have initiated an inflammatory response and thereby led to the overproduction of ROS by the activated phagocytes and epithelial cells. However, prolonged accumulation of ROS might pose a detrimental effect to the host.

Furthermore, we measured the antioxidant enzyme SOD in serum and ileum of piglets. *E. coli* challenge affected the intestinal and serum SOD activity as well. SOD is a major antioxidant enzyme which catalyses the dismutation of super oxide ($O^{2}-$) radical into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) (Valko et al., 2006; Valko et al., 2007). Therefore, SOD is a major component in the endogenous antioxidant defense system. In this study, SOD activity levels were significantly decreased in serum as well as in the ileum of piglets at seven days after *E. coli* challenge. This might be due to the reason that SOD was used up for quenching the over produced free radicals induced by *E. coli* infection or the expression of SOD protein was attenuated. These observations suggest that *E. coli* infection increases the

generation of free radicals and attenuates the antioxidant enzyme defense system in piglets. It supports our hypothesis, i.e. weaned piglets that were subjected to *E. coli* challenge, suffered from oxidative stress. Furthermore, it prevailed until one week after *E. coli* challenge i.e. the end of the experimental duration, indicating prolonged oxidative stress in piglets.

6.1.2. Antioxidant effects of dietary supplementation of antibiotics and red osier dogwoods plant extracts

In the swine industry, antibiotics have been widely used as a growth promoter and immune modifier to mitigate *E. coli* infection after weaning (Heo et al., 2013). Recently, there have been concerns for antibiotic usage in animal diets due to development of bacterial resistance to antibiotics. In the present study, we observed that antibiotic supplementation in the piglet diet had an inhibitory effect on oxidative stress, as indicated by lower levels of serum and intestine TBARS levels as well as restored levels of SOD enzyme activity in antibiotic supplemented piglets. This might be due to prevention of bacterial infection in the piglets by the antibiotics. When the bacterial infection was prevented, the generation of free radicals was also attenuated. However, due to the recent concerns of usage of antibiotics in animal feed, the need for finding alternatives to replace antibiotics has been raised. More attention has been given to natural herbs and plant extracts to be included in animal diets as alternatives to antibiotics as well as antioxidant supplements (Wu et al., 2006; Zhong and Zhou, 2013). In recent years, researchers have been exploring the properties of Asian and native American traditional herbal plants, which have been known for centuries to play a vital role in health and nutrition (Bye and Linares, 1999). Polyphenols and secondary metabolites that are present in these plants have been reported

to show beneficial effects in food products and in mammalian metabolism (Wenk, 2003; Zhong and Zhou, 2013; Wu et al., 2014).

Red osier dogwoods (*Cornus stolonifera/ Cornus sericea*) has been used as a traditional medicine by native Americans to treat diarrhea, fever and skin diseases (Ritch-Krc et al., 1996; Obomsawin, 2007; Tushingham and Eerkens, 2016). This plant materials (barks and leaves) have been demonstrated to contain total phenolic concentrations ranging from 40 to 220 mg/g depending on the season, highest in the month of August, and these include anthocyanins, gallic acid, ellagic acid, quercentin, kaempferol and cyanidin (Isaak et al., 2013). Gallic acid has been shown to have antioxidative capacity in mice (Rasool et al., 2010) whereas ellagic acid has induced apoptosis in cancer cells (Losso et al., 2004). Moreover, quercetin has been reported to reduce inflammation and oxidative damage caused by *Helicobacter pylori* in the mucosa of guinea pigs (González-Segovia et al., 2008) whereas anthocyanins has shown antioxidative properties in piglets (Mason et al., 2005). A dried ground mixture of barks and leaves was used in this study as the plant supplement. The composition of barks and leaves in the mixture were 40% and 60%, respectively. These levels were determined based on the levels used in our previous study, to achieve the similar phenolic content as the previous study (Isaak et al., 2013). The dogwoods levels (2% and 4%) were determined as dietary supplementation levels based on previous studies that used plant extracts in piglet diets (Manzanilla et al., 2004; Manzanilla et al., 2009). We observed that the dietary polyphenolic concentration of above mentioned studies ranges from 0.1% up to around 1%. The levels of polyphenolics in 2% and 4% dogwoods supplemented diets in the present study were 0.18% and 0.36% respectively. These calculations were done based on the polyphenolic concentration of the dogwoods powder comprised of barks (40%) and leaves (60%) as reported in our previous study (Isaak et al., 2013). According to that,

polyphenolic content of the dogwoods mixture was calculated as 90mg/g of air-dried sample which was the polyphenolic content of the dogwoods mixture in September, because the dogwoods plants we used in this study also were harvested in September 2015.

We observed in the present study that dietary addition of red osier dogwoods plant material had inhibitory effects on oxidative stress indicated by lower levels of MDA and restored levels of SOD activity in the treated piglet groups. However, the mechanisms responsible for these changes are yet to be identified in future studies. Supplement of 4% red dogwoods in the diet appeared to have a favourable effect in tackling oxidative stress. Comparatively higher levels of quercetin and ellagic acid that were detected in dogwoods added diets in the present study with highest in 4% dogwoods added diet corresponded well with these observations (data not shown). Even though 2% dogwoods supplemented diet also contained higher levels of quercetin and ellagic acid when compared to non-supplemented diets, these levels appeared to be less effective in alleviating oxidative stress in *E. coli* challenged piglets. This study can be extended in future by supplementing diets with polyphenolic extractions. As 4% plant mixture in the diet is too higher as a feed additive, specifically in industrial situations, water extracts of the polyphenolics of this plant can be used in future studies to reduce the amount of additives included in diets. The polyphenolic compounds from plants have also displayed antimicrobial properties in many animal models (Aziz et al., 1998; Rauha et al., 2000; González-Segovia et al., 2008). However, in the present study, we did not examine the antimicrobial effects of polyphenols in dogwoods extracts. Further studies are warranted to investigate antimicrobial effects of polyphenolics in dogwoods extracts.

Furthermore, dietary supplementation of this plant material instead of antibiotics did not alter the feed intake, growth performance of piglets during the experimental period. Therefore, this

plant is a good source of antioxidants and shows the potential as an alternative for in-feed antibiotics to be used in the diets of the weaned piglets. Moreover, in the present study, we mainly focused on the polyphenolic compounds that were present in the plant mixture. Apart from the polyphenolic compounds, the rest of the compounds in the mixture such as chlorophylls, starch, fiber could have certain impact on alleviating infection or oxidative stress in the piglet gut. The antioxidant potential of other components in this plant remains to be investigated in future studies.

6.2. Study 2 – Effect of dietary supplementation of epidermal growth factor on oxidative status of *E. coli* challenged weanling piglets

Due to the current trend in animal production that favor elimination of in feed antibiotics, developing alternative strategies to stimulate growth, health and performances of livestock has become imperative. In swine industry, antibiotics played a major role in diets of post weaning piglets by preventing infections such as *E. coli* an organism that causes remarkable losses in the swine industry due to post weaning diarrhea. Therefore, restrictions and concerns for antibiotic usage in piglet diets have caused a huge challenge in controlling weaning related health complications. Per many recent studies, supplementation of epidermal growth factor to weanling piglets is showing promising evidences in growth and health promotion of weanling piglets. Epidermal growth factor (EGF) is a single chain polypeptide comprising 53 amino acids with a molecular weight of 6kDa. Maternal colostrum and milk are the main sources of intestinal EGF and sow milk contains around 124 µg/ L of EGF. Weaning can cause decreased intake of EGF due to the removal of piglets from sow milk. This could be one of the causes for depressed digestive and absorptive functions and decreased mucosal defenses in weanling piglets.

Systemic or oral administration of EGF to new born or weanling piglets regulated enterocyte differentiation as indicated by increased jejunal lactase and sucrose enzyme activities (Jaeger et al., 1990). Supplementation of EGF to piglets has been shown to facilitate recovery from rotavirus intestinal infection (Donovan et al., 1994). It has been suggested that EGF stimulates DNA and protein synthesis in enterocytes (Chang and Chao, 2002). Furthermore, it has been demonstrated that EGF protects the neonatal intestine by inhibiting apoptosis, correcting intestinal and liver tight junction protein alteration induced by proinflammatory cytokines (Khailova et al., 2009). It has also been reported that EGF facilitates protection against intestinal pathogens (Buret et al., 1998). They reported that orally administered EGF protected against enterotoxigenic *E. coli* infection in rabbits. It appears that EGF has many beneficial effects over intestinal function and protection from infection. Therefore, among other alternatives which are being investigated to replace antibiotics in swine diets, EGF is becoming a potential candidate.

As direct administration of recombinant EGF is very costly, an alternative method to deliver EGF to the intestine has been developed by generating EGF-expressing *Lactococcus lactis* (EGF-LL) using recombinant technology (Kang et al., 2010). When these were orally gavaged to early weaned mice, EGF-LL also survived throughout the intestinal tract and enhanced intestinal structure and body weight gain (Cheung et al., 2009). When gavaged to early weaned piglets, it also survived in gastrointestinal tract of pigs and enhanced intestinal development (Kang et al., 2010). In the present study, we investigated how this EGF-LL supplementation to early weaned piglets could affect the oxidative stress in *E. coli* challenged weanling piglets. *E. coli* infection induced the free radical generation in the piglets. The elevated lipid peroxidation prevailed until the end of the experiment as indicated by higher levels of TBARS in the ileum and serum of *E. coli* challenged group when compared to the unchallenged group. Dietary supplementation of

EGF-LL showed some inhibitory effects against lipid peroxidation as indicated by lower TBARS levels. It seems that EGF-LL has some inhibitory effects on oxidative stress. This might be due to the prevention or attenuation of *E. coli* infection in piglets. However, the effect was more obvious at the later stages of the experiments i.e. on day 6 and on day 7 after *E. coli* challenge. One earlier study reported that EGF could protect against *E. coli* infection in rabbits (Buret et al., 1998). Therefore, one possible mechanism could be that EGF in the diet might prevent *E. coli* infection in piglets. EGF might have promoted the cell growth and differentiation in the intestine; therefore, the piglets might have well developed and proper functioning intestinal immune cells such that they became less susceptible to *E. coli* infection. Therefore, there was no excessive generation of ROS due to *E. coli* challenge. Another possibility could be that EGF might alter the expression of certain enzymes that prevent ROS generation or enhance the antioxidant defense mechanism in piglets. The actual mechanism that causes these effects is yet to be identified in further studies. The effect was not obvious at the early stages may be because the supplemented concentrations of EGF-LL was not sufficient enough to exert more obvious effects when the infection was severe at early stages. Also, the mode of supplementation i.e. dietary supplementation might not be effective as the oral gavage used in previous studies (Buret et al., 1998; Kang et al., 2010). Therefore, future studies are warranted with different EGF- LL delivering modes to the piglets or the use of higher dosages.

CHAPTER VII

CONCLUSION AND FUTURE PERSPECTIVES

7.1. Study 1 - Antioxidant effects of dietary supplementation of antibiotics and Red osier dogwoods plant extracts

Results from study 1 demonstrated that *E. coli* infection in weaning piglets elicited intestinal and systemic oxidative stress. Dietary supplementation of red osier dogwoods plant materials or antibiotics can alleviate oxidative stress developed due to the *E. coli* infection. Future studies are warranted to explore the potential of plant extracts as a dietary additive in the swine industry.

Several limitations were observed in the present study as well. First, we were unable to determine the lipid peroxidation levels in the intestinal cells at each time point, as no intestinal samples were collected at different time points. We had limited number of animals per experimental group so that it was challenging to euthanize animals at different time points. Therefore, in future experiments, it is worthwhile to collect intestinal samples at different time points as done for the blood to examine any changes in lipid peroxidation levels in the intestinal cells due to *E. coli* infection. Secondly, *E. coli* challenge induced oxidative stress in piglets shortly after inoculation. This induced oxidative stress seems persistent as we could detect increased levels of oxidative stress markers even after 1 week of inoculation. However, piglets were challenged with *E. coli* only once, but increased number of viable pathogens due to their multiplication in the intestinal epithelium might have induced the activation of immune cells to produce more reactive species. Therefore, it is important to investigate exactly for how long this oxidative stress can prevail in piglets with the *E. coli* inoculation and what effects it can cause.

Dietary dogwoods supplementation showed inhibitory effects against free radicals as indicated by altered levels of oxidative stress markers in the supplied groups. Piglets consumed the diets daily so that they received the dogwoods extract treatment every day. Therefore, dogwoods extract in the diet might have either supressed bacterial growth or multiplication, inhibited

generation of reactive species or promoted antioxidant activity. Future experiments are warranted to investigate whether these inhibition of oxidative stress is due to antibacterial effects or antioxidative effects of dogwoods compounds

7.2. Study 2- Effect of dietary supplementation of epidermal growth factor on oxidative status of *E. coli* challenged weanling piglets

In this study, it further confirmed that *E. coli* infection causes systemic and local oxidative stress in weaned piglets and it was prolonged until the end of the experiment. However dietary supplementation with EGF-LL seemed to have beneficial effects on oxidative stress in piglets that are challenged with *E. coli*. The effects of EGF-LL supplementation were not obvious in early stages. Therefore, further studies are needed such as increasing the dosage of EGF-LL in the diets of piglets and by applying different delivering methods to the piglets such as oral gavage and gastric intubation to obtain more obvious effects.

CHAPTER VII
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CHAPTER IX

APPENDIX

Research Protocol - Evaluating antimicrobial and antioxidative potential of EGF on *E. coli* challenged weanling piglets

EGF Supernatant provided

1. EGF supernatant in 7 tubes (10 mL each, 1 tube each day) for week-1 of the trial and 7 tubes (10 mL each, 1 tube each day) for week-2 of the trial (7 tubes with red cap and 7 tubes with blue cap to avoid confusion). One additional tube with EGF supernatant is provided as a backup.

2. Supernatant without EGF i.e. control (160 mL, 40 mL in each tube) for Trt-1 and Trt-4 (you may discuss and decide that Trt-4 need to feed with Antibiotic + supernatant or only Antibiotic).

3. Store the tubes at -80C/-20C after receiving.

Making working supernatant and mixing with feed

1. On the day 1 of the trial, take one tube labeled W-1 and thaw at room temperature (you may transfer one tube from -80C/-20C to 4C one day before use to thaw).

2. The treatments will be given at a fixed time (9-9.30 am) with the morning diet (100 g/d/pig for wk 1 and 150 g/d/pig for wk 2). Evening diet (2.30-30 pm) will be a regular one without any treatment.

3. After thawing, shake well for mixing and aliquot the 4 mL of the EGF supernatant for Trt-2 (120 µg/kgBW/day) and make up the volume to a certain amount (say 90 mL) with distilled water. This final volume 90 mL will be used to mix with the feed of 9 pigs (Trt-2). We have already adjusted the concentration of EGF in our lab.

4. There are two ways one can mix the supernatant to the feed: (i) we may mix 10 mL to the feed of individual pig (90 mL for 9 pigs) or (ii) we can mix 90 mL to the feed of 9 pigs and then equally distribute the feed to individual pigs. The final make-up volume could be increased or decreased as per need, please just keep in mind that the supernatant is uniformly mixed in the feed and also it should not be too much to wet the feed in excess, as piglets don't like feed if the feed is too moist.

5. Rest 6 mL of the EGF supernatant will be used for Trt-3(180 µg/kgBW/day) and feed to the 9 pigs as discussed in point 3 and 4.

6. For Trt-1 and 4, five mL supernatant without EGF for each treatment can be used and fed to pigs as discussed in point 3 and 4.

7. In summary, for each day 5 mL supernatant without EGF(control) for Trt-1, 4 mL EGF supernatant for Trt-2, 6 mL EGF supernatant for Trt-3 and 5 mL supernatant without EGF (control) for Trt-4 plus antibiotic will be used.

d 1-7	Pigs/Trt	EGF tube type/cap	Dose	Preparation	Comments
Trt 1	7	Blue cap (Supernatant without EGF)	5 mL/Trt/day	5 mL + 85 mL Distilled water =90 mL (Use only 70 mL)	With morning feed (10 mL/pig/day)
Trt 2	7	Blue cap (Supernatant with EGF)	4 mL/Trt/day	4 mL + 86 mL Distilled water =90 mL (Use only 70 mL)	With morning feed (10 mL/pig/day)
Trt 3	7	Blue cap (Supernatant with EGF)	6 mL/Trt/day	6 mL + 84 mL Distilled water =90 mL (Use only 70 mL)	With morning feed (10 mL/pig/day)
Trt 4	7	Blue cap (Supernatant without EGF) + 0.25% Antibiotic	5 mL/Trt/day	5 mL + 85 mL Distilled water =90 mL (Use only 70 mL)	With morning feed (10 mL/pig/day)
d 8-14					
Trt 1	7	Blue cap (Supernatant without EGF)	5 mL/Trt/day	5 mL + 85 mL Distilled water =90 mL (Use only 70 mL)	With morning feed (10 mL/pig/day)
Trt 2	7	Red cap (Supernatant with EGF)	4 mL/Trt/day	4 mL + 86 mL Distilled water =90 mL	With morning feed (10 mL/pig/day)

				(Use only 70 mL)	
Trt 3	7	Red cap (Supernatant with EGF)	6 mL/Trt/day	6 mL + 84 mL Distilled water =90 mL (Use only 70 mL)	With morning feed (10 mL/pig/day)
Trt 4	7	Blue cap (Supernatant without EGF) + 0.25% Antibiotic	5 mL/Trt/day	5 mL + 85 mL Distilled water =90 mL (Use only 70 mL)	With morning feed (10 mL/pig/day)
On d 7= *E. coli* Challenge On d 14= Euthinization of pigs					