Impacts of feeding peroxidized oils on growth and oxidative status in swine and poultry

by

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The student author and the program of study committee are solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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LIST OF ABBREVIATIONS

8-OH-2dG = 8-hydroxy-2’-deoxyguanosine

AAFCO = Association of American Feed Control Officials

ADFI = average daily feed intake

ADG = average daily gain

AMP = adenosine monophosphate

AnV = p-anisidine value

AOCS = American Oil Chemists Society

AOM = active oxygen method

ATP = adenosine triphosphate

BW = body weight

CAT = catalase

CO_2 = carbon dioxide

CoA = Coenzyme A

DDE = 2,4-decadienal

DE = digestible energy

DNA = deoxyribonucleic acid

ESR = electron spin resistance

ETC = electron transport chain

FA = fatty acid

FAD = flavin adenine dinucleotide

$FADH_2$ = flavin adenine dinucleotide reduced

FRAP = ferric reducing antioxidant power

GE = gross energy

G:F = gain-to-feed ratio

GPx = glutathione peroxidase

GSH = glutathione

H_2O_2 = hydrogen peroxide

HCl = hydrochloric acid

HNE = 4-hydroxy-2-trans-nonenal

ISP = F_2-isoprostane

ISU = Iowa State University

ME = metabolizable energy

MUFA = monounsaturated fatty acid

N = nitrogen

NADH = Nicotinamide adenine dinucleotide

NRC = National Research Council

OSI = oil stability index

PC = protein carbonyls

PUFA = polyunsaturated fatty acid

PV = peroxide value

ROS = reactive oxygen species

SFA = saturated fatty acid

SO = soybean oil

SOD = superoxide dismutase

TCA = the citric acid cycle

TBARS = thiobarbituric acid reactive substances

UFA:SFA = unsaturated fatty acid-to-saturated fatty acid ratio

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ABSTRACT

It is common for vegetable oils to be supplemented to livestock diets to improve the energy density of the feed. Vegetable oils, however, have high concentrations of polyunsaturated fatty acids (PUFA). Due to this increased unsaturation, they are predisposed to lipid damage through lipid peroxidation. Lipid peroxidation is a dynamic free radical chain reaction that can be initiated by thermal processing in the presence of oxygen. This reaction progresses based on duration and intensity of thermal processing to produce a wide range of potentially oxidative and toxic compounds. Lipid peroxidation products of interest include peroxide value (PV) which measures hydroperoxides formed, and p-anisidine value (AnV) which is a measure of the molecular weight of aldehydes. Two key aldehydes are also formed including 2,4-decadienal (DDE) and 4-hydroxynonenal (HNE), are formed from the peroxidation of linoleic acid and are of interest because of their reactiveness with lipids, proteins, and DNA. Oils from ethanol, rendering or the restaurant industries may be an economical energy source compared to fresh oil, but at the expense of oil quality as they may be thermally peroxidized.

Consumption of these peroxidized oils may induce increased oxidative stress and antagonize livestock performance. Oxidative stress occurs in the event that oxidative compounds such as free radicals and reactive oxygen species (ROS) overwhelm the antioxidant defense system. Enzymatic antioxidants including superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) function in detoxifying (reducing) oxidative compounds to protect the body from oxidative stress. Oxidative compounds can bind to lipids, proteins, and DNA to stabilize resulting in tissue or cellular damage. Commonly, thiobarbituric acid reactive substances (TBARS) and F_2-

isoprostanes (ISP) are measured as indicators of lipid damage, protein carbonyls (PC) are measured as an indicator of protein damage, and 8-hydroxy-2'-deoxyguanosine (8-OH-2dG) is measured as an indicator of DNA damage. However, there is a poor understanding of the effects of feeding peroxidized oils on growth performance, digestibility, and oxidative status in livestock.

Therefore, the overall objectives of this thesis were to determine the impact of feeding peroxidized oils on growth and digestibility parameters, and whole body oxidative status in growing pigs and poultry. To accomplish these objectives, a series of experiments were conducted and are outlined in three chapters (Chapter 2, 3, and 4). In Chapter 2, an experiment was conducted feeding variable levels of peroxidized soybean oil (SO) on growth, digestibility, and intestinal integrity parameters in growing pigs. In Chapter 3 oxidative stress markers associated with lipid, protein, and DNA damage along with enzymatic antioxidants, were measured in pigs fed variable levels of dietary peroxidized soybean oil. The final chapter (Chapter 4) assessed the effects of feeding multiple fresh and peroxidized oil sources on growth performance and markers of oxidative stress in broilers.

The data herein indicate that thermally processing oils at 90^{o}C for 72 h yielded the most harmful lipid peroxidation products as exhibited by reduced overall growth performance and feed efficiency in swine and poultry. Pigs fed thermally peroxidized SO (heated at 90^{o}C for 72 h, 90^{o}C SO) had reduced ADG, energy and lipid digestibility, and whole body N retention (Chapter 2). Further, these pigs also had increases in liver weight as a percentage of BW and generally had increased oxidative stress as measured by serum PC and GPx, urine ISP, and liver 8-OH-2dG (Chapter 3). To build on Chapters 2 and 3,

Chapter 4 evaluated the effects of feeding fresh and peroxidized palm, soybean, flaxseed, and fish oils on performance and oxidative status in poultry. An interaction between oil source and peroxidation status was noted for ADFI, ADG, G:F, and plasma GPx in broilers where peroxidation status reduced each of these variables in birds fed palm, soybean, and flaxseed oil, apart from birds fed fish oil. In general, oil unsaturation increased plasma TBARS, PC and 8-OH-2dG; furthermore, broilers fed peroxidized oils had increased plasma 8-OH-2dG. An interaction was noted in liver TBARS where broilers fed peroxidized palm oil had increased liver TBARS compared to fresh palm oil, while the opposite was true in broilers fed soybean oil, and no change was noted in broilers fed flaxseed oil and fish oil. An interaction was also noted for liver PC where broilers fed palm, flaxseed, and fish oil had similar liver PC regardless of peroxidation status while broilers fed peroxidized soybean oil had increased liver PC compared to the fresh soybean oil diet. Generally speaking, the unsaturation content of the dietary oil increased liver 8-OH-2dG and CAT activity and peroxidation status increased liver SOD activity.

In summary, this thesis reported that formulating diets for pigs and poultry containing thermally processed oils (5-10% of total diet) were shown to have detrimental effects on performance particularly ADG. In pigs, thermally peroxidized SO also reduced digestibility of energy, ether extract, and nitrogen retention in comparison to pigs fed fresh SO. Additionally, growth performance was decreased in poultry fed peroxidized palm, soybean, and flaxseed oils. Oxidative stress was induced in swine and poultry fed peroxidized oils and different oil sources as measured in urine (pigs only), blood, and liver. Further, this data suggests that PV, DDE or AnV, PTAGS, and total tocopherols are

important lipid peroxidation products that need to be measured as suggested by their consistent correlations with growth performance and oxidative status in swine and poultry. Additionally, these experiments and a review of literature indicate that markers of oxidative stress that should be measured include ISP and 8-OH-2dG in urine (pigs), and PC and GPx in blood (pigs and poultry). Overall, this thesis showed that oil quality should not be underestimated in livestock production. Feeding peroxidized oils can induce oxidative stress and antagonizes growth performance and digestibility in swine and poultry.

CHAPTER 1

LITERATURE REVIEW

Introduction

The ability of livestock to efficiently extract nutrients and energy from feedstuffs to support growth and development is critical for production performance efficiency and profitability. Lipids are added to diets as a concentrated energy source because they provide 2.25 times more energy per gram in comparison to carbohydrates and proteins. In addition to supplementing the diet with energy, lipids are added for many other reasons. These include, but are not limited to decreasing dustiness of feed, improvement of palatability to the diet, and providing essential fatty acids and fat soluble vitamins (Pettigrew and Moser, 1991; Azain, 2001; Lin et al., 2013). However, generally speaking, energy is the most expensive component of livestock diets so it is important that animals are efficient with the energy they are given to optimize growth and development.

Lipids are hydrophobic, organic molecules that primarily function in energy storage and provide structural components of cell membranes. Lipids, in broad terms, include fatty acids, fats, oils, triglycerides, waxes, sterols, and phospholipids. The dietary forms of lipids are typically triglycerides in which three fatty acids are attached to a glycerol backbone and phospholipids that are found in the structure of cell membranes. Lipid digestibility is dependent on many variables including age and health status of the animal, as well as lipid source and fatty acid profile (Cera et al., 1988). Fatty acids are broadly categorized as either saturated or unsaturated fatty acids. Saturated fats contain fatty acids that do not have any carbon-carbon double bonds, are more linear in structure,

higher in melting point and are typically solid at room temperature, and are often of animal origin. Unsaturated fats contain FA with one or more carbon-carbon double bonds and are fats that are liquid at room temperature (i.e. lower melting point), are often of marine and plant origin, and are more digestible than saturated fats (Powles et al., 1995). However, lipid quality can also be affected by the stability of the lipid, which can be compromised through processes such as heating of the lipid and exposure to metallic compounds such as iron or copper, which are catalysts of lipid peroxidation, all in the presence of oxygen. Fats and oils are highly susceptible to becoming peroxidized particularly through excessive heating and processing. Furthermore, polyunsaturated fatty acids (PUFA) are far more prone to lipid peroxidation than saturated fatty acids due to multiple double bonding of carbons in the structure of PUFA, which are more readily able to accept the free radical electron. A free radical is a chemical with an unpaired electron that is highly reactive. The generation of unstable lipid peroxidation compounds have been shown to depress growth performance in swine (Boler et al., 2012; Liu et al., 2014a; Lu et al., 2014) and poultry (Takahashi and Akiba, 1999; Anjum et al., 2004; Tavárez et al., 2011), affect digestibility (DeRouchey et al., 1997), and increase oxidative stress in swine and poultry (Tavárez et al., 2011; Boler et al., 2012; Lu et al., 2014).

Oxidative stress occurs when free radical production overwhelms the antioxidant defense system causing free radicals to bind to proteins, lipids, and DNA. To date, most livestock research involving oxidative stress focuses on measuring thiobarbituric acid reactive substances (TBARS), which is a measure of lipid damage, and glutathione peroxidase (GPx) which is a measure of antioxidant status. There is limited understanding of the effects of increasing peroxidation levels and manipulating lipid

quality on animal growth performance and multiple measures of oxidative stress. This review will briefly cover the characteristics of lipids and the mechanism(s) of absorption and metabolism before focusing on lipid peroxidation, oxidative stress, and the impact of feeding peroxidized lipids to swine and poultry.

Lipids, Fats, and Oils

Lipids are organic compounds that are made up of fatty acids and are not soluble in water (hydrophobic). Simple lipids are organic compounds that contain esters of fatty acids with various alcohols which include fatty acids, monoglycerides, diglycerides, triglycerides, and waxes. Fatty acids are the simplest form of lipids and are composed of a hydrocarbon chain with a carboxylic acid group on the terminating end. Fatty acids contain a polar hydrophilic head and a non-polar hydrophobic tail end. Fatty acids vary in chain length and can contain anywhere from a 2 carbon chain length up to a 24 carbon chain length which determines the characteristics of the lipid. Triglycerides are made up of three fatty acids attached to a glycerol backbone and are the digestible form of lipids, usually composed of an even number of carbons. More complex lipids that contain fatty acid esters with alcohols along with additional groups are termed compound lipids and include phospholipids, glycolipids, lipoproteins, and sterols which are important in cell membrane formation and function.

Unsaturated fatty acids are more digestible than saturated fatty acids because of their greater ability to form a micelle compared to saturated fatty acids (Wiseman and Salvador, 1991; Powles et al., 1995). Dietary lipids come from several sources and have diverse degrees of unsaturation and fatty acid profiles (NRC, 2012, Table 1.1). Fatty

acids are categorized as saturated or unsaturated depending on the composition of double bonds and carbon chain length. Short chain fatty acids have a lower melting point which increases as chain length increases. Saturation of fatty acids also affects melting point. Saturated fatty acids do not contain any carbon-carbon double bonds, whereas unsaturated fatty acids contain one or more double bond, most commonly in cis configuration. Unsaturated fatty acids can be further categorized as monounsaturated meaning it has a single double bond or polyunsaturated which have multiple double bonds. The location of the double bonding also plays a role in the characterization of the lipid. Due to these double bonds, unsaturated fatty acids have a lower melting temperature, meaning that they are typically liquid at room temperature and these lipids are often of marine and plant (vegetable) origin.

Vegetable oils are acquired by extraction of oil from the seeds or fruits which can then be processed for consumption (AAFCO, 2011). Some examples of unsaturated oils include soybean oil, corn oil, canola oil, and fish oil. In contrast, saturated fats contain fatty acids that do not contain carbon-carbon double bonds and are solid at room temperature and are often of animal origin. Animal fats are harvested from tissue during commercial rendering processes (AAFCO, 2011). Some examples of fats that are primarily composed of saturated fatty acids include choice white grease, tallow, and poultry fat. A list of common lipids are described in Table 1.1.

Table 1.1. Composition of common lipid sources (Adapted from Kerr et al., 2015).

Lipid Source	Major Fatty Acid	Saturated fatty acid (%)	Monounsaturated fatty acid (%)	Polyunsaturated fatty acid (%)
Algae oil	C18:0	27.84	15.46	56.70
Canola oil	C18:1	6.17	62.34	31.49
Coconut oil	C12:0	91.96	6.24	1.80
Corn oil	C18:2n6	14.02	28.77	57.22
Flaxseed oil	C18:3n3	9.83	21.13	69.04
Lard	C18:1	40.69	46.72	12.59
Menhaden oil	C20:5n3	34.87	27.38	37.74
Olive oil	18:1	14.09	75.00	10.91
Palm oil	16:0	50.10	39.38	10.52
Poultry fat	18:1	30.62	47.32	22.06
Soybean oil	18:2n6	15.45	24.22	60.33
Sunflower oil	18:1	10.47	47.64	41.88
Tallow	18:1	52.42	43.60	3.98

Physiological Functions and Characteristics of FA

Physiologically, lipids play many important roles in the body. A major function of lipids is in the fat surrounding tissues and organs to provide protection from damage and insulates the body. Additionally, lipids provide energy reserves primarily in the form of triglycerides. A major role of fatty acids in the body is to maintain the phospholipid bilayer in cell membrane structure. Furthermore, fatty acids are the major components of prostaglandins which play a role in inflammation (Dowhan and Bogdanov, 2002).

Dietary lipids have many properties and functions that set them apart from other nutrients. A characteristic of lipids that makes them a unique nutrient is that they contain fat soluble vitamins including vitamins A, D, E, and K which can be stored in tissues. Vitamin E or tocopherols are an important class of antioxidants, most common being alpha tocopherol, though there are other isoforms of tocopherols including beta, delta, and gamma tocopherols. Vitamin E originates from plants and is found in high concentration in soybeans and corn. The importance of vitamin E will be discussed in

further detail in the antioxidant section of this review. Dietary lipids also provide essential fatty acids which are required in the diet because the body is unable to synthesize them or produces them in insufficient quantities to meet their metabolic demands. In swine, the major essential fatty acids include an omega-6 fatty acid called linoleic acid (C18:2n6) and an omega-3 fatty acid called linolenic acid (C18:3n3), and in some species arachidonic acid (C20:4n6). Essential fatty acids are necessary for normal tissue function and deficiencies can lead to decreased growth rates and immune function and tissue abnormalities (Hwang, 1989). Fatty acids that are synthesized by the body and are not required in the diet are termed nonessential. In order to fully understand the impact that lipids have on livestock, it is important to have knowledge of dietary fat metabolism.

Lipid Digestion and Absorption

Lipid digestion and absorption is a complex process that differs among livestock species. For the purpose of this review, lipid digestion and absorption in monogastrics will be discussed. Unlike carbohydrate and protein digestion, there is limited lipid digestion and absorption in the mouth and stomach in pigs because lipids are hydrophobic. Although minimal, lipid digestion in swine is initiated in the mouth where lingual lipases begin the degradation of the lipid (Carey et al., 1983). In contrast, there are no reports of lingual lipase in poultry species so digestion begins in the proventriculus (Krogdahl, 1985). Once in the stomach (or proventriculus in poultry), gastric lipases further hydrolyze lipids and results in the release of fatty acids. Although gastric lipase

hydrolyzes lipids, about 70% of ingested triglycerides enter the duodenum, making the small intestine the major site of dietary lipid digestion.

In the small intestine, bile and pancreatic juice work to further hydrolyze and emulsify the dietary lipids before they are absorbed. Bile salts that are produced in the liver and stored in the gall bladder emulsify triglycerides to increase surface area for lipases to act, and also signals for the activation pancreatic lipase. In the lumen, activated pancreatic lipases cleave the ester bond at the Sn-1 and Sn-3 positions of the triglyceride resulting in two free fatty acids and one monoglyceride containing the remaining Sn-2 fatty acid. Once this occurs, these products are formed into micelles, with the concentration of digested lipids at the brush border membrane of enterocytes allowing for them to passively diffuse down a concentration gradient through the unstirred water layer and into the enterocyte. Inside the enterocyte, the monoglycerides and free fatty acids enter the endoplasmic reticulum where they are re-esterified back to a triglyceride before going to the Golgi apparatus for export as a chylomicron. The chylomicron undergoes exocytosis to the villi of the small intestine into the lymphatic system and then to the blood stream. Once in circulation, these lipids can either be metabolized by cells and completely oxidized for energy or stored as triglycerides in adipose tissue.

Metabolism

Lipids are important because they are an efficient energy source that can be easily stored and utilized for ATP by monogastric animals. They also provide 9 kcal GE/g in comparison to carbohydrates and proteins which only provide 4 kcal GE/g. Catabolism of triglycerides produces ATP via the citric acid (TCA) cycle and the electron transport

chain (ETC). Energy production from triglycerides begins with cleavage of carbon-carbon bonding yielding 2 ATP molecules by the oxidation of $FADH_2$ and 3 ATP molecules via NADH oxidation and acetyl CoAs are oxidized to CO_2 and water by the TCA cycle. For example, palmitate (C16:0) will undergo 7 carbon cleavages, each yielding 5 ATP. Palmitate will also produce 8 acetyl CoA molecules which will undergo complete oxidation in the TCA cycle and ETC producing 12 ATP per cycle for a total of 131 ATP produced. However, 2 ATP molecules are required for activation of the TCA cycle so net ATP production of palmitate is 129 ATP. This is in comparison to amino acids and glucose which generate 2 ATP from glycolysis, net 2 ATP from the TCA cycle, and 34 from the ETC for a total of 38 ATP. Thus, lipids provide concentrated amounts of energy resulting from cleavage of carbon bonds and acetyl CoA oxidation.

Adenosine triphosphate (ATP) can be generated from fatty acid metabolism via beta oxidation in cells. Beta oxidation is a catabolic process occurring in the mitochondria and peroxisomes in which fatty acids generate coenzymes used in the electron transport chain. Beta oxidation is initiated when fatty acids bind to CoA ligase to form a fatty acyl adenylate and inorganic pyrophosphate which then reacts with coenzyme A to generate a fatty acyl-CoA ester and adenosine monophosphate (AMP). If the fatty acyl-CoA contains a short chain (6 or less carbons) the fatty acid can diffuse through the mitochondrial membrane. However, long chain fatty acids require the carnitine shuttle to transport from the cytosol into the mitochondria. Acyl-CoA is transferred to the hydroxyl group of carnitine by carnitine palmitoyltransferase I. Acyl-carnitine is shuttled inside by a carnitine-acylcarnitine translocase as carnitine is shuttled outside. Acyl-carnitine is converted back to acyl-coA by carnitine palmitoyltransferase II

in the inner mitochondrial membrane which allows the acyl-carnitine to enter into the mitochondrial matrix.

Once the fatty acid is inside the mitochondrial matrix, beta oxidation occurs by cleaving two carbons in the form of acetyl-CoA every cycle from the fatty acyl CoA. The first step of beta oxidation is dehydrogenation by acyl CoA dehydrogenase to change the configuration of the fatty acid from *cis* to *trans* which uses FAD as an electron acceptor and is reduced to $FADH_2$ to generate trans-delta2-enoyl CoA. This is then hydrated at the *trans* double bond by enoyl-CoA hydratase before being dehydrogenated again. The first two carbons are released by the attack of coenzyme A and the process continues until all of the carbons are turned into acetyl CoA. Acetyl CoA enters into the citric acid cycle to produce ATP. Odd numbered carbons go through beta oxidation until there are three carbons left in the fatty acid in which it forms propionyl-CoA and succinyl-CoA. Propionyl-CoA is catalyzed my methylmalonyl-CoA mutase to form succinyl-CoA which can enter into the citric acid cycle eventually forming ATP.

Mechanism of Lipid Peroxidation

Lipids are susceptible to degradation through lipid peroxidation which negatively affects lipid quality. Lipid peroxidation is the destruction of fatty acids due to the formation of free radicals and reactive oxygen species affecting lipid quality. In the presence of oxygen, lipid peroxidation can be initiated in a number of ways. One way is through exposure to transition minerals such as copper and iron. Copper or iron salts in a biological system can cause site specific formation of free radicals when they attach to a lipid (Gutteridge, 1995). Lipids can also become peroxidized through exposure to light

through photo-oxidation (Girotti, 1990). Additionally, lipid peroxidation can be initiated through subjection of lipids to heat which produces peroxidation compounds increasing in severity throughout the lipid peroxidation reaction (Shurson et al., 2015).

The process of lipid peroxidation progresses as a chain reaction that is caused by oxygen free radicals resulting in the production of peroxide compounds (Gutteridge, 1995). Lipid peroxidation is more commonly observed in PUFA, because as the degree of unsaturation increases, oxygen free radicals have a higher affinity to bind carbon (Holman, 1954). Therefore, PUFA have more potential to become peroxidized than monounsaturated and saturated fatty acids (Halliwell and Chirico, 1993). Lipid peroxidation can also occur *in vivo* (Halliwell and Chirico, 1993) as well as through consumption of peroxidized lipids.

The chain reaction of lipid peroxidation produces free radicals and other harmful products and occurs in three phases: 1) initiation, 2) propagation, and 3) termination. This reaction series cannot be broken unless antioxidants accept the oxygen of free radicals thereby discontinuing the peroxidation reaction. A number of influences including heat, irradiation, and metal ions can begin the initiation phase of the peroxidation process of lipids. Lipid peroxidation begins with the detachment of a hydrogen atom from an unsaturated fatty acid, resulting in the formation of a lipid radical. The initiation phase includes the formation of free radicals and the development of hydroperoxides. These free radicals are formed by the attack of an oxygen molecule on the carbon-carbon double bonds of an unsaturated fatty acid, transferring an electron from the carbon chain forming a free radical. Following the initiation phase, free radicals continue to form as a chain reaction leading into the propagation phase. Hydroperoxides formed in the initiation

phase degrade into more toxic compounds in the propagation phase. As the propagation phase (phase 2) progresses, oxygen free radicals continuously form to produce aldehydes and acids before eventually ending in the termination phase (phase 3). In this phase, the fatty acid becomes completely hydrogenated and free radicals are no longer able to bind, yielding tertiary peroxidation compounds including polymers and polar compounds. Examples of polar compounds include ammonia, sulfur dioxide, and hydrogen sulfide. An outline of the lipid peroxidation scheme is shown in Figure 1.1.

Products that are formed during lipid peroxidation are dependent upon the duration and intensity of thermal processing that the lipid is subjected to. The process of lipid peroxidation yields peroxidation products while diminishing antioxidant compounds (Seppanen and Csallany, 2002). As lipid peroxidation progresses, lipid composition and quality is compromised and unpleasant odors and chemicals are formed which can lead to a decrease in diet palatability (Cabel et al., 1988). Characteristics of lipids are compromised during the process of lipid peroxidation including the degree of unsaturation of fatty acids decreases as peroxidation advances. While degree of unsaturation decreases, viscosity, polymers, polar compounds, peroxides, and free fatty acids all increase as shown in Figure 1.2 (Kerr et al., 2015).

Some lipids that are used in swine diets are derived from rendering facilities that obtain oils originating from restaurants or food processing facilities (Canakci, 2007). Animal fats originating from rendering facilities are commonly processed with steam at approximately 115-145^{o}C for about 90 minutes, but will vary depending on the system (Meeker and Hamilton, 2006). Seppanen and Csallany (2004) thermally processed soybean oil at 185^{o}C for 5 h (to mimic frying oil used in the restaurant industry) as a

model to generate high concentrations of 4-hydroxy-2-trans-nonenal (HNE), a product formed from the oxidation of linoleic acid. Although these thermally treated lipids may be a cost-effective energy source in livestock diets, they may also be less digestible and of lesser nutritional value due to the significant amount of heating during processing (Frankel, 1998).

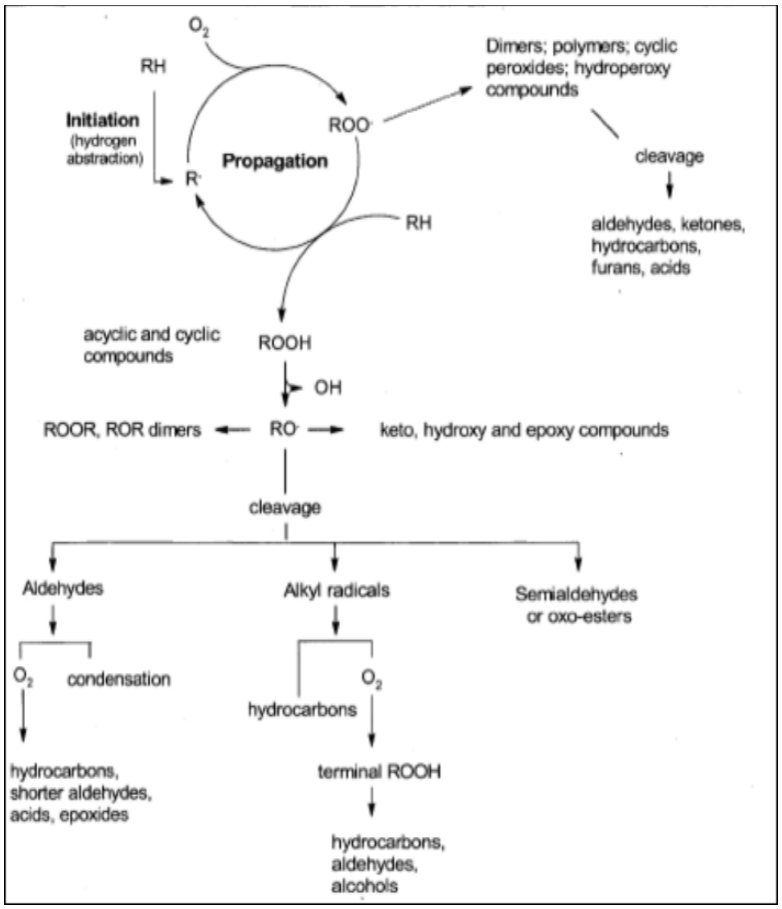

Figure 1.1. General schematic of the lipid peroxidation chain reaction (adapted from Nawar, 1996).

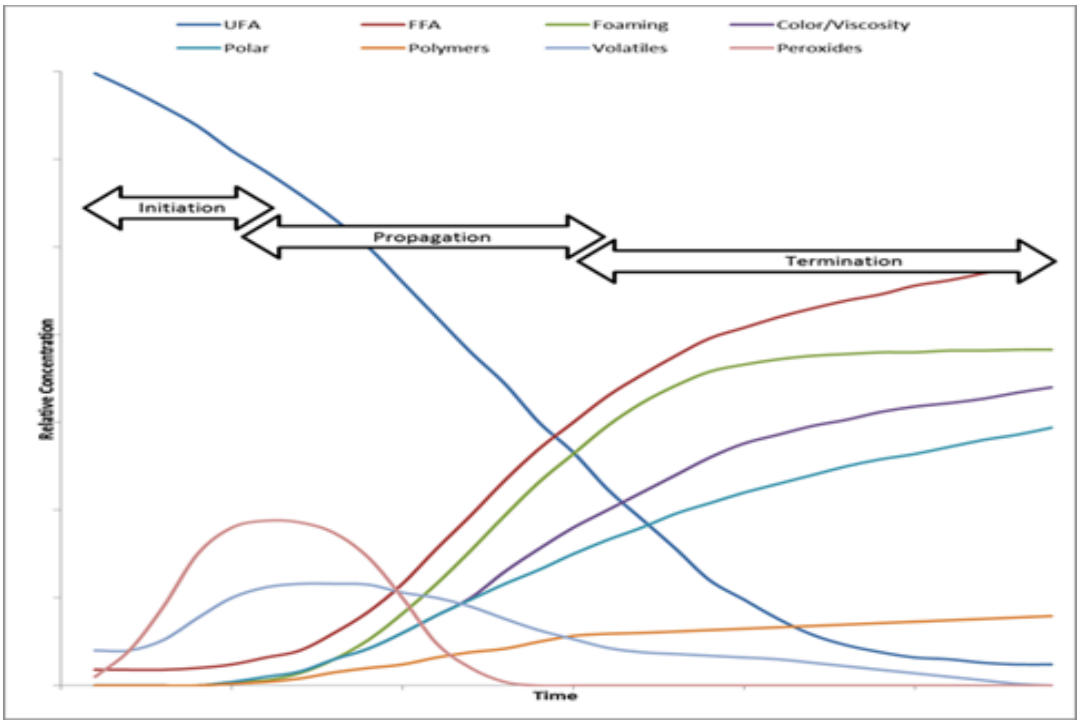

Figure 1.2. Relative production of products formed through lipid peroxidation (Kerr et al., 2015). UFA, unsaturated fatty acid; FFA, free fatty acid.

Measures of Lipid Peroxidation

The severity of lipid damage due to peroxidation can be determined by assessing many peroxidation compounds such as acids, aldehydes, and polymerized fatty acids that are formed throughout the lipid peroxidation process (Kerr et al., 2015). Each phase of lipid peroxidation yields different peroxidants, but because of the high variability of products formed, it is nearly impossible to determine the true damage on the lipid by analyzing a single marker (Shurson et al., 2015). The initiation phase mainly produces hydroperoxides which are measured by peroxide value (PV). Peroxide value measures the concentration of lipid peroxides and hydroperoxides which are generated in the initiation phase and therefore, called primary products. Thus, PV tends to be highest in the initiation phase and drops in the propagation and termination phases (Shurson et al.,

2015). While PV rises in the initiation phase and drops in the propagation and termination phases, other harmful compounds do not arise until the propagation phase. The propagation phase generates secondary peroxidation products including aldehydes, ketones, and acids; and are most commonly measured by p-anisidine value and TBARS. P-anisidine value measures the total molecular weight of unsaturated and saturated aldehydes. Thiobarbituric acid reactive substances are an indirect measure of malondialdehyde which is a compound formed in lipid peroxidation although there are other aldehydes contributing to the TBARS value not specific to lipid peroxidation (Kerr et al., 2015).

The termination phase follows the propagation phase and is thought to yield the most detrimental products of lipid peroxidation relative to DNA, protein, or lipid damage. Along with aldehydes, termination products also include polymers and polar compounds which tend to be indigestible (Márquez-Ruiz et al., 1992). Some of the major aldehydes that are formed include 2,4-decadienal (DDE), 4-hydroxynonenal (HNE), and acrolein. 2,4-decadienal and HNE are aldehydes that are derived from the peroxidation of linoleic and arachidonic acid (Kerr et al., 2015) and are considered two of the most reactive α-β aldehydes (Esterbauer et al., 1991). Acrolein is an aldehyde that is not present in fresh oils but is formed from the thermal degradation of glycerin and has been suggested that free radicals, temperature, and time affect its production (Stevens and Maier, 2008). Polymers are dienes that form a dark residue along the sides of a fryer where the oil and metal fryer come in contact with oxygen from the air (Choe and Min, 2007). Polymers formed in the termination phase of lipid peroxidation can be measured using size exclusion chromatography or viscosity (Kerr et al., 2015).

Lipid peroxidation products including PV, AnV, DDE, HNE, and polymers are considered indicative markers of lipid peroxidation because they suggest the amount of peroxidation that has occurred at a specific time point. These markers can also be utilized to determine the final quality of a lipid. Another method to determine the stability of a lipid includes predictive tests of lipid peroxidation which determines the capacity of a lipid to withstand peroxidation. A couple of common predictive tests include active oxygen method (AOM) and oil stability index (OSI).

Active oxygen method measures the stability of lipids to oxidation by bubbling air through heated material and measuring the formation of peroxides. This is intended to determine the ability of the fat to resist rancidity due to oxidation during storage. Measuring AOM is a very labor intensive process because PV must be measured at regular time intervals throughout the experiment. Stable lipids require significant amount of time to complete the AOM method (upwards of 20 h of heating) (AOCS, Cd 12-57) so researchers are beginning to use alternative methodology to determine lipid quality. The AOM is still used to determine oxidative stability of lipids, however; it is more common to measure final PV.

Another predictive test is OSI that typically replaces AOM because it is labor intensive and has inconsistent repeatability. Oil stability index measures the relative measure of oil resistance to oxidation (AOCS, Cd 12-57). It is a predictive test because it induces lipid peroxidation by ventilating air through a sample at a fixed temperature. All oils and fats are resistant to oxidation depending on the degree of saturation, natural or added antioxidants, presence of pro-oxidants, and prior handling. The OSI method measures the oil's resistance to oxidation which reaches a threshold and degradation

occurs. Oxidation is slow until resistance is overcome, at which point oxidation accelerates and becomes very rapid known as the OSI. Oil stability index is determined through passing air through a lipid under a predetermined temperature at which point volatile acids are decomposed from lipid peroxidation and are driven out by the air and dissolved in water and in doing so increasing its conductivity. Oil stability index is quantified through the conductivity of the water due to volatile fatty acids and is defined as the hours required for the rate of conductivity to reach an established level, so a lower OSI reading indicates more double bonds available to become peroxidized (Kerr et al., 2015).

There are many harmful compounds that are produced in the lipid peroxidation reaction and many ways to measure them. However, to date there is not one single measure or marker of lipid peroxidation that accurately measures each phase of peroxidation and due to the lack of specificity of some analyses, it is advantageous to analyze for multiple markers. Lipid peroxidation forms compounds contributing to free radical production so it can lead to oxidative damage in the body (Montuschi et al., 2004).

Oxidative Stress

Because lipid peroxidation is a free radical producing reaction, consumption of lipid peroxidation products can cause oxidative stress by straining the antioxidant defense system creating an imbalance of free radicals *in vivo*. In addition to free radicals produced by electron leakage in the mitochondria, consumption of peroxidized lipids can overwhelm the antioxidant capacity of the animal with free radicals resulting in a

depletion of antioxidant storage. Uncontrolled leakage of electrons from the ETC in the mitochondria is a major source of free radicals in the cytosol of cells. Free radicals can employ beneficial effects on the immune system and cellular signaling where free radicals influence cell signaling and transformation, smooth muscle control, and immune defense and by acting against antigens during phagocytosis by engulfing adverse bacterial microbes (Dröge, 2002; Forman and Torres, 2002). However, free radicals can also elicit harmful effects that distress body functions by compromising compounds they come in contact with (Finaud et al., 2006). This event is termed oxidative stress which is a disruption in the balance between of free radical production and antioxidant defenses in the body. Oxidative stress in livestock can be caused by a variety of conditions including disease, heat stress, injury, starvation, pregnancy, and consumption of peroxidized lipids. Among free radicals, reactive oxygen species (ROS) are derived from oxygen free radicals. Once antioxidant stores are depleted and enzymatic antioxidant activity is overwhelmed, free radicals bind to proteins, lipids, and DNA which alter their cell membrane and cellular structure and function (Montuschi et al., 2004), and ultimately if severe enough results in tissue damage (Czerska et al., 2015).

Antioxidant machinery and pathways

The severity of oxidative stress directly depends on the balance between ROS and the antioxidant defense system. Enzymatic and non-enzymatic antioxidants are compounds that reduce the harshness or can prevent oxidation of other molecules (Cooke et al., 2002; Poljsak et al., 2013). Antioxidants attract the free radical that causes oxidative stress in order to prevent further oxidation. Antioxidants are present in the body

endogenously and can also be exogenous, mainly through utilization of dietary vitamins. An important enzymatic antioxidant is superoxide dismutase (SOD). The action of SOD is to form hydrogen peroxide from oxygen and hydrogen free radicals as shown in general terms in Equation 1. There are many known classes of SOD including copper, zinc, and manganese SOD (Kalyanaraman, 2013). As shown in Equation 1, SOD suppresses the formation of oxygen free radicals, but does yield hydrogen peroxide (H_2O_2) which is a peroxidant but is less toxic than free radicals.

$$2O_2O_2^{-\bullet} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad \text{(Eq. 1)}$$

Catalase (CAT) is another vital enzymatic antioxidant. Catalase is predominantly found in peroxisomes which are cells that use oxygen to detoxify cells by producing H_2O_2, but CAT also exists in every cell and acts by catalyzing H_2O_2 to water and oxygen shown in Equation 2 (Finaud et al., 2006).

$$2H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2 \qquad \text{(Eq. 2)}$$

Another important enzymatic antioxidant is glutathione peroxidase (GPx). Glutathione peroxidase is similar to CAT because it plays a role in detoxifying H_2O_2 to produce water; however, GPx is more efficient than CAT. As shown in Equation 3, GPx acts on H_2O_2 and glutathione (GSH) to produce oxidized glutathione (GSSG) and water. Glutathione peroxidase is mainly present in the cytosol and mitochondria.

$$H_2O_2 + 2\ \text{GSH} \xrightarrow{\text{GPx}} \text{GSSG} + 2\ H_2O \qquad \text{(Eq. 3)}$$

These enzymatic antioxidants are crucial for the prevention of disease and oxidative stress. Other cellular defenses against oxidative damage include non-enzymatic antioxidants such as tocopherols and ascorbic acid. Vitamin E is present in the cell membrane and is arguably the most important antioxidant in the body because of its

ability to readily react with ROS thereby breaking the chain reaction forming free radicals (Gutteridge, 1995; Finaud et al., 2006; Kalyanaraman, 2013). It is especially useful in protecting membranes from lipid peroxidation by preventing the propagation of free radical formation. However, tocopherols become inadequate at terminating lipid peroxidation at high temperatures (Choe and Min, 2007). Miyagawa et al. (1991) reported that thermally processing a blend of soybean oil and rapeseed oil at frying temperatures (185^{o}C) resulted in a rapid degradation rate for gamma tocopherol followed by delta- and alpha- tocopherol respectively. Based on this data, alpha tocopherol has the greatest retention rate when thermally processed.

Ascorbic acid, or vitamin C, is a water-soluble vitamin that has antioxidant properties and acts in extracellular fluids, but can also be found intracellularly in the cytosol (Finaud et al., 2006; Kalyanaraman, 2013). Ascorbic acid has the ability to neutralize ROS by supporting the action of tocopherols in cells which increases antioxidant potential. The major function of vitamin C is as a reducing agent to reverse oxidation by donating hydrogen atoms to regenerate vitamin E (Carr and Frei, 1999). However, vitamin C can also act as a pro-oxidant when reacting with metals such as copper and iron which then form hydroxyl radicals via the Fenton reaction depicted in Equation 4 (Gutteridge, 1995). Reactive oxygen species are constantly produced in normal metabolism and are safely removed by mechanisms of both enzymatic and non-enzymatic antioxidants to maintain homeostasis among free radicals, ROS and antioxidants. However, when pro-oxidants exceed antioxidant activity, oxidative stress transpires leading to lipid, protein, and DNA damage.

$$H_2O_2 \xrightarrow{Fe^{2+}} HO\bullet + OH^- + Fe^{3+} \qquad \text{(Eq. 4)}$$

Measures of oxidative status

Reactive oxygen species are known to cause lipid, protein, and DNA damage *in vivo*. Because PUFAs are more readily peroxidized than saturated fatty acids (Ayala et al., 2014), they are; therefore, more susceptible to oxidative damage. Oxidative stress can be measured directly or indirectly. Measuring ROS is the only way to directly measure oxidative stress; however, ROS are very unstable making them difficult to properly measure. Reactive oxygen species can be directly measured using electron spin resonance (ESR), and fluorescent probes (Poljsak et al., 2013). More commonly, indirect measurement of damage due to ROS on lipids, proteins, and DNA are measured as accurate measures of oxidative stress because these compounds are more stable than ROS. There are many methods to measure oxidative damage as summarized in Figure 1.3.

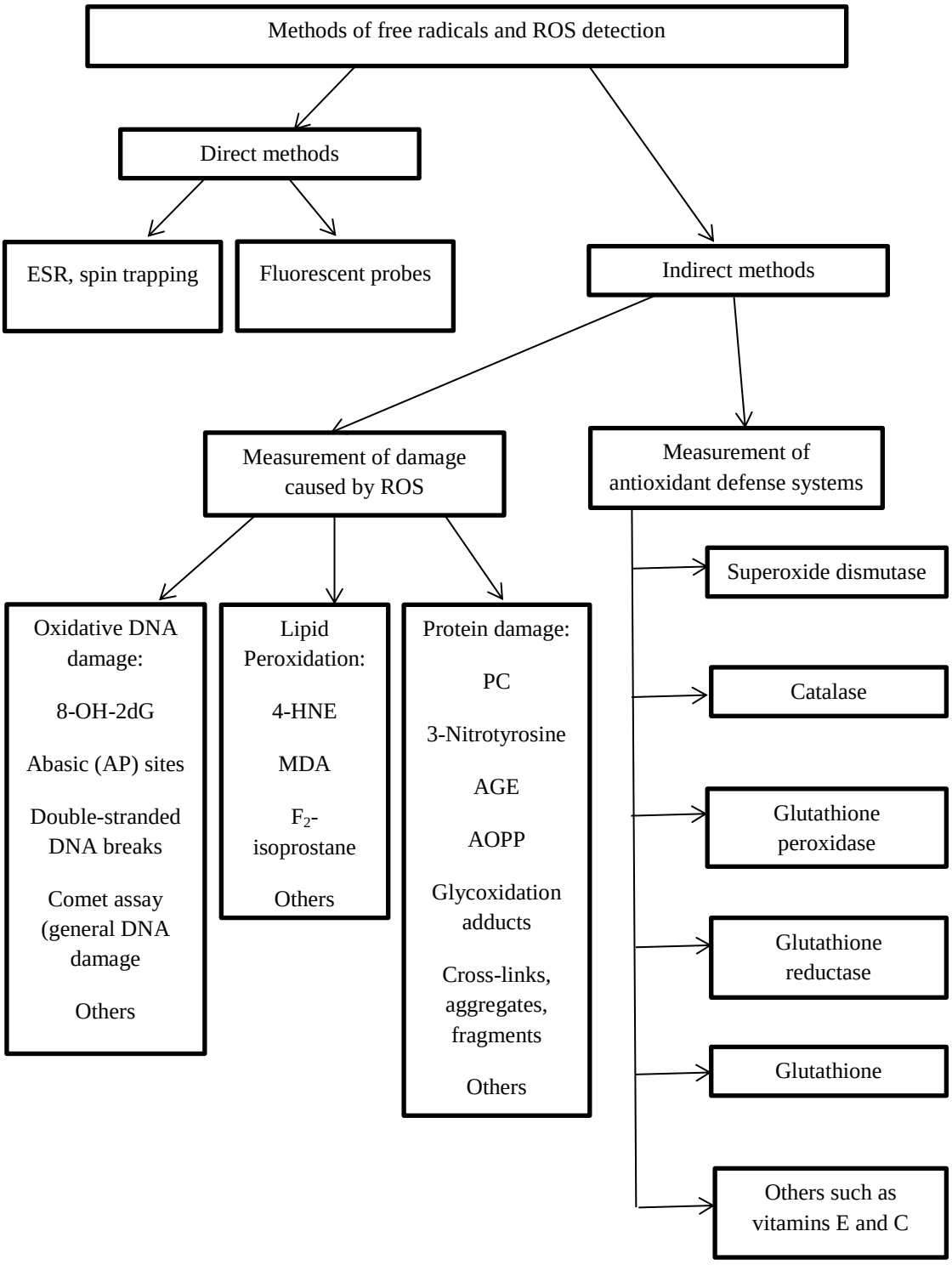

Figure 1.3. Methods to quantify oxidative stress. (Adapted from Poljsak et al., 2013). ESR= electron spin resistance; 8-OH-2dG= 8-hydroxy-2deoxy-guanosine; 4-HNE= 4-Hydroxynonenal; MDA= malondialdehyde; PC= protein carbonyl concentration; AGE= advanced glycation end products; AOPP= advanced oxidation protein products.

Lipid peroxidation

Lipid damage can be measured in multiple ways and TBARS is one of the most common measurements of lipid peroxidation. It measures aldehydes including malondialdehyde (MDA); however, TBARS also measures other aldehydes not specific to lipid peroxidation. Lipids with higher degrees of unsaturation will produce higher values of TBARS because PUFAs are more prone to lipid peroxidation. The TBARS assay is a simple and economical assay that measures MDA which is a major secondary product of lipid peroxidation, although it overestimates MDA concentration due to the non-specificity of the assay. Despite the non-specificity of the TBARS assay, it is the most common assay to evaluate oxidation of lipids because it is a simple and reproducible assay (Halliwell, 2000). Thiobarbituric acid-reacting substances can be measured in any biological fluids including urine, serum or plasma, exhaled breath, and tissue samples. The biggest limitation of the TBARS assay is that it is non-specific due to the fact that it measures MDA and aldehydes that are not specific products of lipid peroxidation. Because of this, the use of this assay alone is problematic (Halliwell, 2000; Monaghan et al., 2008). However, measuring TBARS gives a general idea of the amount of oxidative stress but more specific analysis should be done. For this reason, the TBARS assay should be used in combination with other lipid peroxidation measures.

F_2-isoprostanes is also commonly used to assess lipid peroxidation and oxidative stress in mammalian species. These are prostaglandin-like substances that are produced predominantly by free radical-induced peroxidation of arachidonic acid (Montuschi et al., 2004). There are several other known isoprostane products such as D_2- and E_2-isoprostanes but these are not as commonly measured because they are not as stable as

F_2-isoprostanes (Roberts and Morrow, 2000). F_2-isoprostanes are used as an indicative test for lipid peroxidation because it is specific in measuring strictly products of lipid peroxidation, although it is not a major product of lipid peroxidation (Montuschi et al., 2004). There are many characteristics of F_2-isoprostanes that make it a reliable choice for studying oxidative stress including that it is chemically stable, formed *in vivo*, present in tissues and bodily fluids, and levels increase with increasing levels of oxidative stress (Robertsand Morrow, 2000). F_2-isoprostanes can be measured in plasma and tissue samples; however, they are most stable in urine (Montuschi et al., 2004). Because of this, F_2-isoprostane concentration is a common, noninvasive way to quantify lipid damage due to lipid peroxidation. When used in together, TBARS and F_2-isoprostanes may provide an accurate depiction of lipid oxidative damage.

Protein oxidation

Protein damage could arguably be the most affected by ROS in comparison to lipids and DNA because proteins are catalysts of oxidative damage (Dalle-Donne et al., 2003). Oxidation of proteins modifies the protein structure and impairs protein function with the amount of protein damage depending on the protein structure and the location of the ROS in relation to the protein as well as certain amino acid side chains (Dröge, 2002). All amino acids are susceptible to oxidation by an oxygen radical (Berlett and Stadtman, 1997); however, the susceptibility varies among amino acids. Some of the most notable amino acids that affect the secondary and tertiary structure of protein include tryptophan, tyrosine, histidine, and cysteine (Berlett and Stadtman, 1997; Dröge, 2002). Of the sulfur containing amino acids, cysteine and methionine are the most sensitive to ROS. Cysteine

is converted to disulfides and methionine is readily converted to methionine sulfoxide (Berlett and Stadtman, 1997). Fortunately, most biological systems contain reductases that can convert disulfides and methionine sulfoxide back to their original state; however, these are the only protein repairs currently known (Berlett and Stadtman, 1997). Reactive oxygen species also attack aromatic amino acids. One notable reaction involves tryptophan being oxidized to formylkynurenine and kynurenine, while phenylalanine and tyrosine form a number of hydroxyl derivatives. In addition, histidine can be converted to 2-oxohistidine and asparagine (Berlett and Stadtman, 1997).

Ketones and aldehydes are carbonyl groups that are formed on the side chains of proteins which modify protein function (Dalle-Donne et al., 2003). The amino acid side chains that are predominantly affected include arginine, lysine, proline, and threonine (Dalle-Donne et al., 2003). Aldehydes that are synthesized include 4-hydroxy-2-nonenal, and MDA both of which are produced during lipid peroxidation.

The most general and accepted way to evaluate protein damage is by measuring protein carbonyls. The presence of increased protein carbonyl groups is an indicator of protein oxidation and thus, oxidative damage (Beal, 2002). Protein carbonyls are unable to catabolize in order to rectify amino acids and therefore, blocks proteolysis and oxidized protein accretion (Dröge, 2002) which reduces amino acid recycling and cell integrity. Unlike lipids and DNA oxidation, protein oxidation is not measured in the urine, but best assessed in blood and tissues.

DNA oxidation

Mitochondrial DNA is sensitive to oxidative damage because ROS are generated in the mitochondria (Monaghan et al., 2008). All DNA components are susceptible to base repair damage due to ROS however, out of the nucleic acids, guanine is the most readily oxidized (Cooke et al., 2002; Wu et al., 2004; Mateos and Bravo, 2007) making it the most prevalent measurement for oxidative damage of DNA. DNA is constantly targeted by ROS during normal cellular metabolism (Cooke et al., 2002). Upon oxidation, a hydroxyl group is added to the 8 position of the guanine molecule to produce 8-hydroxy-2'-deoxyguanosine (8-OH-2dG) (Kalyanaraman, 2013). Thus, most DNA damage research is conducted by measuring 8-OH-2dG which is produced by many mechanisms. Some of these mechanisms include the formation of 8-OH-2dG in DNA via oxygen radicals, peroxynitrite, guanine radical cation, and peroxidase (Kasai, 1997). 8-hydroxy-2'-deoxyguanosine is a water soluble DNA damage product that can be measured in many bodily fluids. During DNA repair, products including 8-OH-2dG are ultimately excreted in the urine, and because 8-OH-2dG is water soluble, it is concentrated and stable to measure in urine (Wu et al., 2004). It is also thought that urinary 8-OH-2dG can be an accurate indicator of carcinogenesis in humans (Wu et al., 2004). However, Okamura et al., (2009) reported in humans an increase in urinary 8-OH-2dG does not necessarily mean greater DNA damage. Thus, it could signify decreasing of steady-state levels in tissue DNA possibly due to DNA repair.

Dietary peroxidized lipids on swine and poultry performance

Processed fats and oils from rendering facilities and refined oil sources are fed to pigs and poultry that can negatively impact growth performance and digestibility via lipid peroxides which are capable of impairing cell function and oxidative stability. The influence of thermally peroxidized dietary lipids on swine and poultry performance, digestibility, and oxidative balance is summarized in Table 1.2. Studies have shown that pigs consuming diets containing peroxidized lipids results in reduction of growth. Rosero et al., (2015) conducted an experiment feeding 6% of divergently peroxidized soybean oil (0, 6, 9, 12 d heated at 80^{o}C) to nursery pigs for 35 days, linearly reduced weight gain compared to the controls. Liu et al. (2014a) evaluated slowly and rapidly peroxidized (heated at 95^{o}C and 185^{o}C respectively) corn oil, canola oil, poultry fat, and tallow, which when fed to young pigs at a 10% fat inclusion in the diet resulted in a decrease in ADG by an average of 20.95%,without affecting G:F. These results are in agreement with Lu et al. (2014) who reported that pigs fed peroxidized corn oil without antioxidant decreased ADG by 8.49%.

Secondary lipid peroxidation compounds such as aldehydes which can compromise lipid structure and can lead to the development of rancid odors and flavors of the dietary lipid, thereby impacting diet palatability decreasing feed intake (Shermer and Calabotta, 1985). Rosero et al. (2015) reported that increasing peroxidation of soybean oil linearly decreased feed intake which is in agreement with Liu et al. (2014a) who reported that ADFI was decreased by an average of 15.06% in pigs fed rapidly peroxidized oils compared to fresh oils. Furthermore, Boler et al. (2009) reported pigs fed

peroxidized corn oil without antioxidants decreased ADFI by 5.74% compared to pigs fed fresh corn oil.

Feeding peroxidized lipids has also been shown to affect growth performance and feed intake in broilers. Tavárez et al., (2011) conducted an experiment feeding peroxidized soybean oil to broilers and reported a decrease in weight gain of 6.06% in birds fed peroxidized soybean oil in comparison to the control. Dibner et al. (1996) also determined a decrease in feed efficiency in birds fed peroxidized fat which is supported by Takahashi and Akiba, (1999) who also reported that feeding peroxidized fat suppressed feed intake and feed efficiency and thus, weight gain. Lastly, Anjum et al. (2004) fed peroxidized soybean oil (PV of 50 mEq/kg oil) to broilers for 6 weeks which resulted in depressed average weight gain (4.17%), although no differences in feed intake were noted. Overall, experiments in swine and poultry agree that feeding various peroxidized lipids has a negative impact on growth performance and feed intake.

Consumption of lipid peroxidation products also includes polymers, that have been shown to have a negative effect on energy and nutrient digestibility (Gonzalez-Muñoz et al., 1998). Just as performance has been shown to be linearly decreased with increasing levels of peroxidation, Rosero et al. (2015) also showed that the digestibility of energy and fat decreased in pigs fed peroxidized soybean oil exposed to heat for 9 and 12 d. In contrast, Liu et al. (2014b) measured digestibility of nutrients and energy and concluded that there were no peroxidation effect on dry matter (DM), gross energy (GE), ether extract (EE), and N digestibility among the lipids evaluated. This is in agreement with DeRouchey et al. (1997) who reported no significant differences in DM, GE, EE and

N due to feeding peroxidized choice white grease. Additional experiments are needed to determine the effects of feeding peroxidized oils on energy and lipid digestibility.

In addition to negatively impacting pig and broiler growth performance and digestibility, inclusions of dietary peroxidized lipids may also affect intestinal barrier function and morphology (Dibner et al., 1996; Liu et al., 2014c). Dibner et al. (1996) reported that feeding peroxidized poultry fat (PV of 212.5 mEq kg oil) to swine and poultry reduced enterocyte half-life by about 24 h. In addition, Rosero et al. (2015) reported longer and thinner villi and deeper crypts in nursery pigs fed dietary peroxidized soybean oil. In contrast, Liu et al., (2014c) measured paracellular intestinal permeability via urinary lactulose and mannitol ratios and observed no differences among pigs fed 10% fresh oil compared to peroxidized oil. Lastly, another implication is that feeding peroxidized lipids may affect immune competence and disease resistance (Liu et al., 2014c; Takahashi and Akiba, 1999; Dibner et al., 1996). Because lipid peroxidation occurs as a free radical producing reaction, feeding peroxidized lipids can be a model to induce oxidative stress. Despite this, current experiments have tended to focus on measuring a singular marker of oxidative stress. Boler et al. (2012) concluded that feeding 5% peroxidized corn oil (PV of 7.5 mEq/kg diet) increased TBARS concentration in plasma of finishing pigs which was confirmed by Lu et al. (2014) who reported an increase in TBARS concentration in plasma of pigs fed 5% peroxidized soybean oil compared to their control-fed counterparts (14.76 μm/mL and 3.73 μm/mL respectively) after 55 d. Increased serum TBARS concentration, but not urinary TBARS concentration, was also reported by Liu et al. (2014c) in pigs fed peroxidized lipids compared to fresh lipids. Takahashi and Akiba, (1999) also observed increased plasma

TBARS in chicks fed 5.25% peroxidized soybean oil compared to fresh soybean oil (7.2 nmol MDA/mL and 4.6 nmol MDA/mL respectively) which is supported by Ringseis et al., (2007) who reported that TBARS concentration in enterocyte lysates in pigs fed 90 g/kg peroxidized sunflower oil was approximately double that of in pigs fed fresh sunflower and palm oil blend. This literature highlights that TBARS concentrations increases in tissue homogenates and blood when feeding peroxidized lipids.

Protein oxidation can also occur in response to lipid peroxidation that mainly affects amino acid residues (Fellenberg and Speisky, 2006); most commonly measured via protein carbonyl concentration. Pigs fed 5% peroxidized soybean oil have been shown to have increased plasma protein carbonyl concentration in comparison to pigs fed fresh oil (Lu et al., 2014). Furthermore, protein carbonyl concentrations in the liver were approximately 3 times higher in pigs fed peroxidized soybean oil compared to pigs fed the control soybean oil for 55 d. It could be suggested that protein carbonyl concentration should also be measured in addition to TBARS because protein damage is most affected by ROS in comparison to lipid and DNA damage mechanisms (Dalle-Donne et al., 2003).

Table 1.2: Effects of dietary peroxidized lipids on broilers (n = 26) and pigs (n = 18) performance (Adapted from Hanson, 2014.)

Citation	Species	Source[1]	PV[2]	ADG[3]	ADFI[3]	G:F[3]	Serum Vit E[3]	Serum TBARS[3,4]
Inoue et al., 1984	broilers	soy oil	44.0	49.8	70.1	70.4	23.1	.
Inoue et al., 1984	broilers	soy oil	30.9	60.7	76.4	78.7	50.0	.
L'Estrange et al., 1966	broilers	beef	10.9	68.9	67.8	101.2	.	.
Takahashi and Akiba, 1999	broilers	soy oil	10.0	80.2	90.6	88.5	19.6	174.8
Inoue et al., 1984	broilers	soy oil	22.0	85.8	92.8	92.0	47.8	.
Inoue et al., 1984	broilers	soy oil	15.5	87.4	92.8	94.4	62.3	.
Wang et al., 1997	broilers	poultry fat	26.8	89.3	92.3	91.7	.	.
Engberg et al., 1996	broilers	vegetable	17.2	92.2	92.8	99.4	61.4	.
Takahashi and Akiba, 1999	broilers	soy oil	10.0	92.5	99.1	93.3	15.2	156.5
Anjum et al., 2002	broilers	soy oil	1.5	93.7	98.8	94.6	.	.
Tavárez et al., 2011	broilers	soy oil	6.8	93.9	98.4	95.5	41.5	97.0
Inoue et al., 1984	broilers	soy oil	11.7	94.2	103.2	90.4	105.8	.

Table 1.2 continued

Citation	Species	Source[1]	PV[2]	ADG[3]	ADFI[3]	G:F[3]	Serum Vit E[3]	Serum TBARS[3,4]
Lin et al., 1989	broilers	sunflower	22.0	95.4	98.2	97.1	.	.
Anjum et al., 2004	broilers	soy oil	1.0	95.8	98.6	97.1	.	.
McGill et al., 2011a	broilers	A-V blend	3.5	96.8	99.3	97.8	.	.
Anjum et al., 2002	broilers	soy oil	1.0	97.3	98.9	98.2	.	.
Inoue et al., 1984	broilers	soy oil	5.9	97.5	96.0	101.9	60.9	.
McGill et al., 2011a	broilers	A-V blend	7.1	97.8	99.1	98.4	.	.
McGill et al., 2011b	broilers	A-V blend	7.1	99.3	99.3	98.4	.	.
McGill et al., 2011b	broilers	A-V blend	3.5	99.3	100.0	98.9	.	.
Racanicci et al., 2008	broilers	poultry fat	1.6	100.0	97.8	101.9	.	.
Upton et al., 2009	broilers	poultry fat	3.0	.[5]	.	101.5	.	.
Upton et al., 2009	broilers	poultry fat	6.0	.	.	104.6	.	.
Cabel et al., 1988	broilers	poultry fat	7.2	.	.	96.5	.	.
Cabel et al., 1988	broilers	poultry fat	2.1	.	.	99.4	.	.
Cabel et al., 1988	broilers	poultry fat	4.1	.	.	99.4	.	.
Mean, broilers				**88.9**	**93.4**	**95.4**	**48.8**	**142.8**
Oldfield et al., 1963	pigs	fish oil	6.1	64.6	77.1	83.5	.	.
Liu et al., 2014	pigs	canola oil	23.9	74.6	89.5	84.1	48.9	122.8
Liu et al., 2014	pigs	canola oil	1.2	75.7	78.8	96.8	60.0	120.4
Liu et al., 2014	pigs	corn oil	0.2	76.9	84.4	91.3	18.9	114.8
Liu et al., 2014	pigs	poultry fat	0.2	77.3	87.1	88.6	70.0	103.4
Liu et al., 2014	pigs	tallow	0.3	86.3	89.4	95.7	89.7	102.0
DeRouchey et al., 2004	pigs	CWG	0.1	91.0	90.8	100.3	.	.
Liu et al., 2014	pigs	corn oil	15.1	91.6	88.8	102.9	29.0	121.0
Boler et al., 2012	pigs	corn oil	7.5	93.4	95.0	95.3	46.3	113.1
Oldfield et al., 1963	pigs	fish oil	1.6	93.7	90.0	104.0	.	.
Harrell et al., 2010	pigs	corn oil	7.5	94.7	94.8	99.8	.	.
DeRouchey et al., 2004	pigs	CWG	6.3	95.4	92.1	103.6	.	.
DeRouchey et al., 2004	pigs	CWG	0.1	96.8	91.1	106.3	.	.
DeRouchey et al., 2004	pigs	CWG	2.4	100.2	98.1	102.1	.	.
Liu et al., 2014	pigs	poultry fat	5.7	101.4	102.0	97.1	73.6	107.0
Liu et al., 2014	pigs	tallow	2.9	104.6	109.8	94.3	96.6	103.1
Lu et al., 2014	pigs	soy oil	7.0	55.43	52.17	106.0	.	395.71
Lu et al., 2014	pigs	soy oil	7.0	26.36	33.43	81.58	.	.
Mean, swine				**83.3**	**85.8**	**96.3**	**59.2**	**140.3**
Mean, broilers and swine				84.2	89.9	95.8	53.7	140.9

[1]Source = source of supplemental fat, A-V blend = blend of animal fat-vegetable oil, and CWG = choice white grease.

[2]PV = peroxide value, meq/kg of diet containing peroxidized lipid was calculated as: [fat inclusion level, % x PV of fat source] or reported as stated by researchers.

[3]All response variables were calculated by dividing the mean of the animals fed diets with peroxidized lipids by those fed unperoxidized lipids and multiplying by 100. Therefore, the values presented represent the percentage of the variable relative to controls.

[4]TBARS = thiobarbituric acid reactive substances.

[5]A period indicates that this variable was not reported or estimable from the research reports.

Enzymatic and nonenzymatic antioxidant capacity has also been studied to a limited degree. Boler et al. (2012) reported that Vitamin E concentration in plasma was 56.7% greater in pigs fed fresh corn oil in comparison to pigs fed peroxidized corn oil. Likewise liver vitamin E concentration was 52.8% greater in pigs fed fresh corn oil compared to pigs fed peroxidized corn oil. In contrast, plasma and liver GPx concentrations did not differ among treatment groups (Boler et al., 2012). Ringseis et al. (2007) also reported lower alpha tocopherol concentration in intestinal epithelial cells in pigs fed peroxidized sunflower oil compared to pigs fed a fresh blend of sunflower oil and palm oil with mean values of 0.15 μg/mg protein and 0.10 μg/mg protein respectively.

Summary

Clearly there are many benefits of feeding lipids to swine and poultry such as increasing the energy density of the feed, providing fat soluble vitamins and essential fatty acids, improving diet palatability, while decreasing the dustiness of the feed. However, consumption of lipids which contain peroxidation products can diminish any or all of these benefits. Furthermore, consumption of peroxidized lipids can cause oxidative stress by inducing a free radical reaction and can overwhelm the antioxidant defense system *in vivo*. In order to accurately determine oxidative balance, multiple measures of lipid, protein, and DNA damage in combination with antioxidant status should be measured. However, to date most research on the consumption of lipid peroxidation products has focused on measuring a singular measure of oxidative stress in a single tissue to evaluate oxidative balance, Table 1.2, resulting in a poor understanding of

whole-animal oxidative stress on measures of lipid, protein, and DNA damage and on antioxidant status *in vivo*.

Therefore, the objectives of this thesis are:

1) To evaluate dietary peroxidized lipids on growth performance in growing pigs
2) Investigate whether peroxidized lipids alter oxidative status and to evaluate and compare markers of oxidative stress in pigs
3) To assess several lipid sources and their effects on growth performance and oxidative status in broilers

The results of these experiments will consequently provide information on the use of thermally peroxidized lipids in feed ingredients and diets included in swine and poultry models; and provide direction as to which measures in which tissues should be focused on in future research on the impacts of lipid peroxidation products and oxidative stress.

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CHAPTER 2

INFLUENCE OF FEEDING THERMALLY PEROXIDIZED SOYBEAN OIL ON GROWTH PERFORMANCE, DIGESTIBILITY, AND GUT INTEGRITY IN GROWING PIGS

A manuscript prepared for submission to the Journal of Animal Science

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Abstract

Consumption of peroxidized oils has been shown to affect pig performance and oxidative status through the development of compounds which differ according to how oils are thermally processed. The objective of the current study was to evaluate the effect of feeding varying levels of peroxidized soybean oil (SO) on growth performance, lipid, N, and GE digestibility, and gut integrity in growing pigs. Fifty-six barrows (25.3 ± 3.3 kg initial BW) were randomly assigned to 1 of 4 diets containing either 10% fresh SO (22.5^{o}C) or thermally processed SO (45^{o}C for 288 h, 90^{o}C for 72 h, or 180^{o}C for 6 h), each with an air infusion of 15 L/min. Peroxide values for the 22.5, 45, 90 and 180^{o}C processed SO were 2.0, 96, 145, and 4.0 mEq/kg, respectively; 2,4-decadienal values for 22.5, 45, 90 and 180^{o}C processed SO were 2.11,5.05, 547.62, and 323.57 mg/kg, respectively; and 4-hydroxynonenal concentrations of 0.05, 1.05, 39.46, and 25.71 mg/kg

with increasing SO processing temperature. Pigs were individually housed and fed ad libitum for 49 d to measure growth performance, including a metabolism period to collect urine and feces for determination of GE and N digestibility, and N retention as affected by SO peroxidation status. Following the last day of fecal and urine collection, lactulose and mannitol were fed and subsequently measured in the urine to evaluate gut permeability. Although there were no differences observed in ADFI ($P = 0.19$), ADG was decreased in pigs fed 90^{o}C SO diet ($P = 0.01$), while G:F was increased ($P = 0.02$), in pigs fed 45^{o}C SO diet compared to the other SO diets. Pigs fed the 90^{o}C processed SO had the lowest ($P = 0.01$) DE as a % of GE, whereas ME as a % of DE was lowest ($P = 0.05$) in in pigs fed the 180^{o}C SO and 90^{o}C SO followed by 45^{o}C SO and fresh SO. Ether extract digestibility was lowest ($P = 0.01$) in pigs fed 90^{o}C SO followed by pigs fed 180^{o}C SO, 45^{o}C SO, and fresh SO. The percent of N retained was greatest ($P = 0.01$) in pigs fed fresh SO followed by pigs fed 45^{o}C SO, 180^{o}C SO, and 90^{o}C respectively. There were no differences observed among SO treatments for urinary lactulose:mannitol ratio ($P = 0.60$). These results indicate that the presence of compounds such as PV, DDE, and HNE contained in the 90^{o}C SO diet reduce ADG as well as reduce GE and ether extract digestibility, and N balance, but appear to have no impact on gut permeability.

Key words: digestibility, gastrointestinal integrity, growing pigs, peroxided soybean oil

Introduction

Lipids are added to swine diets as a concentrated energy source (Pettigrew and Moser, 1991; Azain, 2001; Lin et al., 2013). In the United States, soybean oil (**SO**) is a

common lipid source added in swine diets that contains a high concentration of monounsaturated fatty acids (**MUFA**) and polyunsaturated fatty acids (**PUFA**) (NRC, 2012). In contrast to saturated fatty acids (**SFA**), MUFA and PUFA are more susceptible to lipid peroxidation due to the double bonds in their conformation (Holman, 1954). In the swine industry, thermally processed lipids may be an economical alternative to increase dietary energy concentration compared to refined lipid sources, but at the expense of lipid quality. Lipid peroxidation occurs as a free radical chain reaction commonly caused by thermal processing in the presence of oxygen. Products formed through lipid peroxidation include peroxides, aldehydes, polymers, and polar compounds (Gonzalez-Muñoz et al., 1998). In contrast, as lipid peroxidation products are formed, antioxidants in the lipid becomes depleted (Seppanen and Csallany, 2002). In addition to the compounds formed during lipid peroxidation, the unsaturated:saturated fatty acid ratio decreases and FFA concentration increases (Liu et al., 2014b), which may in itself affect the energy value (Wiseman and Salvador, 1991).

Dietary lipid peroxidation products have been shown to decrease growth performance in swine (Boler et al., 2012; Rosero et al., 2015) and poultry (Dibner et al., 1996; Anjum et al., 2004; Tavárez et al., 2011). Furthermore, feeding peroxidized lipids have been linked to decreased digestibility (Liu et al., 2014c). However, limited information is available regarding the effects of feeding various degrees of thermally processed SO in swine nutrition. Therefore, the objective of this study was to evaluate the effect of feeding divergently thermally processed SO to growing pigs on growth performance, digestibility, and intestinal integrity.

Materials and Methods

All animal care and use procedures for this experiment were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Dietary Treatments

Treatments consisted of four diets containing either 10% fresh SO or SO thermally processed at 1) 45^{o}C for 288 h, 2) 90^{o}C for 72 h, or 3) 180^{o}C for 6 h. Each heating process was accompanied with constant air flow (15L/min) using an air pump and a calibrated air flow controller. After thermal processing and before feed mixing, processed oils were stored at -20^{o}C, and no antioxidant was added before or during diet preparation. Diverse analyses including FA profile, oil quality, lipid peroxidation products, and total tocopherols were conducted on each SO treatment as outlined in Table 2.1 to characterize the quality of each SO treatment. Diets (Table 2.2) were formulated to contain 1.30% standardized ileal digestible Lys, with AA ratios, ME, and mineral content adequate for 25 kg pigs according to the NRC (2012).

Experimental Design

A total of 56 barrows (initial BW 25.28 ± 3.31 kg) were housed at the Swine Nutrition Farm at Iowa State University (Ames, IA) for the duration of the study. Pigs were randomly assigned to 1 of 4 dietary treatments, resulting in 14 replications per treatment. Each pig was individually penned (1.8 × 1.9 m) for 49 d and had ad libitum access to feed and water, including 5 d in metabolism crates. Performance data was observed for 49 d with amount of feed added to feeders recorded throughout the

experiment, and pigs and feeders weighed on d 0, 21, d 49 to determine ADG, ADFI, and G:F.

During the performance portion of the experiment, 20, 20, and 16 pigs were moved on d 21, 25, and 29, respectively, to individual metabolism crates for 5 d to collect urine and feces to evaluate N, ether extract (**EE**), and GE digestibility, and for a urine collection to evaluate *in vivo* intestinal permeability. During this period, pigs were fed an amount of diet equivalent to 4% of their average BW twice daily (2% at 0700 h and 2% at 1700 h) with constant access to water. After 2 d for crate adaptation, a 3-d fecal and urine collection period occurred for GE digestibility and for N digestibility and balance. To limit microbial growth and ammonia loss in the collected urine, 15 mL of 6 *N* HCl was added to the collection containers during urine collection. Urine was collected twice daily and stored at -10^{o}C until subsequent analysis. Titanium dioxide was added in the feed as an indigestible marker for digestibility calculations. A subsample of feces were collected during the collection period and stored at -10°C. At the end of the collection period, feces were dried at 70°C for 48 h, weighed, ground through a 2-mm screen, and a subsample from each pig was collected for digestibility analysis.

To determine *in vivo* intestinal permeability, all pigs were subjected to an oral lactulose and mannitol challenge. Following a 12-h fast on the evening of d 4 in the metabolism crates, each pig was fed 100 g of their assigned treatment diet which contained 500 mg/kg BW lactulose and 50 mg/kg BW mannitol (Spectrum Chemical, Gardena, CA). After each pig consumed this portion (within 15 minutes), they were then fed the remaining portion of their respective diet. Plastic containers containing 5 mL chlorhexidine were placed under each metabolism crate to eliminate microbial growth

and urine was collected for the next 12 h (overnight), volume quantified, subsampled, and stored at -20^{o}C for analysis. Following the metabolism experiment, pigs were returned back to their pens for the remainder of the performance portion of the trial, with feed consumption during the metabolism crated period recorded, and added to the total feed intake for the performance trial.

Calculations and Methodologies

Gross energy of the diet, feces, and urine was determined using an isoperibol bomb calorimeter (model 1281, Parr Instrument Co., Moline, IL) using benzoic acid as a standard. Analyses performed on diets and fecal samples were done in duplicates and urine analyses were performed in triplicates. For urine analysis, 1 mL was filtered and added to 0.5 g of dried cellulose and dried for 24 h at 50^{o}C and repeated two times to determine of urinary GE. In addition, GE of cellulose was determined in order to calculate GE of urine by subtracting the GE content of cellulose from the GE content of urine samples.

Treatment diets and feces were analyzed for EE as described previously (Luthria et. al., 2004) for EE digestibility. Briefly, samples were mixed with sand (Fisher #S23-3) to avoid compaction and were added to a 10 mL stainless steel cell. The cell was extracted 3 times in the extraction system at 120^{o}C and pressure using petroleum ether as the solvent. The petroleum ether was added to provide extract for the sample and was collected into a pre-weighed glass vial which was placed in an evaporation system (Multivap Model 118, Organomation Associates, Berlin, MA). The vial was then weighed to determine the residual EE.

Nitrogen was analyzed by thermo-combustion (VarioMAX CNS, Elementar Analysensysteme GmbH, Hanau, Germany). During combustion, gases are converted to individual gases and sorted into adsorption columns and are measured using a thermal conductivity detector. Digestibility coefficients were estimated by marker methodology using titanium dioxide, with DE, EE, and N digestibility calculated as a percentage using the equation $[1- (Ti_{diet} \times GE_{feces}, EE_{feces}, \text{ or } N_{feces})/(Ti_{feces} \times GE_{diet}, EE_{diet}, \text{ or } N_{diet})] \times 100$. Metabolizable energy as a percent of DE was calculated by dividing ME intake/d and DE intake/d. Nitrogen retention was calculated by subtracting excreted N from digested N then taking the ratio of N retained to N digested to report as a percent.

Urinary lactulose and mannitol concentrations were measured via HPLC as an *in vivo* indicator of small intestinal permeability and using the method that has been previously described by Kansagra et al., (2003). The ratio of lactulose:mannitol was calculated back to the total amount of urine collected and reported on a recovery basis.

Statistical Analysis

Data were analyzed as a completely randomized design with individual pig as the experimental unit, using Proc GLM procedure of SAS (version 9.4; SAS, 2009) with means reported and separated using LSMEANS. In addition, relationships between lipid peroxidation measures with growth performance and digestibility variables were evaluated by simple linear correlation (Pearson correlation coefficients) analysis. Differences were considered significant at $P \leq 0.05$, whereas values of $0.05 \leq P \leq 0.10$ were considered statistical trends.

Results and Discussion

Compositional changes of soybean oil due to thermal processing

Lipid peroxidation is a complex process that is typically achieved through thermal processing of a lipid (St. Angelo et al., 1996). Lipid peroxidation is a chain reaction involving free radical formation and propagation of free radical binding to PUFA (Holman, 1954), where its progression is based on the duration and intensity of thermal processing and presence of oxygen. Products formed through thermal processing of lipids decreases the quality of the lipid by increasing peroxides in the initiation phase, which are readily degraded into aldehydes and acids in the propagation phase, ultimately forming polar compounds and indigestible polymers (Gonzalez-Muñoz et al., 1998) in the termination phase. Consequently, understanding lipid quality requires the measurement of multiple lipid peroxidation products.

The current experiment induced differing levels of lipid peroxidation of SO by thermally processing at variable temperatures and durations prior to being mixed in the pig diets. The 45^{o}C SO was heated for 288 h (12 d) which is our estimate of the temperature that feed in a bulk bin could reach during summer months. The 90^{o}C SO was processed for 72 h and was chosen as a doubling of 45°C and was used to resemble the rendering of animal fats where heating temperatures in that industry average 115 to 145^{o}C for approximately 40 to 90 min (Meeker and Hamilton, 2006), and is similar to Liu et al., (2014b) who heated various lipids at 95^{o}C for 72 h. Likewise, Kerr et al. (2015) thermally processed corn oil at 95^{o}C for 72 h which resulted in increased aldehyde production, including 4-hydroxynonenal (**HNE**) and 2,4-decadienal (**DDE**) concentrations. The 180^{o}C SO was chosen to resemble the temperature used for frying in

the restaurant industry. In addition, thermally processing corn oil at 190^{o}C has been shown to yield the greatest concentrations of HNE, DDE, and thiobarbituric acid reactive substances (**TBARS**) when heated for 6 h (Kerr et al., 2015).

The four SO utilized in this study were thoroughly analyzed prior to feed mixing for fatty acid composition along with several lipid peroxidation products (Table 2.1). Peroxide value is a measurement of hydroperoxides formed in the initiation phase of lipid peroxidation and was determined to be highest in the 90^{o}C processed SO followed by the 45^{o}C, 180^{o}C processed SO and fresh SO with values of 145.3, 95.6, 4.0, and 2.0 mEq/kg SO, respectively. We suspect that thermally processing SO at 180^{o}C results in unstable hydroperoxides that have already begun forming secondary and tertiary peroxidation compounds which is in agreement with Wang et al., (2016). In the current experiment, concentrations of DDE and HNE were greatest (547.62, and 39.46 mg/kg respectively) at 90^{o}C. Many other aldehydes were measured to show the complexity of lipid peroxidation although we will focus our discussion on DDE and HNE.

In addition to measuring lipid peroxidation compounds, we were interested in measuring the antioxidant status of SO. Tocopherols are natural antioxidants found in vegetable oils and function in protecting the lipid from degradation (Kamal-Eldin, 2006) so as lipid peroxidation progresses, antioxidant status would be depleted. In the current study, the 90^{o}C processed SO had the lowest total tocopherol (**TOC**) concentration with 405 mg/kg oil followed by 180^{o}C, 45^{o}C, and fresh oil with 609, 620, and 772 mg/kg respectively. Miyagawa et al. (1991) reported that thermally processing a blend of soybean oil and rapeseed oil at frying temperature (185^{o}C) resulted in a rapid degradation rate for gamma tocopherol followed by delta- and alpha- tocopherol respectively. In the

current experiment, the 90°C SO had the lowest gamma tocopherol concentration suggesting that it had the highest degradation rate in comparison to the other SO treatments. This trend was also noted for delta tocopherol. Alpha tocopherol concentration was greatest in the 90°C SO which is again in agreement with Miyagawa et al. (1991) who reported the greatest retention rate for alpha tocopherol when thermally processed.

Growth performance

Performance data were collected over the 49 d trial, including the 5 d while pigs were in the metabolism crates. During the experiment period, 1 pig fed the 45°C SO and 1 pig fed the 180°C SO died due to causes unrelated to dietary SO treatment; therefore, data are reported using 14 observations for 22.5°C and 90°C SO and 13 observations for 45°C and 180°C SO. As shown in Table 2.3, pigs fed the 90°C SO had reduced ADG (P = 0.01) by about 7% in comparison to the other three SO treatment groups, with no differences noted among pigs fed the 22.5°C, 45°C, and 180°C SO treatments. Interestingly, thermal processing of SO did not affect ADFI (P = 0.19). Pigs fed 45°C processed SO had an increased G:F (P = 0.02) by about 3% compared to the other SO treatments, with no differences noted among pigs fed the other SO treatments.

Because growth performance parameters were affected by dietary SO processing, a correlation analysis among various measures of lipid peroxidation products and biological responses were conducted (Table 2.4). We elected to conduct correlations specifically for unsaturated:saturated fatty acid ratio (**UFA:SFA**) and FFA because of their impact on energy digestibility (Wiseman and Salvador, 1991); PV, AnV, TBARS,

and hexanal because they are common measures of lipid peroxidation in the literature (Shurson et al., 2015); acrolein (Kehrer and Biswal, 2000; Abraham et al., 2011), DDE (Wang et al., 2016), and HNE (Esterbauer et al., 1991; Wang et al., 2016), because they are considered highly damaging to DNA, proteins, and lipids; a ratio of 10 aldehydes associated with soybean oil peroxidation which has been suggested to be a good measure of SO peroxidation (Wang et al., 2016); and total tocopherols as they would provide an accurate depiction of the antioxidant status naturally occurring in SO (Seppanen and Csallany, 2002; Kamal-Eldin, 2006). Irrespective of SO diet, PV, AnV, HNE, and DDE all had negative correlations with ADG, while total tocopherols had a positive correlation as shown in Table 2.4. The current experiment did not observe a correlation between TBARS in the SO with ADG, which is in contrast to Liu et al. (2014a) who reported a negative correlation between oil TBARS and ADG. The reduction in ADG in pigs fed the 90^{o}C SO is in agreement with Boler et al. (2012) who reported decreased ADG in finishing pigs fed corn oil processed at 95^{o}C compared to pigs fed a diet containing fresh corn oil, and further confirmed by Liu et al. (2014a) who reported reduced ADG in pigs fed diets containing peroxidized oil (canola oil, corn oil, poultry fat, and tallow) compared pigs fed diets containing fresh oil. Furthermore, Hanson (2014) conducted a detailed review of 16 experiments in swine which were fed peroxidized lipids and reported that pigs fed thermally processed lipids reduced growth by 16% compared to pigs fed fresh lipid sources.

Secondary lipid peroxidation compounds, including many aldehydes, can produce a rancid odor and flavor and have been shown to affect palatability and feed intake in swine and poultry (Dibner et al., 1996; Boler et al., 2012; Liu et al., 2014a). Therefore,

we would have expected a decrease in ADFI as SO processing increased. However, in the current experiment this was not observed (Table 2.3). Regardless, ADFI was negatively correlated with PV (r = -0.28, *P* = 0.04) and TBARS (r = -0.28, *P* = 0.04). These data are in agreement with a review by Hanson (2014) who summarized that pigs fed peroxidized lipids had decreased ADFI (14.2%); however in Hanson's review, ADFI was only correlated with dietary TBARS (r = -0.55, *P* = 0.10).

Interestingly, in the current experiment pigs fed the 45^{o}C SO had the greatest feed efficiency. This is due to 45^{o}C SO fed pigs having numerically higher ADG but a similar ADFI compared to other treatment diets, resulting in increased G:F compared to other SO treatments. Negative correlations with AnV, HNE, and DDE were observed in relation to G:F as shown in Table 2.4. According to the review by Hanson (2014), G:F of pigs fed peroxidized lipids was reduced to 96.6% of pigs fed fresh lipid sources.

Energy and lipid digestibility

Digestibility of lipids is dependent on several factors, one of which being their degree of saturation. Unsaturated fatty acids are typically more digestible because their ability to form a micelle is greater in comparison to saturated fatty acids, with a lower UFA:SFA negatively impacting digestibility (Wiseman and Salvador, 1991). In the current experiment, UFA:SFA was lowest in the 90^{o}C SO (4.64) followed by 180^{o}C SO (5.02), 45^{o}C SO (5.27), and fresh oil (5.35), which confirms that thermal processing increased the saturation of the lipid in the current experiment (Table 2.1). This is further supported by others (DeRouchey et al., 1997; Liu et al., 2014b) who have also shown that lipid peroxidation hydrogenates lipids making them more saturated. In the current

experiment, a positive correlation was noted between UFA:SFA and DE (P = 0.04, r = 0.28). While increased concentrations of FFA may also reduce lipid digestibility (Wiseman and Salvador, 1991), these differences were small in the current study (0.04, 0.07, 0.35, and 0.14% for the 22.5, 45, 90, and 180°C SO diets, respectively) and likely did not have a large impact on lipid digestibility (Kerr and Shurson, 2017). Although the FFA concentrations did not play a large role in digestibility differences, there was a negative correlation between DE and EE with FFA. Digestibility differences could also be due to the increase in lipid peroxidation products as measured by increased PV, AnV, polar compounds and several aldehydes described in Table 2.1.

In the current experiment, DE as a percentage of GE was greatest in the fresh SO group and lowest in the 90°C SO group, with the 45°C and 180°C SO groups being intermediate (P = 0.01). The ME as a percentage of DE also differed among the SO thermal processing procedures, with fresh SO having the greatest ME as a percentage of DE (99.18%), the 90°C and 180°C SO the lowest (98.76 and 98.64%, respectively), and the 45°C SO (99.04%) being intermediate (P = 0.05). Fresh SO had the greatest EE digestibility followed by 45°C SO, 180°C SO, and 90°C SO with values of 83.03%, 82.11%, 81.20%, and 78.35% respectively, P = 0.01 (Table 2.5). Multiple negative correlations were observed between DE and EE with PV, AnV, TBARS, HNE, DDE, and TOC. However, no correlations of these parameters were observed for ME. These results are in agreement with DeRouchey et al. (1997) and Rosero et al. (2015) who reported reductions in nutrient digestibility when pigs were subjected to dietary lipid peroxidation in choice white grease and soybean oil respectively. In contrast, Liu et al. (2014c) did not report changes in DE, ME, and EE digestibility in pigs fed various peroxidized lipids.

The combination of a decreased UFA:SFA, increased FFA content, and lipid peroxidation products such as indigestible aldehydes appear to be responsible for the decreased digestibility in pigs fed the 90°C SO and 180°C SO. The reduction in energy and EE digestibility in pigs fed the 90°C SO also helps explain the reduction in ADG. This could result from the increase in lipid peroxidation products including PV, AnV, polar compounds, DDE, polymers, and saturated fatty acids.

Nitrogen balance

There was a tendency ($P = 0.10$) for N digestibility to be highest for both the fresh SO and the 180°C SO (88.72% and 88.65% respectively) and lowest for the 45°C SO (87.05%), with the 90°C SO (88.19%) being intermediate (Table 2.5). These results are in contrast to DeRouchey et al. (1997) who did not report any statistical differences among N digestibility in nursery pigs fed thermally processed choice white grease. In addition, Liu et al. (2014c) did not report differences in N digestibility in peroxidized lipids in comparison to fresh lipids. Nitrogen digestibility was not correlated to any key lipid peroxidation products (Table 2.6). Dietary treatment did affect N retention ($P = 0.01$) with fresh SO having the highest N retention followed by 45°C SO, 180°C SO, and 90°C SO (90.56%, 88.98%, 86.94%, and 86.04%, respectively). In the current experiment, N retention was negatively correlated to AnV (r = -0.43, $P = 0.01$), HNE (r = -0.43, $P = 0.01$), and DDE (r = -0.42, $P = 0.01$) and positively correlated with total tocopherols (r = 0.39, $P = 0.01$), but this relationship does not necessitate causality. In contrast, Liu et al. (2014c) did not report any differences in N retention. Furthermore, the differences in N

digestibility and retention may suggest that lipid peroxidation affects muscle accretion, although further research is needed to confirm this statement.

Intestinal barrier function

Changes in intestinal integrity may be associated with changes in absorption of nutrients and resistance to pathogens (Wijtten et al., 2011). Therefore, we were interested in measuring the effects of lipid peroxidation products in variably processed dietary SO on intestinal permeability. It has been shown that intestinal permeability is increased when consuming a diet high in saturated FA (Laugerette et al., 2012; Mani et al., 2012; Liu et al., 2014d). The ratio of lactulose to mannitol in urine is commonly used as an *in vivo* indicator of small intestinal paracellular permeability (Wijtten et al., 2011). In the current study, there were no differences in the urinary lactulose to mannitol ratio among SO treatment groups with urinary ratios of lactulose to mannitol on a recovery basis averaging 0.05 ($P = 0.60$). Due to the creation of lipid peroxidation compounds and the increased saturation of thermally processed SO, we would have expected increased intestinal permeability in pigs fed 90^{o}C and 180^{o}C SO. Despite this, our findings are in agreement with Liu et al. (2014d) who also found no significant differences in urinary lactulose:mannitol ratios among lipid peroxidation level. Even though there were no significant differences in urinary lactulose:mannitol ratios, a simple correlation matrix was conducted and, not surprisingly, no significant correlations were found.

In conclusion, thermal processing resulted in reduced UFA:SFA, while increasing FFA and lipid peroxidation compounds including PV, HNE, and DDE developed. The combination of changes in FA profile and formation of toxic lipid peroxidation products

were found to be greatest in the SO thermally processed at 90°C for 72 h in comparison to other treatment groups. This resulted in reduced ADG, energy and EE digestibility, and N retention in pigs fed the 90°C SO. Further, intestinal permeability as measured by urinary lactulose:mannitol was not affected by dietary treatment. The observed correlations between the lipid peroxidation products assessed may provide information implying that a combination of lipid peroxidation products negatively impacts utilization of nutrients.

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Table 2.1. Composition and peroxidation analysis of thermally processed soybean oils

Heating temperature, °C	22.5	45	90	180
Time heated, h[1]	0	288	72	6
Fatty acids, % of total fat[2,3]				
C8:0, Caprylic	ND[4]	ND	0.07	ND
C14:0, Myristic	0.08	0.07	0.09	0.08
C15:0, Pentadecanoic	ND	ND	0.07	ND
C16:0, Palmitic	10.80	10.94	12.25	11.26
C16:1, Palmitoleic	ND	0.08	0.09	ND
C17:0, Margaric	0.11	0.10	0.12	0.11
C18:0, Stearic	3.83	3.88	4.35	4.03
C18:1, Oleic	22.08	22.29	24.28	22.78
C18:2, Linoleic	54.05	53.74	50.96	53.02
C18:3, Linolenic	7.83	7.50	6.27	7.07
C19:0, Nonadecanoic	0.24	0.27	0.20	0.39
C20:0, Arachidic	0.30	0.31	0.34	0.32
C20:1, Gadoleic	0.18	0.19	0.21	0.30
C22:0, Behenic	0.36	0.35	0.23	0.38
C22:5, Docosapentaenoic	ND	0.11	0.12	ND
Other FA[4]	0.13	0.15	0.35	0.20
UFA:SFA[4]	5.35	5.27	4.64	5.02
Free fatty acids, %[2]	0.04	0.07	0.35	0.14
Free glycerin, %[2]	2.68	3.50	2.27	2.44
Moisture, %	0.02	0.02	0.10	0.02
Insoluble impurities, %	0.02	0.02	0.04	0.02
Unsaponifiable matter, %	0.29	0.35	0.53	0.30
Oxidized FA, %[2]	1.3	2.5	3.1	1.4
OSI @ 110 °C, h[2,4]	7.15	3.65	2.70	3.35
p-Anisidine value[2,5]	1.19	8.38	261	174
Peroxide value, mEq/kg[2]	2.0	95.6	145.3	4.0
Polar compounds, %[2]	2.67	7.01	22.65	10.19
TBA value[2,5]	0.10	0.14	0.14	0.09
Aldehydes, mg/kg[6]				
2,4-decadienal	2.11	5.05	547.62	323.57
4-hydroxynonenal	0.05	1.05	39.46	25.71
Acrolein	3.88	3.31	15.82	45.39
Decenal	0.24	0.35	28.17	19.81
Heptadienal	0.12	4.40	85.50	61.86
Heptanal	0.22	3.60	89.12	40.08
Hexanal	2.97	2.71	21.20	16.84
Octenal	0.19	1.10	59.86	21.31
Pentanal	0.28	0.31	1.82	2.50
Undecadienal	0.10	0.18	26.02	15.35
Undecenal	0.27	0.29	27.57	23.38

Table 2.1 continued

Total tocopherols, mg/kg[2]	772	620	405	609
Alpha	56	14	343	197
Beta	< 10	< 10	< 10	< 10
Delta	206	190	15	75
Gamma	510	416	47	337

[1]Thermally processed oils had constant air flow rate at 15L/min.

[2] Analyzed by Barrow-Agee, Memphis, TN.

[3] No other FA were detected besides those listed.

[4]Abbreviations: ND, not detected; FA, fatty acid; UFA:SFA, unsaturated:saturated fatty acid ratio; TBA, thiobarbituric acid; OSI, oil stability index.

[5] There are no units for p-anisidine value or TBA value.

[6]Analyzed by University of Minnesota, St. Paul, MN.

Table 2.2. Ingredient and calculated composition of treatment diets, as-fed basis.

Item, %	Percent
Corn	54.15
Soybean meal	32.40
Soybean oil	10.00
Limestone	1.13
Monocalcium phosphate	0.90
Titanium dioxide	0.50
Sodium chloride	0.35
Vitamin mix[1]	0.25
Trace mineral mix[2]	0.15
L-Lys·HCl	0.13
DL-Met	0.04
TOTAL	100.00

[1]Provided the following per kilogram of diet: vitamin A, 6,125 IU; vitamin D_3, 700 IU; vitamin E, 50 IU; vitamin K, 30 mg; vitamin B_{12}, 0.05 mg; riboflavin, 11 mg; niacin, 56 mg; and pantothenic acid, 27 mg.

[2]Provided the following per kilogram of diet: Cu (as $CuSO_4$), 22 mg; Fe (as $FeSO_4$), 220 mg; I (as $Ca(IO_3)_2$), 0.4 mg; Mn (as $MnSO_4$), 52 mg; Zn (as $ZnSO_4$), 220 mg; and Se (Na_2SeO_3), 0.4 mg.

Table 2.3. Growth performance of pigs fed soybean oil with differing peroxidation levels.

	Processed soybean oil[1]				Statistics	
Parameter	22.5	45	90	180	SEM	P value
ADG, kg	1.02[a]	1.05[a]	0.96[b]	1.03[a]	0.02	0.01
ADFI, kg	2.05	2.00	1.94	2.09	0.05	0.19
G:F	0.50[b]	0.53[a]	0.49[b]	0.50[b]	0.01	0.02

[1]Data are least square mean of 14 observations for 22.5 and 90; and 13 observations for 45 and 180. 22.5 = fresh oil; 45 = oil heated for12 d at 45°C with constant compressed air flow rate at 15L/min; 90 = oil heated for 72 h at 90°C with constant compressed air flow rate at 15L/min; 180 = oil heated for 6 h at 180°C with constant compressed air flow rate at 15L/min. Performance data was collected over 49 d with initial ABW of 25.28 ± 3.31 kg (P = 0.36) and final average BW of 70.80 ± 5.73 kg (P = 0.11).

[2]Superscripts reflect peroxidized soybean oil treatment differences (abc, $P \leq 0.05$).

Table 2.4. Pearson correlation coefficients among lipid composition and peroxidation measures with performance, digestibility, and gut integrity responses[1]

	Soybean oil composition and lipid peroxidation measures[2]										
Criterion	UFA:SFA	FFA	PV	AnV	TBARS	Hexanal	Acrolein	DDE	HNE	Ratio	TOC
ADG	0.44	-0.47	-0.33	-0.39	-	-0.36	-	-0.41	-0.39	-0.33	0.39
	(0.01)	(0.01)	(0.02)	(0.01)		(0.01)		(0.01)	(0.01)	(0.02)	(0.01)
ADFI	-	-	-0.28	-	-0.28	-	-	-	-	-	-
			(0.04)		(0.04)						
G:F	0.27	-0.25	-	-0.32	-	-0.34	-0.26	-0.32	-0.32	-0.35	-
	(0.06)	(0.08)		(0.02)		(0.02)	(0.06)	(0.02)	(0.02)	(0.01)	

[1]Top value represents correlation (*r* value) and bottom value in parenthesis represents significance (*P*-value). If no value is given, it was not found to be significant (-) at $P \leq 0.10$.

[2]UFA:SFA, unsaturated:saturated fatty acid ratio; FFA, free fatty acids; PV, peroxide value; AnV, p-anisidine value; TBARS, thiobarbituric acid reactive substances; DDE, 2,4-decadienal; HNE, 4-hydroxynonenal; Ratio, ratio of aldehydes as described by Wang et al., 2016; TOC, total tocopherols.

Table 2.5. Energy and lipid digestibility, nitrogen balance, and intestinal permeability in growing pigs fed various levels of peroxidized soybean oil.

	Processed soybean oil[1]				Statistics	
Parameter	22.5	45	90	180	SEM	P value[2]
DE, % of GE	88.63^{a}	87.84^{bc}	87.26^{c}	88.44^{ab}	0.24	0.01
ME, % of DE	99.18^{a}	99.04^{ab}	98.76^{b}	98.64^{b}	0.15	0.05
EE digestibility, %	83.03^{a}	82.11^{ab}	78.35^{c}	81.20^{b}	0.57	0.01
Nitrogen digested, %	88.72^{x}	87.05^{y}	88.19^{xy}	88.65^{x}	0.51	0.10
Nitrogen retained, %[3]	90.56^{a}	88.98^{ab}	86.06^{c}	86.94^{b}	0.95	0.01
Lactulose:Mannitol	0.05	0.06	0.05	0.04	0.01	0.60

[1]Data are least square mean of 14 observations for 22.5 and 90; and 13 observations for 45 and 180. 22.5 = fresh oil; 45 = oil heated for12 d at 45^{o}C; 90 = oil heated for 72 h at 90^{o}C; 180 = oil heated for 6 h at 180^{o}C. All oil groups had a constant compressed air flow rate at 15L/min.

[2]Superscripts reflect peroxidized soybean oil treatment differences (abc, $P \leq 0.05$).

[3]Nitrogen retention was calculated by subtracting excreted N from digested N then taking the ratio of N retained to N digested to report as a percent.

Table 2.6. Pearson correlation coefficients among soybean oil composition and peroxidation measures with digestibility responses[1]

	Soybean oil composition and lipid peroxidation measures										
Criterion	UFA:SFA	FFA	PV	AnV	TBARS	Hexanal	Acrolein	DDE	HNE	Ratio	TOC
DE:GE	0.28	-0.32	-0.40	-	-0.36	-	-	-	-	-	0.33
	(0.04)	(0.02)	(0.01)		(0.01)						(0.01)
ME:DE	-	-	-	-0.24	-	-0.25	-0.26	-	-0.24	-0.26	-
				(0.09)		(0.07)	(0.06)		(0.09)	(0.06)	
EE digestibility	0.50	-0.51	-0.36	-0.47	-0.23	-0.45	-	-0.48	-0.47	-0.43	0.47
	(0.01)	(0.01)	(0.01)	(0.01)	(0.10)	(0.01)		(0.01)	(0.01)	(0.01)	(0.01)
N retention	0.42	-0.39	-	-0.43	-	-0.43	-0.26	-0.42	-0.43	-0.42	0.39
	(0.01)	(0.01)		(0.01)		(0.01)	(0.06)	(0.01)	(0.01)	(0.01)	(0.01)

[1]Top value represents correlation (r value) and bottom value in parenthesis represents significance (P-value). If no value is given, it was not found to be nonsignificant (-) at $P \leq 0.10$. There were no correlations observed between SO composition with N digested and lactulose:mannitol so they were removed from the table.

[2]UFA:SFA, unsaturated:saturated fatty acid ratio; FFA, free fatty acids, PV, peroxide value; AnV, p-anisidine value; TBARS, thiobarbituric acid reactive substances; DDE, 2,4-decadienal; HNE, 4-hydroxynonenal; Ratio, ratio of aldehydes as described by Wang et al., 2016; TOC, total tocopherols.

CHAPTER 3

INFLUENCE OF FEEDING THERMALLY PEROXIDIZED SOYBEAN OIL ON OXIDATIVE STATUS IN GROWING PIGS

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Abstract

The objectives of this study were to determine whether feeding thermally processed peroxidized soybean oil (SO) induces markers of oxidative stress and alter antioxidant status in pig tissue, blood, and urine. Fifty-six barrows (25.3 ± 3.3 kg initial BW) were randomly assigned to dietary treatments containing 10% fresh SO (22.5^{o}C) or thermally processed SO (45^{o}C for 288 h, 90^{o}C for 72 h, or 180^{o}C for 6 h), each with constant air infusion rate of 15 L/min. Multiple indices of lipid peroxidation were measured in the SO including peroxide value (2.0, 96, 145, and 4.0 mEq/kg for 22.5, 45, 90 and 180^{o}C processed SO, respectively) and p-anisidine value (1.2, 8.4, 261, and 174 for 22.5, 45, 90 and 180^{o}C processed SO, respectively); along with a multitude of aldehydes. Pigs were individually housed and fed ad libitum for 49 d which included a 5 d period in metabolism crates for the collection of urine and serum for measures of oxidative stress. On d 49, pigs were euthanized to determine liver weight and analysis of

liver-based oxidative stress markers. Oxidative stress markers included serum, urinary, and liver thiobarbituric acid reactive substances (TBARS), and urinary F_2-isoprostanes (ISP) as markers of lipid damage; serum and liver protein carbonyls (PC) as a marker of protein damage; and urinary and liver 8-hydroxy-2'-deoxyguanosine (8-OH-2dG) as a marker of DNA damage. Superoxide dismutase (SOD), and catalase activity (CAT) were measured in liver, glutathione peroxidase activity (GPx) was measured in serum and liver, and ferric reducing antioxidant power (FRAP) was measured in serum and urine as determinants of antioxidant status. Pigs fed 90^{o}C SO had greater urinary ISP (P = 0.02), while pigs fed the 45^{o}C SO had elevated urinary TBARS (P = 0.02) in comparison to other treatment groups. Pigs fed 45^{o}C and 90^{o}C SO had increased serum PC concentrations (P = 0.01) and pigs fed 90^{o}C SO had greater (P = 0.01) liver concentration of 8-OH-2dG compared to pigs fed the other SO treatments. Furthermore, pigs fed 90^{o}C SO had reduced serum GPx activity in comparison to pigs fed fresh SO (P = 0.01). In addition, pigs fed 180^{o}C SO had increased liver CAT activity (P = 0.01). Liver GPx and SOD or serum and urinary FRAP were not affected by dietary treatment. These results indicate that dietary peroxidized soybean oil induced oxidative stress by increasing serum PC while diminishing serum GPx, increasing urinary ISP and TBARS, and increasing 8-OH-2dG and CAT in liver.

Key words: growing pigs, oxidative stress, peroxidized soybean oil

Introduction

Soybean oil (**SO**) is a common energy source added to swine diets and is rich in PUFA such as linoleic acid, making it highly susceptible to lipid peroxidation during thermal processing (Holman, 1954). Lipid peroxidation occurs as a free radical chain reaction that progressively forms products including toxic aldehydes, while diminishing antioxidants (Gonzalez-Muñoz et al., 1998). Recent research suggests that consumption of lipid peroxidation products induces oxidative stress in swine and poultry (Tavárez et al., 2011; Hanson, 2014; Lu et al., 2014); however, these experiments have focused on individual markers of oxidative stress, namely thiobarbituric acid reactive substances (**TBARS**). Further, feeding peroxidized lipids to pigs has been shown to negatively impact growth performance (Boler et al., 2012; Rosero et al., 2015).

Endogenous antioxidants including superoxide dismutase (**SOD**), catalase (**CAT**), and glutathione peroxidase (**GPx**) protect the body from damage caused by ROS and free radicals; however, oxidative stress results when free radical production overwhelms antioxidant compounds leading to modification of lipids, proteins, and DNA. Thiobarbituric acid reactive substances and F_2-isoprostane (**ISP**) concentrations are often measured as markers of lipid damage due to lipid peroxidation (Montuschi et al., 2004). Free radicals also bind to proteins altering protein function and can be accessed via protein carbonyl (**PC**) content (Dalle-Donne et al., 2003). Another major target of free radicals is DNA, which upon oxidation, a hydroxyl group is added to guanosine molecule to produce 8-hydroxy-2'-deoxyguanosine (**8-OH-2dG**) (Kalyanaraman, 2013).

Because oxidative stress is not a singular metabolic event, the objectives of this study were to determine the impacts of feeding thermally processed SO on markers of oxidative stress and antioxidant status in pig liver, serum and urine.

Materials and Methods

All animal care and use procedures for this experiment were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Experimental Design

Methodologies regarding lipid peroxidation of SO, formulation of diets, and animal management have been described previously (Chapter 2, Lindblom et al., 2017). In brief, 56 barrows (initial average BW 25.3 ± 3.31 kg) were randomly assigned to one of four dietary treatments that included 10% fresh SO or 10% SO thermally processed at either; 1) 45^{o}C for 288 h, 2) 90^{o}C for 72 h, or 3) 180^{o}C for 6 h. Lipid peroxidation products and quality of SO where assessed and reported previously (Chapter 2, Lindblom et al., 2017). Each pig was individually penned with ad libitum access to feed and water for a 49 d experimental period. Pigs were blocked into groups of 20, 20, and 16 and moved to metabolism crates on d 21, 25, and 29; respectively, for a 5 d period for digestibility analyses (Chapter 2, Lindblom et al., 2017) and for collections of urine and serum for oxidative stress analyses. During this period, pigs were fed their treatment diet equivalent to 4% of their average BW twice daily (2% at 0700 h and 2% at 1700 h) with constant access to water. On d 5 of the metabolism period, urine was collected for 5 h into plastic containers containing 5 mL chlorhexidine to eliminate microbial growth.

Following the collection, urine volume was quantified and stored at -80^{o}C until subsequent analysis of markers of oxidative stress. Immediately following this urine collection, (5 h after morning feeding) approximately 8 mL of blood was obtained via jugular venipuncture using a 10-mL vacuum serum tube, which was then centrifuged at 2,500 × g for 15 min at 4^{o}C and serum was harvested. Serum samples were immediately frozen at -80^{o}C and stored until subsequent oxidative stress analyses. Thereafter, pigs were returned to their assigned pen where they remained for the duration of the experiment. On d 49, all pigs (final average BW 70.80 ± 5.73 kg) were euthanized by barbiturate overdose followed by exsanguination. Livers were excised, weighed, and a sample taken and snap frozen in liquid N, transported on dry ice, and stored at -80^{o}C until subsequent analyses of oxidative stress markers.

Oxidative stress markers

Multiple oxidative stress markers were measured in urine, serum, and liver homogenates. Analyses were measured using commercially available assay kits and are reported in Table 3.1 along with the dilution factors used. Assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI) and were performed according to the recommendations from the manufacturer, with assays run in triplicate in 96-well microplates and intra-assay CV of $\leq$ 5.0%. Thiobarbituric acid reactive substances and ISP concentrations were measured as indicators of lipid damage. Urine, serum, and liver samples were assessed for TBARS where 100 mg of liver tissue was homogenized per mL of RIPA buffer (Cayman Chemical Co., Ann Arbor, MI; #10010263), centrifuged at 1,600 × g for 10 minutes at 4^{o}C then the supernatant was used to run the assay. No

dilutions were necessary and data were reported as µM of MDA. F_2-isoprostanes were only measured in urine with urine diluted 1:10 in sample buffer provided in the assay kit prior to performing the assay. Urinary ISP are reported on a recovery basis of total urine collected so data is reported as a concentration in pg. Urine TBARS were also normalized to the volume of urine excreted by multiplying the concentration of TBARS from the assay by the quantity of urine collected during the collection period. Because proteins are not excreted in urine, protein damage was measured via PC concentration in serum and liver samples. Briefly, 200 mg of liver tissue was homogenized per mL in 50 mM phosphate buffer containing 1 mM EDTA and centrifuged at 10,000 × g for 15 minutes at 4^{o}C then supernatant was assayed to determine PC concentration. Serum and liver samples were analyzed straight on the plates and PC concentration data was expressed as nmol/mL. The nucleic acid guanine is the base that is most prone to DNA oxidative damage and 8-OH-2dG, the form of oxidized guanine, is most commonly studied (Wu et al., 2004; Mateos and Bravo, 2007). Urine and liver 8-OH-2dG was assessed in which DNA was extracted from 25 mg of liver using ZR Genomic DNA- Tissue MiniPrep (Zymo Research, Irvine, CA). Following extraction, DNA yields were determined using Gen5 software on Cytation 5 Imaging Reader (BioTek, Winooski, VT) and DNA yields ranged from 20-40 µg DNA. The DNA was then digested by nuclease P1 (Sigma-Aldrich, St. Louis, MO) to convert double stranded DNA to single stranded DNA, then 1 unit of alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) was added per 100 µg of DNA to convert nucleotides to nucleosides. The supernatant was assayed on the Cayman Chemical assay kit to determine the 8-OH-2dG of liver and no dilution was

required; however, urine was diluted 1:750 in sample buffer prior to assessment. Both urine and liver 8-OH-2dG concentrations were expressed in pg/mL.

Antioxidants act to detoxify oxidatively damaged molecules, with the most common enzymatic antioxidants measured to evaluate oxidative status being SOD, GPx, and CAT. Superoxide dismutase and CAT were measured in liver homogenates and GPx was measured in serum and liver. For SOD activity, 100 mg of liver tissue was homogenized in 1 mL of 20 mM HEPES buffer containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, centrifuged at 1,500 × g for 5 minutes at 4^{o}C. The supernatant was diluted 1:10 in sample buffer before being assayed and expressed as U/mL. For CAT activity, 100 mg of liver tissue was homogenized in 1 mL of 50 mM potassium phosphate containing 1 mM EDTA and centrifuged at 10,000 × g for 15 minutes at 4^{o}C and the supernatant was diluted 1:10,000 in sample buffer and assayed to determine CAT activity (nmol min^{-1}mL^{-1}). Glutathione peroxidase activity (nmolmin^{-1}mL^{-1}) was measured in liver and serum where 100 mg of liver tissue was homogenized in 1 mL of 50 mM Tris-HCl containing 5 mM EDTA and 1 mM DTT, centrifuged at 10,000 × g for 15 minutes at 4^{o}C. Serum and liver homogenates were diluted 1:20 in sample buffer prior to being assayed. Total antioxidant capacity was measured in urine and serum via ferric reducing antioxidant power (**FRAP**) assay. Briefly, the FRAP assay colorimetrically measures the reduction of the ferric ion (Fe^{+3}) to ferrous ion (Fe^{+2}) by the reaction of ferrous-tripyridyltriazine complex in relation to antioxidant based ascorbic acid standards (Benzie and Strain, 1996; Gabler et al., 2005). Urine samples required a 1:10 dilution before being assayed, but serum was run without diluting, both expressed as µM (FRAP value).

Statistical Analysis

Data were analyzed as a completely randomized design with individual pig as the experimental unit, using Proc GLM procedure of SAS (version 9.4; SAS, 2009) with means reported and separated using LSMEANS. In addition, relationships between lipid peroxidation products with oxidative stress markers and relationships between growth performance and digestibility measures with oxidative stress markers were evaluated by simple linear correlation (Pearson correlation coefficients) analysis. Differences were considered significant at $P \leq 0.05$, whereas values of $P \leq 0.10$ were considered statistical trends.

Results and Discussion

Pig performance and diets

The composition of experimental SO have been described in detail previously (Chapter 2, Lindblom et al., 2017) and highlighted in Table 3.2. In brief, thermal processing decreased the unsaturated:saturated fatty acid ratio (**UFA:SFA**) of the dietary FA with a corresponding increase in the production of lipid peroxidation products including peroxide value (**PV**), p-anisidine value (**AnV**), and multiple aldehydes, including 2,4-decadienal (**DDE**), and 4-hydroxynonenal (**HNE**). The production of these lipid peroxidation products was generally greatest in the 90^{o}C processed SO which was further confirmed with the reduced concentration of total tocopherols. During the experimental period, 1 pig fed the 45^{o}C SO and 1 pig fed the 180^{o}C SO died due to causes unrelated to dietary SO treatment; therefore, data are reported using 14

observations for 22.5°C and 90°C SO and 13 observations for 45°C and 180°C SO. The effects of SO peroxidation on pig performance, digestibility and intestinal integrity were previously reported (Chapter 2, Lindblom et al., 2017) where pigs fed the 90°C SO had reduced ADG and N retention, and reduced energy and ether extract digestibility.

In the present study, dietary treatment impacted liver weight as a percentage of BW (Figure 3.1). Liver weights as a percentage of BW were greatest in pigs fed the 90°C processed SO (P = 0.01) followed by pigs fed the 180°C SO, 45°C SO, and 22.5°C with values of 2.88, 2.49, 2.41, and 2.31%, respectively. This is in agreement with others who reported an increase in liver weight as a percentage of BW when feeding various peroxidized lipids (Eder, 1999; Anjum et al., 2004; Liu et al., 2014a; Lu et al., 2014). The increase in liver weight relative to BW may result from the increase in consumption of lipid peroxidation products including peroxidants and aldehydes (Wang et al., 1997). Because a function of the liver is to detoxify pro-oxidants, an increase in consumption of lipid peroxidation products would result in hypertrophy of hepatocytes and therefore increased liver weight.

Oxidative status in serum, urine, liver

Pro-oxidants target lipids, proteins, and DNA when the antioxidant system reaches capacity and can no longer maintain redox balance. Previous studies have shown that consumption of peroxidized oils induces oxidative stress in blood and tissue samples primarily measured by TBARS (Ringseis et al., 2007; Boler et al., 2012; Liu et al., 2014b; Rosero et al., 2015). Consumption of lipid peroxidation products results in the accumulation of aldehydes in the gastric lumen which are then absorbed through the

small intestine where they are metabolized in the liver (Kanazawa and Ashida, 1998). This is supported by Cardoso et al. (2013) who reported that high fat diets fed to mice enhanced ROS release from liver mitochondria. Considering the above literature and because a main function of the liver is detoxification of pro-oxidants, we chose to measure oxidative balance in liver tissue in addition to serum and urine (Table 3.3).

Consumption of peroxidized oils has been shown to increase TBARS in bodily fluids in livestock (Tavárez et al., 2011; Boler et al., 2012; Liu et al., 2014b; Lu et al., 2014). In the current experiment, no differences were noted for serum TBARS ($P = 0.51$) among pigs fed the variable thermally processed SO. In contrast, others (Boler et al., 2012; Liu et al., 2014b; Lu et al., 2014) have shown that feeding peroxidized lipids increases plasma TBARS concentration. Pigs fed the 45^{o}C processed SO treatments tended ($P = 0.08$) to have elevated liver TBARS concentration compared to pigs fed the 22.5^{o}C SO, with intermediate concentrations for pigs fed the 90^{o}C SO and 180^{o}C SO treatments. These data are in agreement with Lu et al. (2014) who observed a similar increase in liver TBARS in pigs fed peroxidized SO for 55 d. Likewise, urinary TBARS was greatest in pigs fed the 45^{o}C SO and intermediate in pigs fed the 90^{o}C SO compared to pigs fed the other SO treatment groups ($P = 0.02$). Because we observed an increase in liver and urinary TBARS and a lack of an effect in the serum, our results suggest that the malondialdehyde (which is measured in the TBARS assay) is getting rapidly absorbed and metabolized in the liver and excreted out in urine without affecting circulating levels of TBARS. While the measurement of TBARS is the most common measurement of lipid damage due to lipid peroxidation, this assay detects aldehydes that are not specific to lipid peroxidation, thereby often resulting in overestimations of lipid peroxidation

(Halliwell and Chirico, 1993; Monaghan et al., 2009). For this reason, we also measured urine ISP concentrations which measures specific products produced in response to lipid peroxidation and because ISP is most stable and concentrated in urine (Montuschi et al., 2004). In the current experiment, urinary ISP was nearly 4 times greater in pigs fed the 90^{o}C SO in comparison to the other treatments (P = 0.02). As reported previously, the 90^{o}C processed SO had the greatest concentration of lipid peroxidation products including PV, AnV, DDE, and HNE (Table 3.2); so because ISP is a specific measure of damage due to lipid peroxidation, this result was expected. While we have no livestock data from which to compare our data to, in human research, urinary ISP concentrations were elevated under smoking-induced oxidative stress (Obata et al., 2000). In the current experiment, the combination of increased TBARS and ISP in the urine, and TBARS in the liver indicates that lipid damage occurred in response to the consumption of lipid peroxidation products.

Proteins and amino acids are another major target of pro-oxidants of which carbonyls are a by-product of oxidatively damaged proteins (Beal, 2002). In the current study, we reported (Table 3.3) serum from pigs fed the 45^{o}C SO and 90^{o}C SO having elevated PC (P = 0.01). This is in agreement with Lu et al. (2014) who also reported an increase in plasma PC in pigs fed peroxidized SO after 55 d. In the current experiment, no differences in liver PC values were detected (P = 0.81) which is in contrast to Lu et al. (2014) who observed an increase in liver PC in pigs fed peroxidized SO. This was unexpected because pigs were fed for a similar duration and with a peroxidized SO with a similar PV (180 mEq/kg oil compared to 145.3 mEq/kg oil in the current experiment), so

we would have expected a similar outcome because lipid peroxidation has been shown to stimulate protein oxidation (Fellenberg and Speisky, 2006).

DNA is especially sensitive to oxidative damage of which, guanine is the most readily oxidized nucleic acid (Wu et al., 2004; Mateos and Bravo, 2007). Therefore, we chose to measure 8-OH-2dG in the urine and liver in the current experiment. During DNA repair, products such as 8-OH-2dG are excreted in the urine, and because 8-OH-2dG is water soluble, and concentrated and stable to measure in urine (Wu et al., 2004). Feeding pigs the variable SO treatments did not affect urinary excretion of 8-OH-2dG (P = 0.15). In contrast, liver 8-OH-2dG was affected by dietary treatments, where it was observed that liver concentration of 8-OH-2dG was greatest in pigs fed the 90^{o}C SO (P = 0.01) in comparison with other treatment groups. This may be explained by the decreased CAT activity in pigs fed the 90^{o}C processed SO (Table 3.3) which shifts free radical binding from the antioxidant defense system to DNA, possibly causing oxidative damage. We cannot explain why we observed treatment differences in liver 8-OH-2dG but not urine 8-OH-2dG, and to our knowledge, we have no literature from which to compare our data to.

Antioxidant machinery and peroxidized soybean oil

Antioxidant enzyme activities including SOD, GPx, and CAT were measured to determine antioxidant status in the liver and are presented in Table 3.3. The biochemical reactions of SOD, GPx, and CAT are well known (Kalyanaraman, 2013). In the current experiment, liver SOD activity was not affected by dietary treatment (P = 0.22). These results are similar to data in broilers where oxidative status was measured in response to

heat stress where no differences were reported in liver SOD activity between heat stressed and thermal neutral broilers (Lin et al., 2006).

Simplistically speaking, the function of GPx and CAT is to metabolize H_2O_2 to water, but if this cannot be done effectively, H_2O_2 is rapidly converted to the $OH^{\cdot}$ free radical, which is reactive with lipids, proteins, and DNA (Shu et al., 1979). Serum from pigs fed the 90^{o}C SO had reduced GPx activity (P = 0.01) in comparison to pigs fed fresh SO and the 45^{o}C processed SO, with the GPx activity of pigs fed the 180^{o}C SO being intermediate. This is similar to results reported by others who also observed a decrease in GPx activity in plasma of pigs fed peroxidized oils (Yuan et al., 2007; Boler et al., 2012).

Liver GPx was not affected by dietary treatment (P = 0.83) which is supported by Boler et al. (2012) who observed no differences liver GPx between pigs fed peroxidized corn oil in comparison to pigs fed fresh corn oil, and also in agreement with Di Giancamillo et al. (2015) who measured oxidative status in piglets fed sunflower oil in comparison to pigs fed sunflower with an antioxidant. While liver SOD and GPx were not affected by dietary treatment in the current experiment, liver CAT was increased in pigs fed the 180^{o}C SO diet compared to the other SO treatments (P = 0.01). This is in contrast to Di Giancamillo et al. (2015) who did not report changes in CAT activity in piglets fed sunflower oil in comparison to pigs fed sunflower with an antioxidant. The reduction in activity of GPx in serum and CAT in liver could be explained by the lack of pro-oxidant substrates for these enzymes. Because the activity of antioxidant enzymes were reduced, this could be driving the increase in serum PC and the increases in 8-OH-2dG and TBARS in the liver in addition to the increased liver weight in pigs fed the 90^{o}C processed SO.

Lastly, antioxidant capacity was analyzed by assessing FRAP activity in serum and urine. Gabler et al. (2005) demonstrated that feeding antioxidant compounds improves blood FRAP activity. In the current study, serum and urinary excretion of FRAP were not affected by dietary treatment ($P > 0.12$). This is in contrast to Lin et al. (2006) who observed a decrease in plasma FRAP in broilers that were heat stressed to induce disruption of oxidative balance.

Correlations between lipid peroxidation products and oxidative stress markers

Because consumption of lipid peroxidation products in SO was clearly shown to affect oxidative stress markers in the current experiment, we determined that conducting a correlation analysis between lipid peroxidation products and markers of oxidative stress was worthwhile (Table 3.4). Even though correlation analysis does not represent a cause and effect relationship, the lack of comprehensive measures of lipid peroxidation products and oxidative stress measures in the literature and in the current experiment led us to conduct this analysis to provide guidance on research involving lipid peroxidation status and the effects on animal production and oxidative status. Correlations between SO lipid composition and peroxidation products with measures of oxidative status are reported in Table 3.4. There were no significant correlations noted between the SO quality (Table 3.2) with serum TBARS, serum FRAP, urine FRAP, liver PC, liver SOD, and liver GPx (Table 3.3) so they are not reported in Table 3.4. It was interesting to observe that urinary and liver TBARS exhibited very few correlations to the lipid composition and peroxidation measures analyzed in the SO; given that TBARS is one of the most commonly measured compounds for both lipid quality assessment (Shurson et

al., 2015) and as an indicator of animals' response to oxidative stress. However, given that TBARS is a non-descript measure of lipid peroxidation damage (Yin et al., 2011), this was not completely unexpected.

Further, markers of lipid, protein, and DNA damage as measured by ISP in urine, PC in serum, and 8-OH-2dG in liver, respectively, had the greatest relationship to the lipid composition and peroxidation measures analyzed in SO namely PV, TBARS, and total tocopherols in the dietary SO (Table 3.4). It is not surprising that total tocopherol concentrations in the SO treatments were negatively correlated with markers of oxidative stress. Liu et al. (2016) has shown in heat stressed pigs that dietary tocopherols have antioxidant capabilities that alleviate metabolic oxidative stress. As to which tissue is most representative of measuring the overall oxidative balance, is not clear from the experiment herein. Complicating matters is that some oxidative stress metabolites cannot be measured in a specific tissue. For example, because protein excretion is extremely low in urine; therefore, PC could not be analyzed and the kit we utilized is not validated for measuring 8-OH-2dG in serum.

In summary, thermal processing of SO generated lipid peroxidation products. Thermally processing SO at 90^{o}C for 72 h was a successful model to induce oxidative stress in growing pigs; with thermally processing at 180^{o}C for 6 h having a smaller impact and thermally processing at 45^{o}C for 288 h having a minimal effects compared to fresh SO. The induction of oxidative stress by this model was measured by several markers across most of the mediums selected for evaluation; and our data show that markers of oxidative stress in the liver are not well correlated with lipid peroxidation products and suggest that further research should focus on markers of oxidative stress in

blood and urine. However, this experiment was unable to determine a specific marker that should be focused on in future research. The data do, however, suggest that several lipid composition and lipid peroxidation products need to be measured, specifically PV, TBARS, and total tocopherols, and suggests that specific aldehydes may also need to be considered. Moreover, serum, urinary, and liver TBARS were poorly correlated with lipid composition and peroxidation measures analyzed in SO; however, other measures of lipid, protein, and DNA damage (as measured by urinary ISP, serum PC, and liver 8-OH-2dG, respectively) have the greatest relationship to the lipid composition and peroxidation measures analyzed in SO. Collectively, these data provide clear evidence that feeding peroxidized SO sources modulates markers of oxidative stress in growing pigs.

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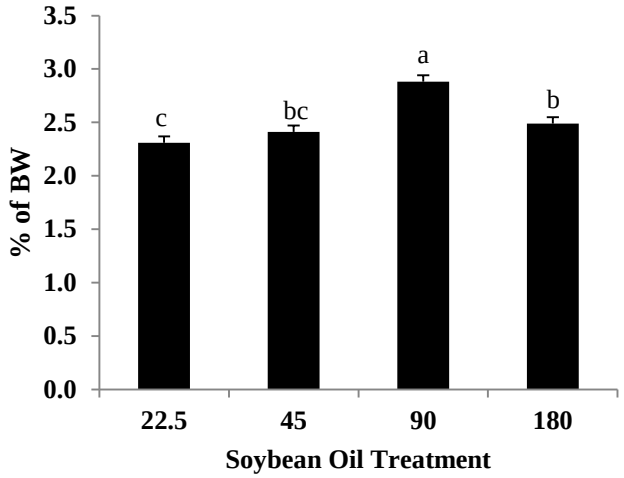

Figure 3.1. Effect of thermally peroxidized soybean oil on liver weight as a percentage of BW in growing pigs. 22.5 = fresh soybean oil as 22.5^{o}C; 45 = soybean oil heated for 288 h at 45^{o}C; 90 = soybean oil heated for 72 h at 90^{o}C; 180 = soybean oil heated for 6 h at 180^{o}C. All processed soybean oil had constant compressed air flow at a rate of 15L/min. Average final BW was 70.80 ± 5.73 kg (P = 0.11). Peroxidation effect P = 0.01, with superscripts reflecting peroxidized soybean oil treatment differences at $P \leq 0.05$.

Table 3.1. Assay kits performed to determine oxidative status in serum, urine, and liver.[1]

Assay Kit[2]	Catalog Number	Serum	Urine	Liver
		Dilution factor	Dilution factor	Dilution factor
TBARS	700870	ND	ND	ND
ISP	516351	NA	1:10	NA
PC	10005020	ND	NA	ND
8-OH-2dG	589320	NA	1:750	ND
SOD	706002	NA	NA	1:10
GPx	703102	1:20	NA	1:20
CAT	707002	NA	NA	1:10,000

[1]All assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI).

[2]TBARS, thiobarbituric acid reactive substances; ISP, F_2-isoprostanes; PC, protein carbonyls; 8-OH-2dG, 8-hydroxy-2'-deoxyguanosine; SOD, superoxide dismutase; GPx, glutathione peroxidase activity; CAT, catalase activity; ND, no dilution; NA, not applicable.

Table 3.2. Compositional and peroxidation analysis of soybean oil (adapted from Lindblom et al., 2017)

Heating temperature, ^{o}C	22.5	45	90	180
Time heated, h[1]	0	288	72	6
UFA:SFA[2]	5.35	5.27	4.64	5.02
Free fatty acids, %	0.04	0.07	0.35	0.14
Peroxide value, mEq/kg	2.0	95.6	145.3	4.0
p-Anisidine value[3]	1.19	8.38	261	174
TBARS[2,3]	0.10	0.14	0.14	0.09
Hexanal, mg/kg	2.97	2.71	21.20	16.84
Acrolein, mg/kg	3.88	3.31	15.82	45.39
2,4-decadienal, mg/kg	2.11	5.05	547.62	323.57
2-hydroxynonenal, mg/kg	0.05	1.05	39.46	25.71
Total tocopherols, mg/kg	772	620	405	609

[1]Thermally processed oils had constant air flow rate at 15L/min.

[2]UFA:SFA, unsaturated to saturated fatty acid ratio; TBARS, thiobarbituric acid reactive substances.

[3]There are no units for p-anisidine value or TBARS value.

Table 3.3. Oxidative status in serum, urine, and liver of pigs fed soybean oil with differing peroxidation levels

	Processed SO[1]				Statistics	
Parameter[2]	22.5	45	90	180	SEM	P value
Serum[3]						
TBARS, μM	8.3	7.3	7.3	7.5	0.6	0.51
PC, nmol/mL	19.5^{b}	26.3^{a}	28.2^{a}	21.4^{b}	1.5	0.01
GPx, nmolmin^{-1}mL^{-1}	1,954^{a}	1,805^{ab}	1,430^{c}	1,529^{bc}	116	0.01
FRAP, μM	188.3	198.1	193.9	205.6	13.6	0.83
Urine[4]						
TBARS, μM	9.0^{c}	18.1^{a}	11.1^{b}	8.6^{c}	2.31	0.02
ISP, pg	5,150^{b}	3,848^{b}	17,812^{a}	4,947^{b}	3,646	0.02
8-OH-2dG, pg/mL	43,232	67,334	53,725	36,991	9,530	0.15
FRAP, μM	1,080	1,588	1,378	1,316	210	0.41
Liver[5]						
TBARS, μM	9.9^{y}	12.7^{x}	11.6^{xy}	11.5^{xy}	0.8	0.08
PC, nmol/mL	57.3	54.9	55.4	51.8	4.0	0.82
8-OH-2dG, pg/mL	104^{b}	145^{b}	257^{a}	163^{b}	27	0.01
SOD, U/mL	325	256	336	297	29	0.22
GPx, nmol min^{-1}mL^{-1}	4,563	5,027	4,718	4,740	375.5	0.84
CAT, nmol min^{-1}mL^{-1}	12,427^{b}	11,255^{b}	9,933^{b}	19,282^{a}	1,672	0.01

[1] Processing temperatures, ^{o}C: 22.5, fresh oil; 45, SO heated for 288 h at 45^{o}C with constant compressed air flow rate at 15L/min; 90, SO heated for 72 h at 90^{o}C with constant compressed air flow rate at 15L/min; 180, SO heated for 6 h at 180^{o}C with constant compressed air flow rate at 15L/min. Superscripts reflect peroxidized soybean oil treatment differences (abc, $P \leq 0.05$; xyz, $P \leq 0.10$).

[2]TBARS, thiobarbituric acid reactive substances; PC, protein carbonyls; GPx, glutathione peroxidase activity; ISP, F_2-isoprostanes; 8-OH-2dG, 8-hydroxy-2'-deoxyguanosine; SOD, superoxide dismutase; CAT, catalase activity.

[3]Serum obtained after a 17 h fast.

[4]Urine collected and quantitated for 5 h following a 12 h fast.

[5]Liver obtained on d 49 from pigs in a fed state.

Table 3.4. Pearson correlation coefficients among SO composition and lipid peroxidation products with measures of oxidative status[1]

	SO composition and peroxidation measures[2]									
Criterion	UFA:SFA	FFA	PV	AnV	TBARS	Hexanal	DDE	HNE	Ratio	TOC
Serum PC	-0.38	0.41	0.57	0.29	0.54	0.25	0.31	0.30	-	-0.50
	(0.01)	(0.01)	(0.01)	(0.04)	(0.01)	(0.08)	(0.03)	(0.04)		(0.01)
Serum GPx	0.46	-0.43	-0.27	-0.47	-	-0.47	-0.47	-0.47	-0.47	0.44
	(0.01)	(0.01)	(0.06)	(0.01)		(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
Urinary TBARS	-	-	0.25	-	0.34	-	-	-	-	-
			(0.09)		(0.02)					
Urinary ISP	-0.42	0.44	0.36	0.38	0.27	0.35	0.39	0.38	0.33	-0.39
	(0.01)	(0.01)	(0.02)	(0.01)	(0.08)	(0.02)	(0.01)	(0.01)	(0.03)	(0.01)
Urinary 8-OH-2dG	-	-	0.24	-	0.30	-	-	-	-	-
			(0.10)		(0.04)					
Liver TBARS	-	-	-	-	0.24	-	-	-	-	-
					(0.10)					
Liver 8-OH-2dG	-0.51	0.51	0.41	0.47	0.28	0.45	0.48	0.47	0.42	-0.51
	(0.01)	(0.01)	(0.01)	(0.01)	(0.04)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
Liver CAT	-	-	-0.39	-	-0.45	-	-	-	-	-
			(0.01)		(0.01)					

[1]Top value represents correlation (*r* value) and bottom value in parenthesis represents significance (*P*-value). If no value is given, it was not found to be nonsignificant (-) at $P \leq 0.10$. There were no correlations observed between SO treatment and serum TBARS, serum FRAP, urinary FRAP, liver PC, liver SOD, and liver GPx; the only correlations for acrolein were with serum GPx (r = -0.31, $P = 0.03$) and liver CAT (r = 0.49, $P = 0.01$) and were removed from the table.

[2]UFA:SFA, unsaturated:saturated fatty acid ratio; FFA, free fatty acids; PV, peroxide value; AnV, p-anisidine value; TBARS, thiobarbituric acid reactive substances; DDE, 2,4-decadienal; HNE, 4-hydroxynonenal; Ratio, ratio of aldehydes as described by Wang et al., 2016; TOC, total tocopherols; PC, protein carbonyls; GPx, glutathione peroxidase activity; ISP, F_2-isoprostanes; 8-OH-2dG, 8-hydroxy-2'deoxyguanosine; CAT, catalase activity.

CHAPTER 4

EFFECT OF OIL SOURCE AND PEROXIDATION STATUS ON GROWTH PERFORMANCE AND OXIDATIVE STATUS IN YOUNG BROILERS

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Abstract

Oil source has been shown to affect broiler performance and oxidative status. Lipid peroxidation may also affect animal performance and oxidative status through the generation of peroxidation compounds which differ according to how oils are thermally processed. The objectives of this study were to evaluate the effect of oil source and peroxidation status on broiler performance and markers of oxidative stress. Broilers (initial BW 85.1 ± 7.8 g) were allotted to 40 battery cages containing 5 birds/cage in a completely randomized design. The 4 × 2 factorial arrangement of treatments consisted of oil source (palm oil, soybean oil, flaxseed oil, and fish oil) and peroxidation status (fresh or peroxidized) with peroxidation achieved by heating oils at 90^{o}C for 72 h with a constant air flow of 3 L/min. Broilers were fed their respective diets for 20 d to measure growth performance. On d 21, plasma and liver samples were harvested from 2 broilers/cage for analysis of oxidative status including thiobarbituric acid reactive

substances (TBARS), protein carbonyls (PC), 8-hydroxy-2'-deoxyguanosine (8-OH-2dG), and glutathione peroxidase activity (GPx) in plasma and liver, superoxide dismutase (SOD) and catalase (CAT) activity in liver, and ferric reducing antioxidant power (FRAP) in plasma. An interaction was noted between oil source and peroxidation status where broilers fed peroxidized oils had reduced ($P \leq 0.04$) ADFI, ADG, G:F, and plasma GPx in all oil sources except for broilers fed fish oil. Plasma 8-OH-2dG was increased by feeding the peroxidized oils ($P = 0.01$). An interaction was noted in liver TBARS where broilers fed peroxidized palm oil had greater liver TBARS compared to fresh palm oil, while the opposite was true in broilers fed soybean oil, and no change was noted in broilers fed flaxseed oil and fish oil ($P = 0.09$). An interaction was also noted for liver PC where broilers fed palm, flaxseed, and fish oil had similar liver PC regardless of peroxidation status while broilers fed peroxidized soybean oil had increased liver PC compared to the fresh soybean oil diet ($P = 0.04$). Oil source affected plasma TBARS and 8-OH-2dG ($P = 0.01$), plasma PC ($P = 0.09$), liver 8-OH-2dG ($P = 0.08$), and liver CAT ($P = 0.02$). In conclusion, the degree of polyunsaturation and peroxidation status of dietary oils affected growth performance, and increased plasma and liver markers of oxidative stress in poultry.

Key words: broilers, growth, oxidative stress, peroxidized oils

Introduction

Poultry diets commonly contain added oils which are high in PUFA to increase the energy density of the feed. While the fatty acid profile of a dietary oil is known to affect digestibility (Wiseman et al., 1998), it also plays a role in the susceptibility of oils

to peroxidize where PUFA are more prone to lipid peroxidation than saturated fatty acids (Holman, 1954). Lipid peroxidation occurs as a chain reaction that produces and consumes compounds such as peroxides, aldehydes, and polar compounds, while diminishing its antioxidant capacity, differing in severity based on the temperature and duration of thermal processing. Because there is no single measurement that depicts the overall peroxidation status of oils, it is advantageous to measure multiple markers of peroxidation.

Feeding peroxidized oils to poultry negatively impacts growth performance (Takahashi and Akiba, 1999; Anjum et al., 2004; Tavárez et al., 2011) and has the potential to induce oxidative stress *in vivo* (Tavárez et al., 2011; Boler et al., 2012). Oxidative stress occurs *in vivo* when reactive oxygen species (**ROS**) and free radical products outweigh antioxidant enzymes and antioxidants resulting in free radicals binding to lipids, proteins, and DNA, leading to tissue damage (Kalyanaraman, 2013). Typically, thiobarbituric acid reactive substances (**TBARS**) are measured as a marker of lipid damage, protein carbonyl concentration (**PC**) is measured for protein damage, and 8-hydroxy-2'-deoxyguanosine (**8-OH-2dG**) is measured to indicate DNA damage. Antioxidants work to combat and detoxify ROS and are commonly measured by superoxide dismutase (**SOD**), catalase (**CAT**), and glutathione peroxidase (**GPx**) activities. Because there is limited data available regarding the effects of feeding thermally processed oils on markers of oxidative stress in poultry, the objectives of this study were to evaluate the effect of oil source and peroxidation status on broiler performance and markers of oxidative stress.

Materials and Methods

All animal care and use procedures for this experiment were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Animals and housing

Day-old, Ross 308 (Aviagen Group, Huntsville, AL) chicks were brooded in raised wire battery pens (Petersime Incubator Co., Gettysburg, OH) located within an environmentally controlled room. The chicks received supplemental heat starting at 35º C on 1 d of age, decreasing 2ºC every week with light provided at 23L:1D. Chicks were allowed 3 d of acclimation to the batteries and fed a common starter diet during this acclimation period. On d 4 (d 0 of the experiment), chicks were individually wing banded, weighed (initial BW 85.1 ± 3.5 g), and randomly assigned to 1 of 8 dietary treatments. The experimental unit consisted of a battery cage of 5 broilers with 5 replications for each treatment allotted to 40 battery cages for a total of 200 broilers. Broilers had ad libitum access to their experimental diets and water for the duration of the 20 d experimental period.

Diets

Broilers were allotted to a 4 × 2 factorial arrangement of dietary treatments in a completely randomized design. Factors consisted of four oil sources including palm oil, soybean oil, flaxseed oil, and fish oil at a 5% inclusion level, in combination with peroxidation status (fresh or peroxidized oil). Prior to diet manufacturing, lipid peroxidation was achieved by placing 2.5 L of each oil into a 5 L round-bottom glass

flask and heating each oil with an electric heating mantle with a power controller. Oils were thermally processed at 90^{o}C for 72 h with a continuous infusion of air at a rate of 3L/min, while the fresh oils had no thermal or air infusion treatment. Various lipid peroxidation products were measured to determine the quality of the oils as shown in Table 4.1. Diets (Table 4.2) contained 5% of their respective fresh or peroxidized oil and were formulated to meet or exceed NRC (1994) recommendations.

Data collection

Broiler chicks were monitored daily over the duration of the experiment. On d 0, 7, 14, and 20, all broilers were individually weighed and ADG was calculated. Feed disappearance was measured on d 7, 14, and 20 to calculate ADFI and used to calculate feed efficiency (**G:F**, gain:feed). During the experiment, one bird fed the peroxidized soybean oil, fresh flaxseed oil, peroxidized flaxseed oil, fresh fish oil, and peroxidized fish oil diets died and two birds fed the peroxidized palm oil diets died (all causes unrelated to dietary treatment) so growth performance data was corrected accordingly for mortality. On d 21, broilers were euthanized via carbon dioxide asphyxiation and blood was obtained via cardiac venipuncture using a 10-mL vacuum tube containing sodium heparin, which was then centrifuged at 2,500 × g for 15 min at 4^{o}C and plasma was harvested. Plasma samples were immediately frozen at -80^{o}C and stored until subsequent analysis of oxidative stress markers. In addition, liver tissues were excised and snap frozen in liquid nitrogen, transported on dry ice, and stored at -80^{o}C until analysis of oxidative stress markers.

Oxidative stress markers

Multiple oxidative stress markers were measured in plasma and liver from two randomly selected birds/cage using commercially available assay kits, each carried out according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI). A complete listing of assay kits and dilution factors performed are described in Table 4.3. Assays were performed according to the recommendations from the manufacturer, with assays run in triplicate in 96-well microplates and intra-assay CV of ≤ 5.0%. All liver tissues were homogenized in their respective buffers and the supernatants were stored at -80^{o}C and assayed within a month of homogenization. Thiobarbituric acid reactive substances were measured as an indicator of lipid damage. Plasma and liver samples were assessed for TBARS where 100 mg of liver tissue was homogenized per mL of RIPA buffer (Cayman Chemical Co., Ann Arbor, MI; #10010263), centrifuged at 1,600 × g for 10 minutes at 4^{o}C then the supernatant was used to run the assay or 100 µL of plasma was used in assays. No dilutions were necessary and were reported as µM of MDA. Protein damage was measured via PC concentration in plasma and liver samples. Briefly, 200 mg of liver tissue was homogenized per mL in 50 mM phosphate buffer containing 1 mM EDTA and centrifuged at 10,000 × g for 15 minutes at 4^{o}C then supernatant was assayed to determine PC concentration. No dilutions were required for plasma and liver quantification of PC with results expressed in nmol/mL.

Guanine is the nucleic acid base that is most prone to oxidative damage with 8-OH-2dG from DNA being the form of oxidized guanine that is most commonly studied (Wu et al., 2004; Mateos and Bravo, 2007). Therefore, in the current experiment we measured 8-OH-2dG in plasma and liver where 25 mg of liver was homogenized then

DNA was extracted using ZR Genomic DNA-Tissue MiniPrep (Zymo Research, Irvine, CA). Following DNA extraction, DNA yields were determined using Gen5 software on Cytation 5 Imaging Reader (BioTek, Winooski, VT) and DNA yields ranged from 20-40 µg DNA. DNA was then digested by nuclease P1 (Sigma-Aldrich, St. Louis, MO) to convert double stranded DNA to single stranded DNA then 1 unit of alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) was added per 100 µg of DNA to convert nucleotides to nucleosides and was stored at 4^{o}C until use. The supernatant was assayed to determine the 8-OH-2dG of liver and no dilution was required; however, plasma was diluted 1:75 in sample buffer prior to assessment and both plasma and liver 8-OH-2dG is expressed in pg/mL.

To assess antioxidant machinery, we evaluated SOD, GPx, and CAT activities. Superoxide dismutase and CAT were measured in liver homogenates while GPx was measured in plasma and liver. For SOD, 100 mg of liver tissue was homogenized in 1 mL of 20 mM HEPES buffer containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, and centrifuged at 1,500 × g for 5 minutes at 4^{o}C. The supernatant was diluted 1:10 in sample buffer before being assayed and expressed as U/mL. For CAT, 100 mg of liver tissue was homogenized in 1 mL of 50 mM potassium phosphate containing 1 mM EDTA and centrifuged at 10,000 × g for 15 minutes at 4^{o}C and the supernatant was diluted 1:10,000 in sample buffer and assayed to determine CAT and expressed as nmolmin^{-1}mL^{-1}. Glutathione peroxidase activity was measured in liver and plasma where 100 mg of liver tissue was homogenized in 1 mL of 50 mM Tris-HCl containing 5 mM EDTA and 1 mM DTT, and centrifuged at 10,000 × g for 15 minutes at 4^{o}C. Plasma and liver homogenates were diluted 1:20 in sample buffer prior to being assayed and were

expressed as nmol $min^{-1}mL^{-1}$. Lastly, total antioxidant capacity was measured in plasma via ferric reducing antioxidant power (**FRAP**). Briefly, the FRAP assay colorimetrically measures the reduction of the ferric ion (Fe^{+3}) to ferrous ion (Fe^{+2}) by the reaction of ferrous-tripyridyltriazine complex in relation to ascorbic acid standards (Benzie and Strain, 1996; Gabler et al., 2005). No dilution was necessary and results are expressed as µM (FRAP value).

Statistical Analysis

The battery cage was considered the experimental unit for broiler performance (5 birds/cage) and for the analysis of oxidative status in the plasma and liver (2 birds/cage) for a total of 5 replications per treatment. Data were analyzed using the MIXED procedure of SAS (SAS, 2009) with means reported and separated using LSMEANS. The main effect of oil source and peroxidation status, and their interactions were fixed effects, with cage as a random effect. In addition, relationships between oil composition with growth performance and oxidative stress markers and relationships between growth performance and oxidative stress markers were evaluated by simple linear correlation (Pearson correlation coefficients) analysis. Differences were considered significant at $P \leq 0.05$ and trends were noted at $0.05 \leq P \leq 0.10$.

Results and Discussion

Peroxidation status and diet composition

Lipid peroxidation can be achieved through thermal processing of oils rich in PUFA which results in a decrease in unsaturated FA, an increase in saturated FA, and

increased generation of lipid peroxidation products (Shurson et al., 2015). Lipid peroxidation occurs in three consecutive phases: initiation, propagation, and termination (Gutteridge, 1995) which consumes and produces a variety of lipid peroxidation products, some of which may cause pathophysiological effects in cells and tissues (Esterbauer et al., 1991).

The poultry industry utilizes a variety of oil sources such as palm, soybean, flaxseed, and fish oil to diet formulation as an energy source; however, oil quality can be compromised through lipid peroxidation. Therefore, the current study was designed to test oils with widely differentiating fatty acid compositions and differing in susceptibility to peroxidation based on the ability to uptake oxygen (Holman, 1954) on broiler performance and oxidative status. As expected, FA analysis of the four oil sources (Table 4.1) show that palm oil was high in palmitic acid (C16:0), soybean oil was high in linoleic acid (C18:2n6), flaxseed oil rich in linolenic acid (C18:3n3), and fish oil containing high amounts of the omega-3 long chain FA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (C20:5n3 and C22:6n3, respectively). Palm oil contained about 50% palmitic acid (C16:0), however, fish oil also contained a moderate amount of palmitic acid (20%). Although PUFA are susceptible to lipid peroxidation, omega 3 fatty acids linolenic acid, EPA and DHA have been shown to mitigate oxidative stress and have beneficial effects on inflammation (Song and Horrobin, 2004; Gomaa and Abd El-Aziz, 2016). Because of this, we were interested in evaluating how palm, soybean, flaxseed, and fish oils (fresh or thermally peroxidized) modulated *in vivo* markers of oxidative stress. To thermally peroxidize our oils, temperature and duration of thermal processing was based off of past experiments including Liu et al. (2014b) who thermally

processed various lipids at 95^{o}C for 72 h. Likewise, Kerr et al. (2015) thermally processed corn oil at 95^{o}C for 72 h which resulted in increased peroxide values (**PV**) and aldehydes such as 4-hydroxynonenal (**HNE**) and 2,4-decadienal (**DDE**).

As shown in Table 4.1, compared to their peroxidized counterpart each fresh oil (i.e., 22.5°C) had a higher unsaturated to saturated fatty acid ratio (**UFA:SFA**) where the UFA:SFA went from 0.91 to 0.81 for palm oil, 5.32 to 3.41 for soybean oil, 9.96 to 7.80 for flaxseed oil, and 1.65 to 1.36 for fish oil, respectively. Although the UFA:SFA ratio is a fairly crude measure related of the degree of unsaturation and thus susceptibility to peroxidation, we also determined the degree of FA unsaturation in each oil (i.e. total number of double bonds; Table 4.1), and similar to the change in UFA:SFA, there was a consistent decrease in the total number of double bonds due to thermally processing each oil (Table 4.1). This was expected because the progression of lipid peroxidation involves the hydrogenation of FA, thereby decreasing the number of double bonds available for peroxidation (Holman, 1954; Yin et al., 2011).

Prior to diet formulation, we also characterized the peroxidation status of each oil by measuring common lipid peroxidation products (Table 4.1). As expected, PV, which measures hydroperoxides formed in the initiation phase of lipid peroxidation, increased for all thermally processed oil treatments compared to their fresh oil counterparts where PV increased by 592.3, 601.2, 117.0, and 5.4 mEq/kg for palm, soybean, flaxseed, and fish oil respectively. The same trend was noted for p-anisidine value (**AnV**) which measures the molecular weight of saturated and unsaturated aldehydes. In addition, individual aldehydes including hexanal, which measures major secondary lipid peroxidation products produced from the termination phase during peroxidation of

linoleic acid, DDE, an aldehyde derived from the peroxidation of linoleic acid, were both increased due to thermal processing. Additionally, total polar compounds which measures monoglycerides, diglycerides, and free fatty acids; and polymerized triglycerides (**PTAGS**) which measures the molecular size of polymeric compounds formed in the tertiary phase of lipid peroxidation, were also increased due to thermal processing, supporting the statement that the oils evaluated were peroxidized. These analyses of our peroxidized oils where similar to the changes in fatty acid composition and lipid peroxidation products reported by others (Engberg et al., 1996; Kerr et al., 2015) due to thermal processing.

Total tocopherols were also measured as an indication of antioxidant status of each oil source. Depending upon the degree of processing, vegetable oils have natural antioxidants including tocopherols which protect the oil from autoxidation (Seppanen and Csallany, 2002). In the current experiment (Table 4.1), fresh soybean oil had the highest concentration of total tocopherols (802 mg/kg) followed by flaxseed oil (267 mg/kg), fish oil (119 mg/kg), and palm oil (58 mg/kg). Thermally processing the treatment oils virtually depleted total tocopherol concentrations aside from fish oil, where the total tocopherol concentration of fresh fish oil was 119 mg/kg and 125 mg/kg in the thermally processed oil. Miyagawa et al. (1991) reported that thermally processing a blend of soybean oil and rapeseed oil at frying temperature (185^{o}C) resulted in a rapid degradation rate for gamma tocopherol followed by delta- and alpha- tocopherol respectively. Based on these data, alpha tocopherol has the greatest retention rate when thermally processed. This could explain why we did not see a drop in alpha tocopherol level in processed fish oil and a less dramatic drop in the other oils.

Broiler performance

The effects of oil source and peroxidation status on broiler performance over the 20 d performance period are reported in Table 4.4. An interaction ($P = 0.01$) between oil source and peroxidation status was noted for ADG where broilers fed peroxidized palm oil, soybean oil, and flaxseed oil had a 13.08%, 23.45%, and 22.95% reduction respectively in ADG compared to their fresh oil counterparts. However, peroxidation status did not affect ADG in broilers fed fish oil, thus driving the interaction. The observed reduction in ADG in broilers fed fish oil compared to the other oils tested in the current experiment is in contrast to Scaife et al., (1994) who reported similar weight gain in 56 d old broilers fed fresh (e.g., un-peroxidized) marine oil in comparison to broilers fed tallow, rapeseed oil, and soybean oil. The reduction in ADG for broilers fed peroxidized oils in the current experiment is in agreement with Anjum et al. (2004) who reported a decrease in ADG of 4.2% in broilers fed peroxidized soybean oil in comparison to those fed fresh soybean oil. Our data are also in agreement with a review of 26 broiler experiments by Hanson (2014), who reported that broilers fed peroxidized oil had reduced ADG of 11.1% compared to the control groups.

A similar interaction ($P = 0.01$) between oil source and peroxidation status was noted for ADFI (Table 4.4), in which birds fed the peroxidized soybean oil and flaxseed oil exhibited reduced ADFI (16.3% and 12.5% respectively) compared to broilers fed the fresh oil counterparts. However, the depression in ADFI was not as great in birds fed palm oil (6.6%), and peroxidation status did not affect ADFI in broilers fed fish oil, so similar to ADG, broilers fed fresh fish oil appear to be the main factor driving the

interaction we observed. The reduction in feed intake, especially in the fish oil treatment, could be a result in an increase in the dietary UFA:SFA. Crespo and Esteve-Garcia (2001) determined that increasing UFA:SFA in the dietary oils resulted in increased feed intake. Further, broilers fed diets containing 4.2% redfish oil has been shown to have reduced ADFI compared to a control group and groups fed redfish meal (Hulan et al., 1988). However, these results are in contrast to Scaife et al. (1994) who did not report differences in ADFI in broilers fed 5% fresh oils containing differing fatty acid profiles. Additionally, Lelis et al. (2009) reported that layers fed a diet containing 4% fish oil had similar feed intake in comparison to layers fed other oil sources including soybean, canola, and linseed oil. Peroxidation status also contributed to this interaction where broilers fed peroxidized oils had decreased ADFI, aside from broilers fed fish oil. This is in agreement with other studies that have shown decreased feed intake in birds fed peroxidized oils (Wang et al., 1997; Anjum et al., 2004; Tavárez et al., 2011). In the Hanson (2014) review, ADFI of broilers fed peroxidized oils was reduced by 6.6% in comparison to broilers fed fresh oil groups. While Hanson (2014) concluded that the reason ADFI decreased in broilers fed peroxidized oils was due to the PV of the oil, other lipid peroxidation products such as aldehydes, acids, ketones, and polymerized triglycerides could also contribute to the decreased ADFI as suggested by Shermer and Calabotta (1985).

An interaction ($P = 0.01$) was observed for G:F where broilers fed peroxidized palm, soybean, and flaxseed oils all had reduced G:F in comparison to their fresh oil counterparts, while broilers fed fish oil had decreased G:F regardless of peroxidation status. We would have expected broilers fed fish oil to respond similarly to the other

treatment groups but because this was not the case, the interactions observed are driven by broilers fed fresh fish oil. It is possible that the inclusion rate of 5% fish oil had a negative effect on diet palatability accounting for the reduction in growth performance in broilers fed fish oil. The oil source effect on G:F is supported by others (Pumchasov and Nir, 1992; Scaife et al., 1994; Crespo and Esteve-Garcia, 2001), who reported increased G:F when dietary PUFA intake increased in broiler diets. Additionally, Hulan et al. (1988) also reported reduced feed efficiency in broilers fed 4.2% redfish oil. Furthermore, the effect of peroxidation on G:F is in agreement with others (Wang et al., 1997; Anjum et al., 2004; Tavárez et al., 2011) where broilers fed peroxidized oil sources had reduced G:F. This is supported in the review by Hanson (2014) who reported a 4.6% reduction in G:F in broilers fed peroxidized oil sources. Secondary lipid peroxidation compounds including many aldehydes can produce a rancid odor and flavor and have been shown to affect palatability and feed intake in swine and poultry (Dibner et al., 1996; Boler et al., 2012; Liu et al., 2014a) which could account for the reduction in growth performance observed in broilers fed peroxidized oil sources.

Because growth performance parameters were affected by oil source and peroxidation status, a correlation analysis among multiple measures of lipid peroxidation products and performance parameters were conducted (Table 4.5). The UFA:SFA was positively correlated with ADFI, ADG, and G:F, which was expected because as the unsaturation of fatty acids increases, they are more digestible because of the ability to form a micelle compared to saturated fatty acids (Wiseman and Salvador, 1991). This was also expected because as each oil was thermally stressed, the UFA:SFA ratio decreased across all oils evaluated. In contrast to Hanson (2014), we did not find that PV

was correlated to any growth performance parameters, while AnV, DDE, polar compounds, and polymerized triglycerides were all negatively correlated to each growth parameter. This was expected because these lipid peroxidation products were are all increased due to thermal processing of the oils. Lastly, total tocopherols were positively correlated to ADG, ADFI, and G:F, which again was expected because thermal processing reduced tocopherol content in all oils evaluated, except for fish oil.

Markers of oxidative stress

To further assess oil source and quality, we evaluated broiler oxidative status. Increased oxidative stress has been shown to antagonize animal performance (Takahashi and Akiba, 1999; Tavárez et al., 2011; Boler et al., 2012). Oxidative stress occurs when free radical production overwhelms antioxidant production causing damage to lipids, proteins, and DNA. Because fatty acid profile (Liu et al., 2014b) and peroxidation status (Takahashi and Akiba, 1999) have been shown to affect oxidative status, we were interested in measuring indices of lipid, protein, and DNA oxidative damage, as well as multiple enzymatic antioxidants in plasma (Table 4.6) and liver (Table 4.7). It has been shown that consumption of lipid peroxidation products results in the accumulation of aldehydes in the gastric lumen which are then absorbed through the small intestine where they are concentrated and metabolized in the liver (Kanazawa and Ashida, 1998). For this reason, and because a major function of the liver is detoxification of pro-oxidants, we chose to measure oxidative balance in liver tissue in addition to plasma. Similar to the performance data, a correlation analysis was performed between oil composition and lipid peroxidation measures with oxidative stress markers in the plasma and liver (Table

4.8). No correlations for plasma FRAP, liver 8-OH-2dG, and liver GPx were observed and are therefore not reported in Table 4.8.

No significant interactions ($P \geq 0.11$) were observed between dietary oil source and peroxidation status on plasma TBARS (Table 4.6). Plasma TBARS were affected by oil source ($P = 0.01$), where broilers fed fish oil had the greatest plasma TBARS concentration followed by flaxseed oil, soybean oil, and palm oil (17.5, 15.7, 13.4, and 11.9 μM/mL, respectively). We hypothesize that plasma TBARS increased with increasing FA unsaturation of the oils of the treatment diets because the more polyunsaturated an oil is, the more susceptible it is to peroxidation (Holman, 1954; Yin et al., 2011). This was confirmed by a correlation analysis between plasma TBARS and double bonds in the dietary oil (r = 0.55, $P = 0.01$, data not shown). In comparison, Lin et al. (2006) induced oxidative stress in broilers via heat stress and observed increases in plasma TBARS. While we would have expected broilers fed peroxidized oils to have increased plasma TBARS, the numerical increase observed in the current trial was not found to be significant ($P = 0.39$, Table 4.6). Our results are similar to Tavárez et al. (2011) who reported no differences in plasma TBARS concentration between broilers fed fresh and peroxidized soybean oil. Furthermore, Liu et al. (2014c) reported that oil source influenced serum TBARS in pigs where pigs fed corn oil and canola oil had increased serum TBARS in comparison to pigs fed poultry fat and tallow. However, this is in contrast to other studies which have reported increases in plasma TBARS in broilers (Engberg et al., 1996; Takahashi and Akiba, 1999) and pigs (Liu et al., 2014c) fed peroxidized oils compared fresh oil treatments. These discrepancies may be a result of differences in the extent of peroxidation, particularly with secondary and tertiary lipid

peroxidation products including DDE, PTAGS, and polar compounds which were not shown to be related in the current experiment (Table 4.8). Despite the lack of effect of peroxidation status on plasma TBARS, lipid peroxidation compounds in the dietary oil including PV, AnV, and hexanal were correlated to plasma TBARS (Table 4.8).

There tended ($P = 0.09$) to be an interaction between oil source and peroxidation status for liver TBARS (Table 4.7). In general, broilers fed peroxidized palm oil had increased liver TBARS in comparison to broilers fed fresh palm oil (9.6 and 13.0 µM/mL, respectively), while the opposite effect was observed in broilers fed soybean oil (15.4 and 11.2 µM/mL for broilers fed fresh and peroxidized soybean oil, respectively), and no difference in liver TBARS were noted in broilers fed flaxseed oil and fish oil due to thermal processing. There was a tendency ($P = 0.10$) for broilers fed flaxseed oil to have increased liver TBARS in comparison to broilers fed palm oil and fish oil with broilers fed soybean oil as intermediates (Table 4.7). Due to the amount of unsaturation of the flaxseed and fish oil in our study, the rate of peroxidation and peroxidation products in these diets was expected. As expected, the broilers fed flaxseed and fish oil diets had the greatest liver TBARS because TBARS measures lipid damage due to lipid peroxidation. This observation is supported by a positive correlation between UFA:SFA and liver TBARS ($r = 0.39$, $P = 0.02$) as shown in Table 4.8. This is in contrast to plasma TBARS where we observed an increase in TBARS with increasing number of double bonds present in the dietary oil. The main effect of peroxidation status did not affect liver TBARS ($P = 0.98$). Our data agrees with others (Takahashi and Akiba, 1999; Tavárez et al., 2011) who also observed similar liver TBARS concentrations in broilers fed peroxidized and fresh soybean oil in broilers.

Increased ROS and PC concentration in mitochondria of breast muscle has been linked to low feed efficiency in broilers (Bottje et al., 2002; Iqbal et al., 2004). In the current study, there was no interaction between oil source and peroxidation status on plasma PC ($P = 0.19$). However, plasma concentration of PC (Table 4.6) tended to differ among the oil sources ($P = 0.09$), with broilers fed fish oil having the greatest plasma PC concentration (15.6 nmol/mL) and broilers fed palm oil having the lowest plasma PC concentration (11.1 nmol/mL), with flaxseed oil and soybean oil having intermediate concentrations (12.1 and 13.9 nmol/mL, respectively). This result was similar to that of plasma TBARS leading us to speculate that the number of double bonds in the dietary oil played a role in the increase in plasma PC because lipid peroxidation can lead to protein oxidation (Fellenberg and Speisky, 2006). Surprisingly, no correlation between plasma PC and UFA:SFA was noted (Table 4.8). While peroxidation status numerically increased plasma PC, it was not significant ($P = 0.15$). This result is in contrast to experiments in swine where consumption of highly peroxidized soybean oil (PV = 180 mEq/kg oil) increased plasma PC in comparison to pigs fed fresh soybean oil (Lu et al., 2014). Despite not being significant, however, plasma PC was correlated to lipid peroxidation products including AnV, DDE, and PTAGS (Table 4.8).

There was an interaction between oil source and peroxidation status observed in liver PC ($P = 0.04$; Table 4.7), where PC concentrations were increased by 25.7% in broilers fed peroxidized soybean oil and 18.2% in broilers fed peroxidized fish oil in comparison to the fresh oil counterparts; however, no changes were observed in broilers fed peroxidized palm oil and flaxseed oil. Interestingly, the main effects of oil source and peroxidation status on liver PC were not found to be significant ($P = 0.32$ and $P = 0.29$,

respectively). Lu et al. (2014) determined that feeding pigs peroxidized soybean oil results in an increase in liver PC so we would have expected a similar outcome. We have no explanation for these responses and to our knowledge, have no literature on liver PC in poultry from which to compare our data with. Similar to plasma PC, liver PC were correlated with compounds including DDE and PTAGS in addition to polar compounds in the dietary oil.

All cellular DNA components are susceptible to base repair damage due to ROS however, out of the nucleic acids, guanine is the most readily oxidized (Wu et al., 2004; Mateos and Bravo, 2007) making 8-OH-2dG the most prevalent measurement for oxidative damage of DNA. No interactions between oil source and peroxidation status were observed for plasma 8-OH-2dG ($P = 0.15$). Plasma 8-OH-2dG was affected by both oil source ($P = 0.01$) and peroxidation status ($P = 0.01$). Broilers fed flaxseed oil had the greatest plasma 8-OH-2dG concentration (17,162 pg/mL) in comparison to broilers fed palm oil and fish oil (11,571 and 12,182 pg/mL, respectively), with broilers fed soybean oil being intermediate (15,048 pg/mL) (Table 4.6). We hypothesize that the UFA:SFA of the dietary oil may have played a role in the increase in plasma 8-OH-2dG which is supported by our correlation analysis where plasma 8-OH-2dG was correlated to UFA:SFA ($r = 0.37$, $P = 0.02$). Furthermore, broilers fed peroxidized oil diets had greater plasma 8-OH-2dG concentration (15,818 pg/mL) compared to broilers fed fresh oils (12,163 pg/mL). This outcome was also expected because DNA is sensitive to oxidative damage and is supported by the observation of multiple correlations among lipid peroxidation products and plasma 8-OH-2dG as described in Table 4.8, namely secondary and tertiary lipid peroxidation products including DDE ($r = 0.48$, $P = 0.01$) and

polar compounds (r = 0.55, *P* = 0.01). Unfortunately, there is no current available data in poultry regarding plasma 8-OH-2dG from which to compare our data with.

There was no interaction observed between oil source and peroxidation status for liver 8-OH-2dG (*P* = 0.74); however, there was a tendency (*P* = 0.08) for oil source to increase 8-OH-2dG. Broilers fed fish oil and flaxseed oil had the greatest liver 8-OH-2dG (198.2 and 193.4 pg/mL, respectively) in comparison to soybean oil (145.3 pg/mL), with palm oil being intermediate (164.5 pg/mL). We hypothesize that the degree of unsaturation of the dietary oil influenced liver 8-OH-2dG. In contrast to plasma samples, peroxidation status did not affect liver 8-OH-2dG (*P* = 0.33). Interestingly, there were no correlations noted among oil composition and lipid peroxidation products with liver 8-OH-2dG (data not shown). These data suggest that the antioxidant defense system was functioning properly as there was minimal oxidative damage of lipids, proteins, and DNA in the liver.

We were also interested in examining SOD, GPx, and CAT activities to indicate total antioxidant balance. There was no interaction noted between oil source and peroxidation status (*P* = 0.98) and no oil source effect (*P* = 0.61) on liver SOD. There was a peroxidation effect (*P* = 0.03) on liver SOD where broilers fed peroxidized oils had a 19.7% increase in SOD activity in comparison to broilers fed fresh oils. This is in agreement with Altan et al. (2003) who reported a 47.4% increase in plasma SOD in broilers that were exposed to heat as an inducer of oxidative stress in comparison to broilers under thermal neutral conditions. This is in contrast to Lin et al. (2006) who induced oxidative stress via heat exposure to broilers and reported no differences in liver SOD. Surprisingly, the only correlation for liver SOD was with total tocopherols (r = -

0.30, $P = 0.07$; data not shown). We would have expected correlations between liver SOD and lipid peroxidation measures because the main effect of peroxidation status increased liver SOD, but this was not the case and is a reminder that correlations are not a cause-and-effect relationship.

Glutathione peroxidase is a common enzymatic antioxidant studied in livestock (Altan et al., 2003; Boler et al., 2012). In the current study, there was an interaction between oil source and peroxidation status ($P = 0.04$) on plasma GPx activity, where plasma GPx activity was decreased in broilers fed peroxidized palm oil, soybean oil, and flaxseed oil in comparison to their fresh oil counterparts; while peroxidation status had no effect on plasma GPx in broilers fed fish oil. Interestingly, this mimics the changes noted in total tocopherol concentrations of each oil and as supported by the correlation between total tocopherols and plasma GPx ($r = 0.36$, $P = 0.03$). Overall, oil source did not affect plasma GPx activity ($P = 0.44$) while feeding peroxidized oils reduced ($P = 0.01$) plasma GPx activity. This was expected because consuming lipid peroxidation products activates the antioxidant systems *in vivo* until the antioxidant system becomes overwhelmed which explains why GPx activity was decreased in broilers fed peroxidized oils. This is in agreement with an experiment conducted in swine where pigs fed peroxidized oils had reduced plasma GPx activity (Boler et al., 2012); however, the opposite effect occurred in the plasma GPx activity of heat stressed broilers (Altan et al., 2003).

In the current experiment, there was no interaction between oil source and peroxidation status ($P = 0.78$) on liver GPx activity. Similarly, the main effects of oil source and peroxidation status had no effect on liver GPx activity ($P \geq 0.70$). Due to this lack of effect, it was not surprising that no correlations were noted between liver GPx

activity with oil composition and lipid peroxidation products (data not shown). Because of the differential effects of peroxidation status on markers of oxidative stress in the liver, it may have been expected that the antioxidant defense system was not majorly affected.

Cooperativity between GPx and CAT is required for adequate clearing of H_2O_2 (Baud et al., 2004). In the current experiment, there was no interaction observed between oil source and peroxidation status for liver CAT (P = 0.95). Likewise, there was no effect of peroxidation status on liver CAT (P = 0.40); however, liver CAT was affected by oil source (P = 0.02). This is in contrast to Altan et al. (2003) who reported a 36.8% increase in plasma CAT activity in heat stressed broilers compared to birds at a thermal neutral temperature. The only correlation noted for liver CAT was with UFA:SFA (r = 0.48, P = 0.01) which was somewhat expected as the main effect of oil source affected liver CAT. Because GPx was not affected by peroxidation status in the liver, it makes sense that CAT was also not affected. Catalase is similar to GPx in that it detoxifies H_2O_2 to produce H_2O, however it is more efficient in comparison to GPx when H_2O_2 concentration is high (Finaud et al., 2006) suggesting that the antioxidant system was functioning properly. To our knowledge, there is no available data regarding the effect of oil source on catalase activity from which we can compare our data with.

Lastly, we measured FRAP in the plasma as a general indicator of total antioxidant capacity. There was no interaction noted between oil source and peroxidation status (P = 0.81) and plasma FRAP was not affected by either oil source or peroxidation status ($P \geq 0.12$). This is in contrast to Lin et al. (2006) who observed a decrease in plasma FRAP when broilers were under oxidative stress conditions due to exposure to

heat. We would have expected FRAP to decrease in broilers fed peroxidized oils because of the observed increase in 8-OH-2dG and decrease in GPx activity in plasma.

Relationship between growth performance and markers of oxidative stress

To further relate the effects of dietary lipid quality to oxidative stress and broiler performance, we conducted an additional correlation analysis between broiler growth performance and markers of oxidative stress (Table 4.9). Interestingly, there were no correlations between growth performance and markers of oxidative stress in the liver apart from liver TBARS which tended to be correlated to ADFI (r = 0.28, P = 0.09). Therefore, markers of oxidative stress in liver were not reported in Table 4.9. Correlations were observed between growth performance and oxidative stress markers in plasma suggesting that feeding peroxidized oils elicits a response on oxidative stress in plasma which thereby affects animal performance. Plasma PC were negatively correlated and GPx was positively correlated with all measurements of growth performance, whereas plasma TBARS were correlated to ADG (r = -0.35, P = 0.03) and ADFI (r = -0.42, P = 0.01) while plasma 8-OH-2dG was not correlated with any growth performance measures.

Conclusions

Results in the present study reveal that oil source and peroxidation status differentially affected broiler growth performance where broilers fed 5% peroxidized palm, soybean and flaxseed oils decreased ADFI and ADG which then resulted in decreased feed efficiency. In contrast, feeding peroxidized fish oil did not affect broiler

performance, thus driving many of the interactions noted in this experiment. We would have expected broilers fed fresh fish oil to respond similarly to the other fresh oil groups as suggested by others (Korver and Klasing, 1997; Farhoomand and Checaniazer, 2009; Lelis et al., 2009), but this was not the outcome. Broilers fed fresh fish oil resulted in a significant interaction in plasma GPx; however, it was not responsible for the observed interactions for liver TBARS and PC. Oil source impacted markers of oxidative stress in plasma and liver whereupon we hypothesize that the UFA:SFA and the degree of unsaturation of the dietary oils played a role explaining the results we obtained. Furthermore, peroxidation status increased plasma 8-OH-2dG and liver SOD, but decreased plasma GPx suggesting that collectively, the antioxidant system was moderately affected by peroxidation. The observed correlations between oil composition with growth performance and oxidative stress markers imply that it is worthwhile to measure multiple lipid peroxidation products including AnV, polar compounds, and PTAGS as they were most highly correlated to both growth and oxidative stress. Collectively, these data provide insight on the implications of feeding multiple oil sources and peroxidation state on successfully inducing metabolic oxidative stress, and consequently negatively affecting growth performance.

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Table 4.1. Compositional analysis of fresh and thermally processed oils.

	Palm oil		Soybean oil		Flaxseed oil		Fish oil	
Heating temperature, °C[1]	22.5	90	22.5	90	22.5	90	22.5	90
FA, % of total oil								
C14:0, Myristic	1.34	1.43	0.08	0.11	0.04	0.03	10.00	11.33
C16:0, Palmitic	46.41	49.07	11.09	15.83	5.21	6.55	20.16	22.80
C16:1, Palmitoleic	0.19	0.22	0.00	0.00	0.00	0.00	13.06	14.48
C 18:0, Stearic	4.56	4.80	4.51	6.45	3.84	4.74	3.78	4.28
C18:1, Oleic	38.48	39.17	23.89	30.52	15.70	18.66	5.75	6.36
C18:2, Linoleic	8.73	5.06	52.72	42.14	16.24	16.76	1.79	1.85
C18:3, Linolenic	0.29	0.24	6.86	3.73	58.56	52.89	1.97	1.92
C20:5, EPA[2]	0.00	0.00	0.00	0.00	0.00	0.00	16.91	14.27
C22:6, DHA[2]	0.00	0.00	0.00	0.00	0.00	0.00	16.55	13.18
UFA:SFA[2]	0.91	0.81	5.32	3.41	9.96	7.80	1.65	1.36
Double bonds[3]	57	50	150	126	224	211	212	181
PV, mEq/kg[2]	15.1	607.4	15.0	616.2	11.2	128.2	15.0	20.4
AnV[2,4]	3.9	87.0	0.8	384.1	ND	ND	38.4	436.6
Hexanal, mg/kg[2]	12.3	311.2	< LOQ[2]	108	< LOQ	8.3	< LOQ	5.1
DDE, mg/kg[2]	13.8	131.5	29.1	686	6.1	174	9.2	52.5
Polar compounds, %[2]	13.2	29.6	4.7	61.4	10.7	50.2	5.3	30.0
PTAGS, %[2]	0.00	4.9	0.00	31.7	0.00	21.8	0.00	9.9
Total tocopherols, mg/kg	58	< 10	802	37	267	< 10	119	125
Alpha	58	< 10	71	37	21	< 10	119	125
Beta	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Delta	< 10	< 10	196	< 10	< 10	< 10	< 10	< 10
Gamma	< 10	< 10	535	< 10	246	< 10	< 10	< 10

[1]The temperature of 22.5°C was fresh oil and not subjected to heat whereas 90°C oils were heated for 72 h with constant air flow (3L/min).

[2]Abbreviations: EPA, eicosapentaneoic acid; DHA, docosahexaenoic acid; UFA:SFA, unsaturated fatty acid:saturated fatty acid ratio; PV, peroxide value; AnV, p-anisidine value; DDE, 2,4-decadienal; PTAGS, polymerized triglycerides; LOQ, limit of quantification; ND, p-anisidine value could not be detected in flaxseed oil because of color interference. Samples analyzed at the USDA-ARS, Peoria, IL.

[3]The double bonds present in the experimental oils were calculated taking the number of double bonds in a FA multiplied by the analyzed FA composition.

[4]There is no unit for p-anisidine value.

Table 4.2. Ingredient and calculated composition of treatment diets, as-fed basis.[1]

Item, %	Percent
Corn	47.06
Soybean meal	43.41
Oil[1]	5.00
Dicalcium phosphate	1.83
Limestone	1.10
Vitamin mineral premix[2]	0.63
Sodium chloride	0.50
Methionine hydroxy analog	0.34
Choline chloride 60	0.10
L-threonine	0.03
TOTAL	100.00

[1]Oil treatments included fresh and peroxidized palm oil, soybean oil, flaxseed oil, and fish oil, with composition described in Table 4.1.

[2]Provided the following per kilogram of diet: selenium, 250 µg; vitamin A, 8,250 IU; vitamin D_3, 2,750 IU; vitamin E, 17.9 IU; menadione, 1.1 mg; vitamin B_{12}, 12 µg; biotin, 41 µg; choline, 447 mg; folic acid, 1.4 mg; niacin, 41.3 mg; pantothenic acid, 11 mg; pyridoxine, 1.1 mg; riboflavin, 5.5 mg; thiamine, 1.4 mg; iron, 282 mg; magnesium, 125 mg; manganese, 275 mg; zinc, 275 mg; copper, 27.5 mg; iodine, 844 µg.

Table 4.3. Assay kits used to determine oxidative status in plasma and liver.[1]

Assay Kit[2]	Catalog Number	Plasma	Liver
		Dilution factor	Dilution factor
TBARS	700870	ND	ND
PC	10005020	ND	ND
8-OH-2dG	589320	1:75	ND
SOD	706002	NA	1:10
GPx	703102	1:20	1:20
CAT	707002	NA	1:10,000

[1]All assay kits were purchased from Cayman Chemical Company (Ann Arbor, MI).

[2]TBARS, thiobarbituric acid reactive substances; PC, protein carbonyls; 8-OH-2dG, 8-hydroxy-2'-deoxyguanosine; SOD, superoxide dismutase; GPx, glutathione peroxidase activity; CAT, catalase activity; ND, no dilution; NA, not applicable.

Table 4.4. Effect of oil type and peroxidation status on broiler growth performance.[1]

Oil		ADFI	ADG	G:F
Source	Peroxidation[2]	g	g	g:g
Palm	22.5	56.5^{ab}	46.8^{a}	0.829^{a}
Palm	90	52.8^{b}	40. 7^{b}	0.769^{b}
Soybean	22.5	57.4^{a}	47.0^{a}	0.819^{a}
Soybean	90	48.1^{bc}	36.0^{c}	0.749^{bc}
Flax	22.5	56.1^{a}	47.3^{a}	0.845^{a}
Flax	90	49.0^{bc}	36.5^{c}	0.744^{bc}
Fish	22.5	46.9^{c}	34.0^{c}	0.726^{c}
Fish	90	46.4^{c}	34.5^{c}	0.746^{bc}
Statistics				
	SEM	1.26	1.20	0.015
	Oil × Peroxidation	0.01	0.01	0.01
	Oil	0.01	0.01	0.01
	Peroxidation	0.01	0.01	0.01
Main Effects				
Palm		54.6^{a}	43.7^{a}	0.799^{a}
Soybean		52.7^{a}	41.5^{a}	0.784^{a}
Flax		52.6^{a}	41.9^{a}	0.794^{a}
Fish		46.6^{b}	34.3^{b}	0.736^{b}
	Fresh	54.19	43.78	0.805
	Peroxidized	49.06	36.90	0.752

[1]Data are means of 5 replications per treatments with 5 broilers/cage except for palm oil-22.5^{o}C which represents 4 replications. Average initial BW, 85.1 ± 7.8 g. The trial lasted 20 d. Superscripts reflect dietary treatment differences (abc, $P \leq 0.05$; xyz, $P \leq 0.10$).

[2]The temperature of 22.5^{o}C was fresh oil and not subjected to heat whereas the 90^{o}C oils were heated for 72 h with constant air flow (3L/min).

Table 4.5. Pearson correlation coefficients among oil composition and lipid peroxidation products with growth performance[1]

	Oil composition and lipid peroxidation products							
Criterion	UFA:SFA	PV	AnV	Hex	DDE	Polar	PTAGS	TOC
ADG	0.32	-	-0.59	-	-0.35	-0.49	-0.50	0.52
	(0.04)		(0.01)		(0.03)	(0.01)	(0.01)	(0.01)
ADFI	0.27	-	-0.60	-	-0.32	-0.45	-0.47	0.50
	(0.10)		(0.01)		(0.05)	(0.01)	(0.01)	(0.01)
G:F	0.33	-	-0.42	-	-0.30	-0.42	-0.43	0.42
	(0.04)		(0.01)		(0.07)	(0.01)	(0.01)	(0.01)

[1]Top value represents correlation (*r* value) and bottom value in parenthesis represents significance (*P*-value). If no value is given, it was not found to be significant (-) at $P \leq 0.10$.

[2]UFA:SFA, unsaturated:saturated fatty acid ratio; PV, peroxide value; AnV, p-anisidine value; hex, hexanal; DDE, 2,4-decadienal; PTAGS, polymerized triglycerides; TOC, total tocopherols.

Table 4.6. Effect of oil type and peroxidation status on broiler oxidative stress in plasma.[1]

Oil		TBARS[2]	PC	8-OH-2dG	GPx	FRAP
Source	Peroxidation[3]	μM/mL	nmol/mL	pg/mL	$nmolmin^{-1}mL^{-1}$	μM
Palm	22.5	12.0	10.1	10,161	$2,733^{abc}$	257.2
Palm	90	11.9	12.0	12,981	$2,074^{bc}$	192.8
Soybean	22.5	13.3	10.8	11,063	$2,555^{ab}$	169.2
Soybean	90	13.5	17.0	19,034	$1,734^{c}$	162.5
Flax	22.5	15.3	11.3	17,265	$3,149^{a}$	195.4
Flax	90	16.1	12.9	17,059	$1,798^{c}$	165.8
Fish	22.5	16.4	16.5	10,165	$2,067^{bc}$	229.7
Fish	90	18.6	14.6	14,199	$2,119^{bc}$	162.9
Statistics						
	SEM	1.27	1.86	1,768	247	36.8
	Oil × Peroxidation	0.80	0.19	0.15	0.04	0.81
	Oil	0.01	0.09	0.01	0.45	0.44
	Peroxidation	0.39	0.15	0.01	0.01	0.12
Main effects						
Palm		11.9^{c}	11.1^{y}	$11,571^{b}$	2,228	225.0
Soybean		13.4^{bc}	13.9^{xy}	$15,048^{ab}$	2,144	165.8
Flax		15.7^{ab}	12.1^{xy}	$17,162^{a}$	2,473	180.6
Fish		17.5^{a}	15.6^{x}	$12,182^{b}$	2,093	196.3
	Fresh	14.2	12.2	12,163	2,538	212.9
	Peroxidized	15.0	14.1	15,818	1,931	171.0

[1]Data are means of 5 replications per treatments with 2 broilers/cage except for fresh palm oil which represents 4 replications. Superscripts reflect dietary treatment differences (abc, $P \leq 0.05$; xyz, $P \leq 0.10$).

[2]TBARS, thiobarbituric acid reactive substances; PC, protein carbonyls; 8-OH-2dG, 8-hydroxy-2'deoxyguanosine; GPx, glutathione peroxidase activity.

[3]The temperature of 22.5°C was fresh oil and not subjected to heat whereas 90°C oils were heated for 72 h with constant air flow (3L/min).

Table 4.7. Effect of oil type and peroxidation status on broiler oxidative stress in liver.[1]

Oil		TBARS	PC	8-OH-2dG	SOD	GPx	CAT
Source	Peroxidation	μM/mL	nmol/mL	pg/mL	U/mL	nmolmin^{-1}mL^{-1}	nmolmin^{-1}mL^{-1}
Palm	Fresh	9.6^{z}	96.5^{abc}	163.7	165.9	2,592	26,248
Palm	Peroxidized	13.0^{xyz}	93.6^{abc}	165.2	207.2	2,364	32,670
Soybean	Fresh	15.4^{x}	87.2^{bc}	138.4	140.5	2,175	43,653
Soybean	Peroxidized	11.2^{yz}	117.4^{a}	152.2	167.6	2,160	43,341
Flax	Fresh	14.0^{xy}	112.0^{ab}	192.0	151.0	2,134	51,372
Flax	Peroxidized	14.7^{xy}	93.9^{abc}	194.9	197.8	2,499	61,454
Fish	Fresh	11.2^{yz}	79.6^{c}	175.21	152.4	2,081	25,907
Fish	Peroxidized	11.2^{yz}	97.3^{abc}	221.24	187.5	2,292	31,802
Statistics							
	SEM	1.44	8.87	22.79	24.06	302.96	9,232
	Oil × Peroxidized	0.09	0.04	0.74	0.98	0.78	0.95
	Oil	0.10	0.32	0.08	0.61	0.73	0.02
	Peroxidized	0.98	0.29	0.33	0.03	0.70	0.40
Main effects							
Palm		11.3^{y}	95.1	164.5^{xy}	186.6	2,478	29,459^{b}
Soybean		13.3^{xy}	102.3	145.3^{y}	154.0	2,168	43,497^{a}
Flax		14.4^{x}	103.0	193.4^{x}	174.4	2,316	56,413^{a}
Fish		11.2^{y}	88.5	198.2^{x}	170.0	2,187	28,854^{b}
	Fresh	12.6	93.8	167.3	152.5	2,245	36,795
	Peroxidized	12.5	100.57	183.4	190.0	2,329	42,317

[1]Data are means of 5 replications per treatments with 2 broilers/cage except for palm oil-fresh which represents 4 replications. Superscripts reflect dietary treatment differences (abc, $P \leq 0.05$; xyz, $P \leq 0.10$).

[2]TBARS, thiobarbituric acid reactive substances; PC, protein carbonyls, 8-OH-2dG, 8-hydroxy-2-deoxy-guanosine; SOD, superoxide dismutase; GPx, glutathione peroxidase activity; CAT, catalase activity,

Table 4.8. Pearson correlation coefficients among oil composition and peroxidation measures with oxidative stress markers[1]

	Oil composition and lipid peroxidation products[2]						
Criterion[3]	UFA:SFA	PV	AnV	DDE	Polar	PTAGS	TOC
Plasma							
TBARS	-	-0.34 (0.04)	0.36 (0.05)	-	-	-	-
PC	-	-	0.35 (0.07)	0.32 (0.05)	-	0.31 (0.06)	-
8-OH-2dG	0.37 (0.02)	0.33 (0.05)	0.58 (0.01)	0.48 (0.01)	0.55 (0.01)	0.56 (0.01)	-
GPx	0.28 (0.09)	-0.34 (0.04)	-0.34 (0.07)	-0.39 (0.02)	-0.47 (0.01)	-0.46 (0.01)	0.36 (0.03)
Liver							
TBARS	0.39 (0.02)	-	-	-	-	-	0.33 (0.04)
PC	-	-	0.40 (0.03)	0.35 (0.03)	0.30 (0.06)	0.30 (0.07)	-

[1]Top value represents correlation (*r* value) and bottom value in parenthesis represents significance (*P*-value). If no value is given, it was not found to be significant (-) at $P \leq 0.10$.

[2]UFA:SFA, unsaturated:saturated fatty acid ratio; PV, peroxide value; AnV, p-anisidine value; DDE, 2,4-decadienal; Polar, percentage of polar compounds; PTAGS, polymerized fatty acids; TOC, total tocopherols; TBARS, thiobarbituric acid reactive species; PC, protein carbonyls; 8-OH-2dG, 8-hydroxy-2'deoxy-guanosine; GPx, glutathione peroxidase activity.

[3]No correlations for plasma FRAP, liver 8-OH-2dG and GPx were observed. The only correlation for liver superoxide dismutase was with TOC (r = -0.30, *P* = 0.07), the only correlation for liver catalase was with UFA:SFA (r = 0.48, *P* = 0.01), and the only correlation with hexanal was with plasma TBARS (r = -0.38, *P* = 0.02) and were removed from the table.

Table 4.9. Pearson correlation coefficients of broiler performance with measures of oxidative stress in plasma.

	TBARS	PC	8-OH-2dG	GPx
ADG	-0.35	-0.47	-	0.44
	(0.03)	(0.01)		(0.01)
ADFI	-0.42	-0.43	-	0.45
	(0.01)	(0.01)		(0.01)
G:F	-	-0.45	-0.04	0.33
		(0.01)	(0.83)	(0.04)

[1]Top value represents correlation (*r* value) and bottom value in parenthesis represents significance (*P*-value). If no value is given, it was not found to be significant (-) at $P \leq 0.10$.

[2]TBARS, thiobarbituric acid reactive species; PC, protein carbonyls; 8-OH-2dG, 8-hydroxy-2'deoxy-guanosine; GPx, glutathione peroxidase activity.

[3]The only correlation noted in the liver oxidative stress markers was for liver TBARS was with ADFI (r = 0.28, $P = 0.09$) so all liver oxidative stress markers were removed from the table.

CHAPTER 5

GENERAL DISCUSSION

Livestock diets commonly contain added lipids to increase the energy density of the feed, improve palatability, and for dust control in the barn. In Midwestern diets, vegetable oils, specifically soybean oil, is the most commonly added vegetable oil, however, palm oil is the most prevalently used oil worldwide (Carter et al., 2007). Vegetable oils have high concentrations of polyunsaturated fatty acids (**PUFA**) which make them predisposed to lipid peroxidation, especially in the presence of oxygen, light, metals, and exposure to heat (Girotti, 1990). Lipid peroxidation is a free radical chain reaction that occurs in three general phases. The initiation phase can be quantified measuring hydroperoxide production via peroxide value (**PV**). The secondary phase is the propagation phase which degrades peroxides into ketones, acids, and aldehydes. This phase is commonly measured via p-anisidine value (**AnV**) or individual aldehydes including 2,4-decadienal (**DDE**), 2-hydroxynonenal (**HNE**), and hexanal. Finally, the termination phase occurs when the fatty acid becomes completely hydrogenized and free radicals can no longer bind to the fatty acid and is commonly measured by aldehydes, total polar compounds, and polymerized triglycerides (**PTAGS**).

Lipid peroxidation is a dynamic reaction that produces various compounds that have been associated with reduced growth (Tavárez et al., 2011; Boler et al., 2012; Liu et al., 2014a) and disrupting antioxidant status (Takahashi and Akiba, 1999; Tavárez et al., 2011) in swine and poultry. Oxidative stress is commonly understood to be an imbalance between free radical and reactive oxygen species (**ROS**) within the animal with the antioxidant defense system including superoxide dismutases (**SOD**), glutathione

peroxidase (**GPx**), and catalase (**CAT**). If the antioxidant system becomes overwhelmed, free radicals begin to bind to lipids, proteins, and DNA causing cell damage. The only way to directly measure oxidative stress is by measuring ROS; however, because ROS are unstable, it is far more common to measure damage caused by ROS on lipids, proteins, and DNA. Commonly, thiobarbituric acid reactive substances (**TBARS**) is measured as an indicator of lipid damage; however, TBARS is a nondescript measure of lipid damage due to lipid peroxidation so measuring F_2-isoprostanes (**ISP**) is a more valid measure because it is a specific measure of lipid peroxidation. Protein carbonyl (**PC**) concentration is commonly measured as a marker of protein damage while 8-hydroxy-2'deoxyguanosine (**8-OH-2dG**) is measured as a marker of DNA damage.

To date, most research has focused on analyzing a single product of lipid peroxidation and an individual marker of oxidative damage. However, as mentioned above, lipid peroxidation is a dynamic reaction and oxidative stress is not a singular metabolic event. Therefore, the overall objective of this thesis was to determine the impacts of feeding peroxidized oils on growth, digestibility, and total body oxidative stress in growing swine and poultry. The overarching hypothesis of this thesis was that feeding oil that has been peroxidatively modified by thermal processing would induce oxidative stress in pigs and poultry, and thus reduce their growth performance. To test this hypothesis, three experimental research papers where conducted (Chapters 2-4).

In the first experiment (Chapter 2), we studied the impacts of feeding thermally peroxidized soybean oil (**SO**) on growth performance and digestibility in growing pigs. Treatments included fresh SO, SO processed at 45^{o}C for 288 h, SO processed at 90^{o}C at 72 h, and SO processed at 180^{o}C for 6 h, each with a constant air flow rate of 15L/min,

and each fed at 10% in the total diet. Based on previous research (Boler et al., 2012; Liu et al., 2014a; Rosero et al., 2015), we hypothesized that feeding highly peroxidized SO would result in reduced growth performance, feed efficiency, and nutrient digestibility. Growth performance was collected over a 49 d period including 5 d in metabolism crates for fecal and urine collections to acquire digestibility data. As expected, ADG was reduced in pigs fed the 90^{o}C processed SO; however, lipid peroxidation had no effect on ADFI and, surprisingly, pigs fed the 45^{o}C SO had improved G:F in comparison to the other treatment groups. During the process of lipid peroxidation, aldehydes and PTAGs are generated which may negatively impact digestibility (Gonzalez-Muñoz et al., 1998). Analysis of the processed SO at 90^{o}C revealed increased fatty acid saturation which was predicted to reduce digestibility (Wiseman and Salvador, 1991). As expected, pigs fed the 90^{o}C processed SO also had reduced DE as a percentage of GE, EE digestibility, and N retention (Chapter 2). To further understand how thermally peroxidized SO may modulate intestinal function and integrity, at the end of the digestibility study we assessed *in vivo* intestinal permeability by feeding lactulose and mannitol and measuring these products in urine excretion. The recovered ratio of these carbohydrates in urine is indicative of small intestinal paracellular permeability where a high recovery of lactulose indicates poor intestinal barrier function (Wijtten et al., 2011). Results from these pigs in Chapter 2 indicated that peroxidized SO had no impact on intestinal permeability. This outcome was supported by Liu et al. (2014b) who also reported no differences between pigs fed peroxidized oils compared to fresh oil sources. Correlation analyses between SO quality and growth performance and digestibility measures revealed that PV, individual aldehydes (such as DDE or HNE), or AnV had strong negative correlations while total

tocopherols were positively correlated. In summary, Chapter 2 provided evidence that consumption of highly peroxidized SO results in a depression in growth performance and digestibility of nutrients.

Because the consumption of lipid peroxidation products has been shown to induce oxidative stress (DeRouchey et al., 1997; Boler et al., 2012; Rosero et al., 2015), we evaluated the impacts of feeding variable levels of thermally peroxidized SO on markers of oxidative stress in growing pigs (Chapter 3). For this study, our overarching hypothesis was that feeding peroxidized SO would increase markers of oxidative stress in growing pigs. This experiment was conducted on the same pigs as described in Chapter 2. Because the 90^{o}C processed SO produced the greatest concentration of lipid peroxidation products (Chapter 2), in Chapter 3 we hypothesized that pigs fed the 90^{o}C SO diet would be under the most oxidative stress. On d 25, 29, and 34, urine was collected for 5 h and following this collection, blood was collected for subsequent analysis of markers of oxidative stress. After 49 d on SO treatments, pigs were euthanized and liver samples were obtained to assess markers of lipid, protein, and DNA oxidative damage, and measured enzymatic antioxidant function. Results from Chapter 3 indicated that pigs fed thermally processed so at 90^{o}C for 72 h exhibited the greatest amount of oxidative stress as determined by increases in serum PC, urinary ISP, and liver 8-OH-2dG, and a decrease in serum GPx activity. The data in swine (Chapters 2 and 3) suggest that PV, AnV, and total tocopherols are important lipid peroxidation products that should be analyzed to determine oil quality as these compounds were most correlated to markers of oxidative stress. Furthermore, based on the data in Chapter 3 we were able to conclude that

thermally processing SO at 90^{o}C for 72 h induced oxidative stress as measured by urinary ISP, serum PC and GPx activity, and liver 8-OH-2dG in growing pigs.

Due to the impacts of consuming lipid peroxidation products found in the 90^{o}C processed SO on growth performance and oxidative stress in growing pigs (Chapters 2 and 3), we also wanted to evaluate the susceptibility of poultry. Likewise, others have shown that growth performance is affected by oil source (Scaife et al., 1994; Crespo and Esteve-Garcia, 2001; Lelis et al., 2009) and peroxidation status (Takahashi and Akiba, 1999; Anjum et al., 2004; Tavárez et al., 2011) in broilers. The objectives of Chapter 4 were to determine the impacts of multiple oil sources and peroxidation status on growth performance and markers of oxidative stress in broilers. This experiment studied the effects of palm, soybean, flaxseed, and fish oil either fresh or thermally processed at 90^{o}C for 72 h on broiler performance and markers of oxidative stress in plasma and liver. Because oils with a high degree of unsaturation (as measured by unsaturated:saturated FA and the total number of double bonds) are more vulnerable to lipid peroxidation, we hypothesized that birds fed peroxidized fish and flaxseed oil would be most affected by peroxidation status. Broilers were fed the experimental diets (oil included at 5% of the total diet) ad libitum for 20 d to collect growth performance data and plasma and liver samples were collected on d 21 for subsequent analysis of oxidative stress markers. In Chapter 4, we reported an interaction between oil source and peroxidation status on ADFI, ADG, and G:F over the 21 d trial. Broilers fed peroxidized palm, soybean, and flaxseed oils had reduced performance in comparison to their fresh oil counterparts; however, peroxidation status had no impact on performance in broilers fed fish oil, thus driving the interaction. We would have expected broilers fed fresh fish oil to respond

similarly to the other fresh oil groups as suggested by others (Korver and Klasing, 1997; Farhoomand and Checaniazer, 2009; Lelis et al., 2009), but this was not the outcome. It is possible that the 5% inclusion rate of fish oil to the diet may have caused detrimental effects on palatability, resulting in reduced growth performance; and that peroxidation of fish oil had no additional effect.

Research evaluating oxidative stress in broilers has been induced by various models that have included heat stress (Altan et al., 2003; Lin et al., 2006), and lipid peroxidation (Takahashi and Akiba 1999; Tavárez et al., 2011) have been studied. In agreement with Takahashi and Akiba, (1999) and Tavárez et al. (2011), we reported that thermally oxidized oils and source modulated oxidative stress markers (Chapter 4). Herein, an interaction was observed for plasma GPx activity where broilers fed fresh palm, soybean, and flaxseed oils had greater GPx activity than the peroxidized oil groups; while peroxidation status of fish oil had no effect on GPx activity. Additionally, degree of oil unsaturation increased TBARS, 8-OH-2dG, and PC in plasma; furthermore, broilers fed peroxidized oils had increased plasma 8-OH-2dG. An interaction was noted in liver TBARS where broilers fed peroxidized palm oil had increased liver TBARS compared to fresh palm oil, while the opposite was observed in broilers fed soybean oil and no changes were noted in broilers fed flaxseed oil and fish oil. Another interaction was noted for liver PC where broilers fed palm oil, flaxseed oil and fish oil had similar liver PC while broilers fed peroxidized soybean oil had increased liver PC compared to the fresh soybean oil diet. Degree of unsaturation also increased liver 8-OH-2dG and CAT; additionally, peroxidation status increased SOD activity in the liver. Because of the differential effects of peroxidation status on markers of oxidative stress between the

plasma and liver were not consistant, it may have been expected that the antioxidant defense system was not greatly affected. A correlation analysis between oil composition and growth performance and oxidative stress markers showed that AnV, DDE, and PTAGS should be considered when determining oil quality. Furthermore, plasma PC and GPx had the greatest correlation to growth performance parameters. Based on the results from Chapter 4, we were able to conclude that oil source and peroxidation status differentially affected broiler growth performance, plasma GPx, and liver TBARS and PC, while degree of oil unsaturation generally increased oxidative damage in plasma and liver. Furthermore, peroxidation status was also shown to largely increase oxidative damage in plasma and liver. Data from Chapter 4 provide insight on the implications of feeding multiple oil sources and peroxidation status on markers of oxidative stress and the subsequent negative effect on broiler growth performance.

Overall, the data reported in this thesis have provided a better understanding of the implications of feeding peroxidized oils to swine and poultry. In pigs, thermally processing SO at 90^{o}C for 72 h was shown to yield the greatest concentrations of lipid peroxidation products and was shown to reduce ADG and nutrient digestibility (Chapter 2) and resulted in the greatest increase in oxidative stress (Chapter 3) which suggests that redox imbalance contributes to reduced growth performance and feed efficiency. Likewise, Chapter 4 confirmed in broilers that consumption of lipid peroxidation products in thermally processed oils at 90^{o}C for 72 h reduced growth performance apart from broilers fed fish oil. In addition, oil source and peroxidation status differentially influenced oxidative stress in broilers.

Some similarities in markers of oxidative stress were noted across experiments (Chapters 3 and 4). In both swine and poultry, plasma TBARS and FRAP were not impacted by dietary treatment; however, we found that blood GPx was reduced. In contrast, serum PC was not affected by SO treatment in pigs but unsaturation of the dietary oil increased plasma PC in poultry. In the liver, PC was not affected by peroxidation status; additionally, dietary treatment did not have an impact on liver GPx in swine and poultry. Furthermore, according to the correlation matrices presented in this thesis, lipid peroxidation products that should be measured to determine the quality of oils include PV, DDE or AnV, PTAGS, and total tocopherols because they were the most correlated to growth performance and oxidative status in swine (Chapter 2, 3) and poultry (Chapter 4).

Thermally processing SO was a successful model to induce oxidative stress in both swine and poultry as measured by PV, multiple aldehydes, AnV, and PTAGS, and should be used in further research to determine the impacts of oil quality on growth performance and oxidative status *in vivo*. Collectively speaking, markers of oxidative stress measured in liver tissues were not well correlated with oil composition and quality indicating that further research should focus on measuring oxidative stress markers in plasma and urine samples.

Consequently, further research should be considered to determine whether a specific measure or a multiple variable model of oxidative stress can be generated that accurately depicts total body oxidative status. The experiments herein were unable to determine a specific marker of oxidative stress to focus on in further experiments; however, data in Chapters 3 and 4 suggests that blood GPx gives an accurate indication

of antioxidant status such that liver SOD, GPx, and CAT are not necessary. Moreover, analysis of oxidative stress should focus on measures in plasma and urine because according to the correlation analyses, markers of oxidative stress in the liver were not well correlated with oil quality. Based on data from Chapters 3 and 4 and a review of literature, *in vivo* markers of oxidative stress that should be measured include ISP and 8-OH-2dG in urine, while PC, and GPx should be measured in blood.

Oxidative stress can be induced in biomedical and agricultural settings in many ways including exercise (Urso and Clarkson, 2003), radiation and UV exposure (Azzam et al., 2012), air pollution (Kelly, 2003), heat stress (Altan et al., 2003; Lin et al., 2006), and via consumption of peroxidized lipids (Tavárez et al., 2011; Boler et al., 2012; Liu et al., 2014b). An influx of free radicals and ROS (and their secondary and tertiary products such as aldehydes) can lead to modifications and mutations of lipids, proteins, and DNA which can antagonize cell signaling, disrupt immune function, increasing susceptibility to carcinogenesis and disease (Berlett and Stadtman, 1997), and neurodegenerative disorders in humans (Hald and Lotharius, 2005). There is ample research regarding oxidative damage due to the consumption of lipid peroxidation products; however, research is needed in order to determine the roles in aggregating the secondary effects of oxidative stress. For example, it is not known how aldehydes, ketones, polar compounds, and polymers formed throughout lipid peroxidation modulate inflammation, immune system function, and disease.

Due to the inconsistencies of research regarding the effects of dietary lipid peroxidation products on feed intake (Anjum et al., 2004; Tavárez et al., 2011; Boler et al., 2012; Liu et al., 2014a) in livestock, further research could be done to determine the

lipid peroxidation products (such as DDE, Chapter 4) that drive feed intake and appetite. Research could also be conducted to determine if the addition of supplemental dietary antioxidants (e.g. vitamin E or synthetic antioxidants) would alleviate the detrimental effects of feeding highly peroxidized oils on feed intake, growth and oxidative status.

In summary, feeding SO that has been peroxidized by thermal processing, especially at 90^{o}C for 72 h, will induce oxidative stress in pigs and poultry, and thus reduce their growth performance. Discrepancies in growth performance may be due to reduced digestibility of nutrients and/or oxidative stress *in vivo*. The information contained in this thesis will ease future research to better understand the biological effects of consumption of lipid peroxidation products on growth, digestibility, and oxidative status.

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APPENDIX A

AUTHORED ABSTRACTS

S. C. Lindblom, G. C. Shurson, W. A. Dozier, B. J. Kerr. (2016) Digestibility of energy and lipids, and metabolic oxidation status in nursery pigs fed various lipids. ASAS-ADSA Midwest Sectional Meeting, Des Moines, IA. March 16-18, Abstract #253.

An experiment was conducted to evaluate the impact of lipid source on energy and lipid digestibility, and on metabolic oxidation status of nursery pigs fed diets containing 10% of soybean oil (SO), choice white grease (CWG), palm oil (PO), or 1 of 2 different sources of distillers corn oil (DCO1 and DCO2). Fifty-four barrows weaned at 28-d of age were fed a common starter diet from d 1 to 7, followed by group feeding their respective experimental diets (either 100% basal or 90% basal + 10% test lipid) from d 8 to 14 to adapt the pigs to their dietary treatments and to optimize subsequent feed intake. For the next 10 d (d 15 to 24), pigs were moved to metabolism crates for continued diet adaptation and to become accustomed to the twice daily feeding regimen in the metabolism crates. Following this period, a 4-d total fecal and urine collection occurred (d 25 to 29, final BW 11.03 ± 0.51 kg) to determine apparent total tract digestibility (ATTD) of energy and lipids, and to determine the DE and ME content of each lipid source. After an overnight fast of 12 h, urine was collected for 5 h, quantified, and subsequently analyzed for thiobarbituric acid reactive substances (TBARS) and isoprostane (IsoP) concentration. Following this collection, serum was obtained and analyzed for TBARS. Soybean oil had greatest ($P < 0.05$) DE (9388 kcal/kg) content compared with DCO1, DCO2, CWG, PO, and SO containing 8001, 8052, 8531, 8293, and 9388 kcal/kg lipid, respectively. Digestible energy as a percentage of GE was greatest ($P < 0.05$) for SO when compared to the other lipid sources ($P < 0.05$). The ATTD of EE averaged 85.0% and varied slightly (84.4% to 85.6%) among treatments. Differences in ME among lipids were similar to that for DE, with ME values for DCO1, DCO2, CWG, PO, and SO containing 7921, 7955, 8535, 8350, and 9408 kcal/kg lipid, respectively. Metabolizable energy as a percentage of DE did not differ between lipid sources. Pigs fed the diets containing lipids had higher ($P < 0.05$) plasma TBARS compared to pigs fed the control diet, but no differences were noted in urinary TBARS excretion among treatments. Urinary IsoP excretion differed among treatments ($P < 0.01$), but was highly variable (34.0 to 104.6 pg/ml). These results indicate that DE and ME values vary among lipid sources and appear to have variable effects on metabolic oxidation measures.

S. C. Lindblom, G. C. Shurson, J. Moser, B. J. Kerr. (2016) Kinetics of lipid peroxidation in fats and oils as affected by lipid source, heating temperature, and length of heating. ASAS-ADSA-CSAS-WSASAS Joint Animal Science Meeting, Salt Lake City, UT. July 19-23, Abstract #960.

Lipid peroxidation is a chain reaction of generation and degradation of peroxidation compounds including acids, aldehydes, ketones, and polymers. However, assays used in farm animal research commonly measure 1 or 2 compounds and do not assess the change in a lipid's peroxidation status over time. Consequently, a laboratory-study was conducted to evaluate the impact of heating temperature and length of heating on the generation of lipid peroxidation products in lipids varying in fatty acid composition. Six lipids were selected based on their divergent fatty acid composition and predicted peroxidizability index (Hammond, 1954). Lipids used in this study included tallow, palm oil, soybean oil, linseed oil, menhaden oil, and a manufactured oil were heated at 4 temperatures to reflect ambient, summertime feed temperature in a bulk bin, an elevated feed processing temperature, and a frying temperature (22.5°, 45°, 90°, and 180°C, respectively). Oils (2.5 L each) were placed in a 5 L round-bottom glass flask and heated with an electric heating mantle with power controller, with bubbling air at 3 L/min. Because tallow and palm oil were solids at room temperature, they were evenly spread into a 30.5 × 45.7 cm pan to allow for air reaction to the lipid. Samples were taken at equally distributed time points within a temperature (22.5°C for 0, 6, 12, 18, 24 d; 45°C for 0, 3, 6, 9, 12 d; 90°C for 0, 18, 36, 54, 72h; and 180°C for 0, 3, 6, 9, 12h). Lipid analysis for composition, quality, and peroxidation indicators were conducted. Depending on the lipid source, heating temperature, and sampling time, peroxide value increased from 11 to 1200 mEq/kg, *p*-anisidine value from 0 to 810, hexanal from 0 to 300 mg/kg, 2,4-decadienal from 0 to 690 mg/kg, polar compounds from 5 to 60%, polymers from 0 to 30%, and OSI from 0 to 22 h at 110°C. The data show that the peroxidizability of lipids varies greatly depending on their fatty acid profile, the degree of heating and time of sampling. Because peroxidized lipids have been shown to impact animal performance, gastrointestinal integrity, gene expression, immune competence, and metabolic oxidative status, the understanding of how and when lipid peroxidation compounds are generated and degraded, and their concentration in a lipid at specific time point is important to their use in livestock feeding programs.

S. C. Lindblom, E. A. Bobeck, and B. J. Kerr (2017) Effect of oil source and peroxidation status on broiler performance and oxidative status. International Poultry Scientific Forum, Atlanta, Georgia. January 30-31, Abstract #M89.

Oil source has been shown to affect broiler performance and oxidative status. Lipid peroxidation may also affect animal performance and oxidative status through the generation and degradation of peroxidation compounds which differ according to oil source and temperature and length of heating. The objective of the study was to evaluate the effect of oil source and peroxidation status on broiler performance and measures of oxidative stress. Broilers (initial BW 85.1 ± 7.8 g) were allotted to 40 battery cages in a completely randomized 4 × 2 factorial arrangement of treatments. Treatments consisted of oil source (palm oil, soybean oil, flaxseed oil, and fish oil) in combination with lipid quality (fresh or peroxidized oil). Peroxidation was achieved by thermally processing each oil at 90^{o}C for 72 h with a constant air flow of 3 L/min. Oils were analyzed for peroxide value, anisidine value, hexanal, 2,4-decadienal, and total polymers as measures of oil peroxidation. Each treatment was replicated 5 times with 5 birds/cage (200 birds), with birds fed their respective diets for 20 d to measure performance criteria. On d 21, plasma was harvested from 2 birds from each cage for analysis of oxidative status, which included thiobarbituric acid reactive species (TBARS), protein carbonyls (PC), 8-hydroxy-2'-deoxyguanosine (8-OH-2dG), and glutathione peroxidase activity (GPx). An interaction between oil source and peroxidation status was noted for ADFI, ADG, and F:G ($P = 0.01$), where birds fed the peroxidized oil reduced ADFI, ADG, and F:G in all oil sources except for birds fed the fish oil. There were no interactions noted between oil source and peroxidation status for TBARS, PC, 8-OH-2dG, or GPx ($P > 0.10$). Oil source increased plasma TBARS and 8-OH-2dG ($P = 0.01$), and tended to increase plasma PC ($P = 0.09$), but did not affect plasma GPx ($P > 0.44$). Although lipid peroxidation had no effect on plasma TBARS or PC ($P > 0.15$), plasma 8-OH-2dG and GPx were affected by lipid peroxidation ($P < 0.01$). In conclusion, oil source and peroxidation status differentially affected growth performance, oil source increased plasma TBARS, PC, and 8-OH-2dG, and lipid peroxidation increased plasma 8-OH-2dG and decreased plasma GPx.

S. C. Lindblom, N. K. Gabler, B. J. Kerr. (2017) Influence of thermally peroxidized soybean oil on growth performance and oxidative status in growing pigs. ASAS-ADSA Midwest Sectional Meeting, Omaha, NE. March 13-15, Abstract #096.

The objective of this study was to evaluate the effect of feeding peroxidized soybean oil (SO) on growth performance and oxidative status in growing pigs. Fifty-six barrows (25.3 ± 3.3 kg initial BW) were randomly assigned to one of four diets containing either 10% fresh SO (22.5°C) or SO exposed to heat (45°C for 288 h, 90°C for 72 h, or 180°C for 6 h), each with an air infusion of 15 L/min. Peroxide values for the 22.5, 45, 90, and 180°C processed SO were 2.0, 96, 145, and 4.0 mEq/kg, respectively. Anisidine values for 22.5, 45, 90, and 180°C processed SO were 1.2, 8.4, 261, and 174, respectively. Pigs were individually housed and fed ad libitum for 49 d to measure growth performance, including a metabolism period to collect urine and serum for analysis of oxidative stress markers. Oxidative stress markers included serum and urinary thiobarbituric acid reactive species (TBARS) and urinary F2 –isoprostanes (ISP) as markers of lipid damage, serum protein carbonyls (PC) as a marker of protein damage, and urinary 8-hydroxy-2'-deoxyguanosine (8-OH-2dG) as a marker of DNA damage. Glutathione peroxidase activity (GPx) was measured in serum, and ferric reducing antioxidant potential (FRAP) was measured in urine as determinants of antioxidant status. Although there were no differences in final BW 70.8 ± 5.7 kg (P = 0.11) and ADFI (P = 0.19), ADG was decreased in pigs fed 90°C SO diet (P = 0.01), while G:F was increased (P = 0.02) in pigs fed 45°C SO diet compared to the other SO diets. Compared to the other treatments, urinary ISP was increased in pigs fed the 90°C SO diet (P = 0.02), while pigs fed the 45°C SO diet had increased urinary TBARS (P = 0.02), with no differences found in urinary FRAP and 8-OH-2dG. Dietary lipid peroxidation had no effect on serum TBARS (P = 0.51); however, pigs fed the 90°C SO diet had significantly higher serum PC (P = 0.01) compared to the other SO diets. In addition, pigs fed the 90°C and 180°C SO diets had significantly lower serum GPx (P = 0.01) in comparison to the 22.5°C and 45°C SO diets. The presence of peroxidation compounds as measured by PV and AnV found in the 90°C SO diet were shown to decrease performance by reducing ADG and metabolically inducing oxidative stress by increasing urinary ISP and TBARS and increasing serum PC while diminishing serum GPx.

M. F. Overholt, G. D. Kim, S. C. Lindblom, B. J. Kerr, D. D. Boler, A. C. Dilger (2017) Growth performance, carcass characteristics, and shelf-life of loin chops of finishing pigs fed peroxidized soybean oil. ASAS-ADSA Midwest Sectional Meeting, Omaha, NE. March 13-15, Abstract #134.

Objectives were to determine the effects of feeding peroxidized soybean oil (SO) to finishing pigs on growth performance, carcass characteristics, and loin chop shelf-life. Individually housed pigs (N = 56; initial BW = 46.7 ± 5.1 kg) were randomly allotted to 1 of 4 diets containing 10% SO treated as follows: 1) unheated (CON), or heated at 2) 45°C for 288 h (45C), 3) 90°C for 72 h (90C) or 4) 180°C for 6 h (180C), each aeriated with 15 L air/min, and fed for 81-d. Data were analyzed as a one-way ANOVA with initial BW used as a covariate for growth performance and carcass characteristics. Feeding 90C reduced ($P \leq 0.03$) ADG compared with pigs fed 45C and 180C by 11.2 and 9.2%, respectively. There was no difference ($P = 0.81$) in ADFI, but G:F of pigs fed 90C was reduced ($P \leq 0.03$) compared with pigs fed 45C and 180C by 8.6 and 6.4%, respectively. Ending BW of pigs fed 90C diet were reduced ($P < 0.01$) compared with pigs fed 45C and 180C by 7.3 and 6.2%, respectively. For ADG, G:F, and ending BW, CON-fed pigs were similar to all other treatments ($P \geq 0.09$). Feeding pigs 90C reduced ($P \leq 0.03$) HCW by 6.3 to 9.0% compared to all other treatments. Livers of 90C-fed pigs were heavier as a proportion of ending BW ($P \leq 0.03$) than those from pigs fed 45C or 180C. Livers of CON-fed pigs were proportionally smaller than ($P \leq 0.01$) those from pigs fed 90C or 180C, but did not differ ($P = 0.15$) from those fed 45C. There was no effect ($P \geq 0.18$) of diet on BF depth or LMA. Clear plate iodine value of 90C-fed pigs was reduced ($P < 0.01$) by 15.1, 14.0, and 11.2 units compared with 45C, CON, and 180C, respectively. Iodine value of 180C-fed pigs was 4.1 units less ($P < 0.01$) than 45C but not different ($P = 0.07$) from CON. There were no diet × storage time interactions ($P \geq 0.44$). Loin chops from 45C-fed pigs had the greatest ($P \leq 0.03$) a*, b*, chroma, and 530/680, but were the most ($P < 0.01$) discolored after 10-d of simulated retail display. In conclusion, feeding SO heated at 180°C reduced growth performance and HCW; whereas, feeding SO heated at 45°C resulted in redder loin chops that discolored more rapidly during simulated retail display.

APPENDIX B

NON-THESIS PEER REVIEWED RESEARCH ARTICLE

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Digestibility of energy and lipids, and oxidative stress in nursery pigs fed commercially available lipids[1]

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ABSTRACT: An experiment was conducted to evaluate the impact of lipid source on GE and ether extract (EE) digestibility, oxidative stress, and gut integrity in nursery pigs fed diets containing 10% of soybean oil (SO), choice white grease (CWG), palm oil (PO), or 2 different distillers corn oils (DCO-1 and DCO-2). Fifty-four barrows weaned at 28-d of age were fed a common starter diet for 7-d, group-fed their respective experimental diets for an additional 7-d, and then moved to metabolism crates and individually fed their respective diets for another 10-d. Following this period, a 4-d total fecal and urine collection period was used to determine apparent total tract digestibility (ATTD) of GE and EE, and to determine the DE and ME content of each lipid source (final BW 11.03 ± 0.51 kg). Following the last day of fecal and urine collection, pigs were given an oral dose of lactulose and mannitol and fed their respective experimental diets to collect urine collected for 12 h. A subsequent urine collection occurred for 5 h later to determine thiobarbituric acid reactive substances (TBARS) and isoprostane (IsoP) concentrations. Following this urine collection, serum was obtained and analyzed for TBARS and endotoxin concentrations. Soybean oil had the greatest ($P < 0.05$) DE (9,388 kcal/kg) content compared to DCO1, DCO2, CWG, PO, and SO (8,001, 8,052, 8,531, 8,293, and 9,388 kcal/kg lipid, respectively). Energy digestibility was greatest for SO compared with the other lipid sources ($P < 0.05$). The ATTD of EE averaged 85.0% and varied slightly (84.4% to 85.6%) among treatments. Differences in ME content among lipids were similar to those reported for DE, with ME values for DCO1, DCO2, CWG, PO, and SO being 7,921, 7,955, 8,535, 8,350, and 9,408 kcal/kg lipid, respectively. Metabolizable energy as a percentage of DE did not differ among lipid sources. Pigs fed lipid diets had greater ($P < 0.05$) serum TBARS compared with pigs fed the control diet, but no

differences were observed in urinary TBARS excretion among the lipid treatments. Urinary IsoP excretion differed among treatments ($P < 0.01$), but was highly variable (34.0 to 104.6 pg). However, no differences were observed among treatments for urinary lactulose:mannitol ratio and serum endotoxin. These results indicate that DE and ME content of SO are greater than all other lipid sources, but feeding these lipids have no effect on gut integrity while producing variable effects on oxidative stress.

Key words: digestibility, distillers corn oil, energy, oxidative stress, pigs

INTRODUCTION

Lipids provide a concentrated source of energy, reduce dust, supply essential fatty acids (**FA**), and improve diet palatability (Pettigrew and Moser, 1991; Azain, 2001; Lin et al., 2013). Lipids are not only variable in FA composition, but lipids such as distiller's corn oil (**DCO**) and by-products from the vegetable oil industry are also available to the livestock industry (Kerr et al., 2015). Lipids are highly digestible (Li et al., 1990; Jorgensen and Fernandez, 2000; Mendoza and van Heugten, 2014) with the apparent total tract digestibility (**ATTD**) of lipids in nursery pigs increasing with age, more so with animal fats compared with vegetable oils (Cera et al., 1988a,b; 1989; 1990). The main factors affecting lipid digestibility and energy value are their FA profile and FFA content (Powles et al., 1995; Wiseman et al., 1998). The FA profile of a lipid and its peroxidation status may also affect intestinal integrity (Mani et al., 2013; Rosero et al., 2015b). Furthermore, lipid source may affect oxidative stress in an animal because of its FA profile (Mani et al., 2013; Liu et al., 2014b) or degree of peroxidation (Liu et al., 2014b; Hanson et al., 2016), creating a potential imbalance between free radical production and antioxidant capacity (Lykkesfeld and Svendsen, 2007; Ho et al., 2013; Kalyanaraman, 2013). Serum endotoxin concentration (Mani et al., 2013) and urinary lactulose to mannitol ratio (Liu et al., 2014b) have been used to measure changes in intestinal integrity, while common measures of lipid-induced oxidative stress include thiobarbituric acid reactive substances (**TBARS**) and isoprostanes (**IsoP**) concentrations (Montuschi et al., 2004; Del Rio, et al., 2005). Because data is lacking on the caloric value of DCO and the impact of lipid source on oxidative stress in young pigs, an experiment was conducted

evaluating the effect of lipid source on ether extract (**EE**) digestibility, intestinal integrity, and oxidative stress status in young pigs.

MATERIALS AND METHODS

All procedures were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Dietary Treatments

Five sources of lipids were obtained, including DCO with approximately 5% FFA (**DCO-1**), distillers corn oil with approximately 10% FFA (**DCO-2**), choice white grease (**CWG**), palm oil (**PO**), and soybean oil (**SO**). Each sample was analyzed for several composition and quality measures at 2 different commercial laboratories as listed in Table 1. A basal diet (Table 2) was formulated to contain 1.30% standardized ileal digestible Lys, with AA ratios, ME, and mineral content adequate for 11 kg pigs according to the NRC (2012). Dietary treatments consisted of a control diet (100% basal diet) and 5 test diets consisting of 90% basal plus 10% of 1 of the 5 lipid sources. Throughout the experiment, all diets were fed in meal form and pigs had access to water from a nipple drinker at all times.

General

Fifty-four weanling barrows were obtained from a commercial farm at weaning (28-d of age), and transported and housed at the Iowa State University Swine Nutrition Farm. For the first 7-d (d 1 to 7), pigs were housed in pens and fed a common starter diet to optimize feed intake during the weaning transition period. For the next 7-d (d 8 to 14), pigs were randomly allotted to 1 of the 6 dietary treatments and fed their respective

experimental diets in groups of 9 pigs to adapt to dietary treatments and to optimize feed intake during the subsequent feeding period. For the next 10-d (d 15 to 24) pigs were moved to individual metabolism crates for continued adaptation to diets, metabolism crates, and the twice daily feeding regimen of 225 g at 0700 h and at 1700 h. After this adaptation period, pigs remained on their respective experimental diets for a 4-d total fecal and urine collection period (d 25 to 29). During the collection period, urine was collected twice daily into a plastic bucket containing 15 mL of 6 *N* HCl and stored at 0°C until the end of the collection period. At the end of the collection period, urine was thawed and weighed, and a subsample was collected and stored at 0°C until subsequent analysis. Feces were also collected twice daily during the collection period and stored at 0°C. At the end of the collection period, feces were dried at 70°C for 48 h, weighed, ground through a 2-mm screen, and a subsample from each pig was collected for digestibility analysis.

On the evening of d 29 following a 12-h fast, each pig was orally administered a 10 mL deionized water solution containing 2.267 g of lactulose and 0.416 g of mannitol (Spectrum Chemical, Gardena, CA). After administration of the sugar solution, pigs were fed their respective experimental diets, and urine was collected for the next 12-h (overnight), quantified, subsampled, and stored at -20^{o}C for analysis. Immediately following this collection, urine was collected for an additional 5 h, quantified, and subsequently analyzed for TBARS and IsoP concentration. Following this urine collection, approximately 8 mL of blood was obtained via jugular venipuncture using a 10-mL vacuum serum tube (BD Diagnostics, Franklin Lakes, NJ). Blood samples were centrifuged at 2,500 × g for 15 min at 4^{o}C and serum was harvested. Serum samples were

immediately frozen and stored at -80^{o}C until analysis. Body weight of individual pigs was obtained at the end of the experiment.

Calculations and Methodologies

The GE content of the basal diet, lipid sources, test diets, feces, and urine was determined using an isoperibol bomb calorimeter (model 1281, Parr Instrument Co., Moline IL), with benzoic acid used as a standard. Duplicate analyses were performed on all diets, lipids, and fecal samples from each pig, whereas triplicate analyses were performed on urine from each pig. For urine, 1 mL of filtered urine was added to 0.5 g of dried cellulose and dried at 50°C for 24 h. Urine addition and subsequent drying was repeated 3 times, for a total of 3 mL, over a 72-h period before urinary GE determination. Gross energy in cellulose was also determined, and urinary GE was calculated by subtracting the GE content of cellulose from the GE content of samples containing both urine and cellulose. Gross energy intake was calculated by multiplying the GE value of the diet fed by feed intake over the 4-d collection period. Apparent DE values were calculated by subtracting fecal GE from feed intake GE, and apparent ME values were calculated by subtracting urinary GE from apparent DE. The apparent DE and ME content of the lipid sources were estimated by difference from the basal diet as described by Adeola (2001).

For EE digestibility, the basal diet, test diets, and feces were analyzed for EE based on methodology described by Luthria et al. (2004) using an accelerated solvent extraction system (model 350, Dionex, Bannockburn, IL) and 10 mL stainless steel cells to accomplish the lipid extraction of the samples. In brief, samples were mixed with sand (Fisher #S23-3) to prevent compaction, which were then loaded into the cell and any

remaining space was filled with additional sand. The cell was then loaded onto the extraction system and extracted 3 times under elevated temperature (120°C) and pressure using petroleum ether as the extraction solvent. The petroleum ether was collected into a pre-weighed glass vial which was placed in an evaporation system (Multivap Model 118, Organomation Associates, Berlin, MA) to remove the petroleum ether (temperature = 65°C, 5 psi of air) and provide an extract from the sample. The vial was re-weighed and the residual EE was determined. Similar to the calculations for energy, ATTD of EE of each lipid source was calculated by subtracting the EE contributed by the basal diet from the EE of the test diet containing a specific lipid source, and the result was divided by the inclusion rate of the lipid source in the diet (i.e. 10%). Digestibility coefficients were determined by dividing grams of component digested by the grams of component consumed and results are reported on a percentage basis.

Serum and urine TBARS concentrations were determined using a TBARS assay kit (TCA Method, Cayman Chemical Company, Ann Arbor, MI). Serum endotoxin was measured using the end point fluorescent assay using the recombinant factor C system (PyroGene Recombinant Factor C Assay, Lonza, Switzerland). Urinary IsoP concentrations were measured using an IsoP kit (8-Isoprostane EIA Kit, Cayman Chemical, Ann Arbor, MI). Lactulose and mannitol concentrations in urine were determined by HPLC and the ratio of lactulose:mannitol was used as an *in vivo* indicator of small intestinal permeability according to the method described by Kansagra et al. (2003).

Statistical Analysis

Data from pigs fed the basal diet were not included in the statistical analysis when comparing differences among the lipid sources, but are reported to provide a reference for pigs fed this type of basal diet in future experiments. Data were analyzed as a completely randomized design with the individual pig as the experimental unit, 9 replications per treatment, using Proc GLM (SAS, 2009) with means reported and separated using LSMEANS. Differences were considered significant at $P \leq 0.05$ and trends were noted at $P \leq 0.10$.

RESULTS AND DISCUSSION

Corn Oil and Diet Composition

The FA profile of CWG, SO, and PO sources evaluated in this study were similar to published values (O'Brien, 2009; NRC, 2012) as shown in Table 1. The 2 DCO samples had FA profiles that resembled refined corn oil (NRC, 2012), and similar to those from recently published data evaluating DCO (Moreau et al., 2011; Winkler-Moser and Breyer, 2011; Kerr et al., 2016). As expected, the FFA content of the 2 refined oils (PO and SO) were low compared to that for CWG and the 2 DCO samples, with the 2 DCO samples having a similar FFA content to those reported previously (Moreau et al., 2010; Kerr et al., 2016). Each lipid source was also analyzed for moisture (**M**), insoluble (**I**), and unsaponifiables (**U**) because of research suggesting that MIU should be considered for improving the accuracy of predicting DE and ME from various lipid sources (Wiseman et al., 1992; Blanch et al., 1996; Vila and Garcia, 1996a,b; Rosero et al., 2015a).

Total tocopherol and carotenoid concentrations are highly variable among corn co-products (Majoni and Wang, 2010; Moreau et al., 2011; Winkler-Moser and Breyer, 2011), both of which have been shown to reduce inflammation and oxidative stress (Singh et al., 2005; Yeum et al., 2009; Kaulmann, 2014). Because the 2 DCO sources were unrefined, we expected them to contain tocopherols. As shown in Table 1, the 2 DCO sources contained moderate amounts of total tocopherols (averaging 678 mg/kg) with γ-tocopherol being the predominant form. These concentrations are greater than those reported by Moreau et al. (2011), but are within the range reported by Firestone (2006), and reflect the relative tocopherol profile reported for lipids extracted from corn distillers dried grains with solubles and other plant sources (Jiang et al., 2001; Moreau et al., 2011). The refined SO and PO sources contained moderate (1,083 mg/kg) and low amounts of total tocopherols (67 mg/kg), respectively, and are also within reported ranges reported by Firestone (2006). The CWG sample contained 253 mg/kg of total tocopherols, which would be expected assuming the pigs had previously been fed distillers dried grains with solubles and diets containing supplemental vitamin E. Xanthophylls (e.g., lutein, zeaxanthin, and β-cryptoxanthin) are oxygenated carotenoids present mainly in the horny endosperm of the corn kernel, with the total xanthophyll content in whole corn being approximately 20 mg/kg (Moros et al., 2002). While the majority of the xanthophyll in whole corn is lutein (Moros et al., 2002), Moreau et al. (2010) recently reported that the carotenoids in distillers corn oil were more evenly distributed among these 3 xanthophylls, with an average total content of approximately 330 mg/kg of oil. In the current study, the 2 DCO samples contained an average of 133.5

mg xanthophylls/kg oil, while PO, SO, and CWG contained no detectable concentrations of xanthophylls.

Lipid peroxidation is a very complex and poorly understood chain of events (Liu et al., 2014a, Kerr et al., 2015; Wang et al., 2016), and the impact of their consumption in animals on performance, and on digestive and metabolic functions is likewise poorly understood. In a review of swine (n = 16 comparisons) and broilers (n = 26 comparisons) studies comparing growth performance differences between pigs or broilers fed diets with and without peroxidized lipids, average reductions in ADG (11%), ADFI (7%), G:F (4%), serum vitamin E (46%) occurred by feeding peroxidized lipids, and serum TBARS concentrations were 120% relative to animals fed unperoxidized lipids, indicating that feeding peroxidized lipids negatively affects pig and broiler growth performance and oxidative stress (Shurson et al., 2015). Although peroxide value (**PV**) and TBARS have been widely used to assess lipid peroxidation and oxidative stress (Shurson et al, 2015), these measures have limited value because of their inherent specificity of measuring only selected peroxidation compounds and the fact that many peroxidation products produced are also decomposed over time (Shurson et al., 2015). As a result, recent research suggests that additional measures need to be used to assess the potential association and causality of feeding peroxidized lipids on phenotypic responses (Liu et al., 2014b; Hanson et al., 2016), and should focus using 4-hydoxynonenal or a ratio of specific aldehydes for more accurate assessment of lipid peroxidation in vegetable oils used to fry foods (Wang et al., 2016). Consequently, we chose to use several peroxidation indicators and predictor measures to analyze the lipids used in this study beyond PV and TBARS, even though the amount of thermal processing of these lipids was well below that of that

used in fried foods. As shown in Table 1, the concentrations of most peroxidation measures were low, which was expected given that the temperature and length of heating was low compared to lipids obtained from the restaurant frying-industry. In general, it appeared that the 2 DCO samples and CWG had greater anisidine values, PO had the greatest oil stability index, and CWG had the greatest concentrations of polar compounds, all of which were expected based on their extent of refinement or FA profile. When the lipid sources were added to experimental diets, the analyzed GE and EE content was similar to the calculated diet concentrations (Table 3).

Energy and Lipid Digestibility

Data typically associated with growth performance-type studies (ADG, ADFI, and G:F) were not measured because this experiment was designed as an energy balance trial. It should be noted, however, that dietary treatment had no effect on final BW (11.13 ± 0.17 kg, $P = 0.19$) when pigs were removed from the metabolism crates at the end of the trial. During the experiment, 1 pig fed the DCO-2 diet and 2 pigs fed the SO diet died. As a result, data reported are based on 7 observations for SO, 8 observations for DCO-2, and 9 observations for all other treatments. The DE for SO (9,388 kcal/kg) was greater ($P < 0.05$) compared with all other lipids, but no differences in DE content were observed among the other lipid sources (DCO-1, 8,001 kcal/kg; DCO-2, 8,052 kcal/kg; CWG, 8,531 kcal/kg; PO, 8,293 kcal/kg). Similarly, DE as a percentage of GE, was greatest for SO ($P < 0.05$), but was not different among the other lipid sources. Metabolizable energy content as a percentage of DE did not differ between lipid sources (average of 99.7%), resulting in a similar trend in responses for ME as observed for DE, where ME content of DCO-1, DCO-2, CWG, PO, and SO was 7,921, 7,955, 8,535, 8,350, and 9,408 kcal/kg

lipid, respectively. Apparent EE digestibility was greatest for DCO-2 and CWG, and lowest for DCO-1 and PO, with the SO being intermediate ($P < 0.01$), and averaged 85.04% among sources (Table 4).

The DE content, DE as a percentage of GE, and ME content determined for SO in the current experiment were substantially higher than reported previously (Cera et al., 1990; Jorgensen and Fernandez, 2000; Kerr et al., 2009; NRC, 2012). We have no explanation for this difference, but it is interesting to note that the ATTD of EE in SO was similar to previously determined values (Cera et al., 1990; Jorgensen and Fernandez, 2000), although lower than that for acid hydrolyzed-EE digestibility reported by Kil et al., (2011). Our DE and ME values for CWG were similar to those reported for lard (Kerr et al., 2009), while our ME value for PO was slightly greater that that reported by Jorgensen and Fernandez (2000). The NRC (2012) provides no GE, DE, or ME estimates for PO, which is surprising given that PO is the most abundant and widely used lipid in the world (USDA, 2016). If the DE and ME values of these lipid sources are adjusted to a 0% MIU basis as suggested by Rosero et al. (2015a), the 2 DCO sources evaluated in the current experiment averaged 8,225 kcal/kg of DE and 8,135 kcal/kg of ME with an ATTD of EE of 85.10%. All of these values are substantially less than the 8,651 kcal/kg of DE, 8,584 kcal/kg of ME, and ATTD of EE of 93.54% for 3 DCO products reported by Kerr et al. (2016). This was surprising given that similar experimental methods (basal diet composition, length of diet adaptation, diet inclusion level of lipids, and general management) were used in the 2 experiments. It is worthy to note, however, that in the current experiment, pigs had slightly lower BW (11.46 vs. 15.53 kg, respectively) and consumed less feed (427 vs. 500 g/d, respectively) compared to pigs used in a previous

experiment (Kerr et al., 2016). Given the differences in BW and ADFI, however, pigs fed the basal diet had similar DE, ME, and ATTD of EE between the 2 experiments.

Energy values of lipids have been predicted based upon the age of the pig, and the unsaturated FA:saturated FA ratio and FFA concentration of the lipids fed (Wiseman et al., 1998). Using the analyzed composition of the lipids evaluated in the current study, the predicted DE of DCO-1, DCO-2, CWG, PO, and SO was 8,893, 8,827, 8,070, 7,892, and 8,931, respectively, compared to the *in vivo* determined values of 8,001, 8,052, 8,531, 8,293, and 9,388, respectively. These results indicate large discrepancies between actual and predicted DE, where the predicted values for the DCO sources were overestimated while the predicted values for CWG, PO, and SO were underestimated. Reasons for the lack of accuracy of using these prediction equations to estimate DE are unclear, and warrant further investigation.

Oxidative Stress Markers

An imbalance between free radical production and antioxidant capacity is the basis for characterizing oxidative stress (Lykkesfeld and Svendsen, 2007; Ho et al., 2013; Kalyanaraman, 2013). Because both FA profile (Mani et al., 2013; Liu et al., 2014a) and the extent of lipid peroxidation (Liu et al., 2014a; Hanson et al., 2016) may affect free radical production and antioxidant status, we chose to measure serum and urinary TBARS and urinary IsoP, the 2 most common measures reported for assessing lipid-induced oxidative stress (Montuschi et al., 2004; Del Rio, et al., 2005). No differences were noted among lipid sources for serum TBARS, but there was a numerical trend for pigs fed the unrefined lipid sources (DCO-1, DCO-2, and CWG; average of 1,882 μM) to have a greater urinary TBARS excretion than pigs fed PO or SO (average of 1,414 μM; *P*

< 0.10). Urinary IsoP was greatest for pigs fed PO and lowest for pigs fed SO and DCO-2, with pigs fed the other lipids being intermediate ($P < 0.05$; Table 5). Unfortunately, these data provide no clear indication of the impact of feeding these lipid sources to young pigs on oxidative stress relative to their FA profile or extent of peroxidation. However, this experiment was not specifically designed to independently determine the impact of FA profile or peroxidation status of lipids on oxidative stress because these factors are confounded within the lipid sources evaluated.

Intestinal Barrier Function

The ratio of lactulose to mannitol in urine is commonly used as an *in vivo* indicator of small intestinal permeability. The FA profile of a lipid has been shown to affect various markers of gastrointestinal permeability (Laugerette et al., 2012; Mani et al., 2013; Liu et al., 2014b; Lam et al., 2015). In general, the consumption of more saturated lipids increases gastrointestinal permeability as measured by inflammatory cytokines and chemokines (Laugerette et al., 2012), increases the ileal apparent permeability coefficient and serum endotoxin concentration (Mani et al., 2013), increases in the lactulose to mannitol ratio (Liu et al., 2014b), and decreases transepithelial resistance (Lam et al., 2015). In the current study, there were no differences in the urinary lactulose to mannitol ratio and serum endotoxin concentrations among the 5 lipid treatments (Table 5). We have no explanation for this lack of an effect, but the lactulose:mannitol ratios in the current experiment were all substantially greater than those reported by Liu et al. (2014b). This is not, however, supported by the serum endotoxin levels which were substantially lower than that reported by Liu et al., (2014b).

In summary, the DE content of SO (9,388 kcal/kg) was greater than the DE content of all other lipid sources tested, where the 2 DCO samples averaged 8,028 kcal/kg (86% of the DE content in SO), 94% of the DE content of CWG (8,531 kcal/kg), and 97% of the DE content of PO (8,293 kcal/kg) when fed to nursery pigs. Although there were no differences noted among the lipid sources for serum TBARS, there was a trend for pigs fed the unrefined lipid sources (DCO-1, DCO-2, and CWG) to have a greater urinary TBARS excretion than pigs fed PO or SO. Even though urinary IsoP excretion differed among the lipid sources, they were highly variable and interpretation of their biological significance, if any, is not clear. Measures of gut permeability as measured by serum endotoxin and urinary lactulose:mannitol ratio did not differ among the lipids evaluated, and were similar to serum and urinary TBARS, but were highly variable. In conclusion, these data indicate that the DE and ME content of SO was greater than that of the other lipid sources and lipid composition appears to have inconsistent effects on gut integrity and measures of oxidative stress.

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Table 1. Composition of lipid samples[1]

Item	DCO-1	DCO-2	CWG	PO	SO
Crude fat, %[2]	98.74	98.22	98.32	98.57	98.45
FFA, %[2]	4.5	10.0	13.4	0.07	0.04
FA, % of total fat[2,3]					
Capric (10:0)	ND	ND	0.07	ND	ND
Lauric (12:0)	ND	ND	ND	0.22	ND
Myristic (14:0)	ND	ND	1.28	0.99	ND
Pentadecanoic (15:0)	ND	ND	ND	0.04	ND
Palmitic (16:0)	12.86	12.88	23.25	43.41	10.74
Palmitoleic (9c-16:1)	0.10	0.10	2.44	0.15	0.08
Margaric (17:0)	ND	ND	0.33	0.10	0.09
Margaroleic (17:1)	ND	ND	0.23	ND	ND
Stearic (18:0)	1.76	1.73	12.54	4.38	4.20
Oleic (9c-18:1)	26.95	26.56	41.38	39.90	23.08
Linoleic (18:2n6)	55.88	56.50	16.52	9.85	53.19
Linolenic (18:3n3)	1.26	1.26	0.55	0.22	7.28
Nonadecenoic (19:1)	0.10	ND	ND	ND	0.31
Arachidic (20:0)	0.39	0.38	0.19	0.37	0.33
Gadoleic (20:1)	0.28	0.25	0.80	0.14	0.20
Eicosadienoic (20:2)	ND	ND	0.74	ND	ND
Homo-y linoleic (20:3)	ND	ND	0.11	ND	ND
Arachidonic (20:4)	ND	ND	0.30	ND	ND
Behenoic (22:0)	0.12	0.14	ND	0.07	0.35
Docosatrienoic (22:3)	ND	ND	0.14	ND	ND
Docosatetraenoic (22:4)	0.12	ND	ND	ND	ND
Docosapentaenoic (22:5)	0.18	0.19	ND	ND	ND
Other Fatty Acids	ND	ND	ND	0.15	0.16
Moisture, %[2]	0.68	0.54	0.24	0.02	0.02
Insolubles, %[2]	0.18	0.04	0.22	0.02	0.02
Unsaponifiables, %[2]	1.53	1.86	0.63	0.21	0.33
Free glycerin, %[2]	0.85	0.53	0.58	0.74	0.31
Total tocopherols, mg/kg[2]	730	626	253	67	1,083
Alpha	51	62	50	67	77
Beta	15	15	< 10	< 10	< 10
Delta	29	15	< 10	< 10	189
Gamma	635	534	203	< 10	817
Xanthophylls, mg/kg[2]	92	175	< 1	< 1	< 1
Anisidine value[2,4]	30.76	21.47	23.26	11.22	5.87
2,4-decadienal, mg/kg[5]	26.4	ND	17.6	ND	6.2
Hexanal, μg/g[5]	ND	ND	14.7	ND	ND

OSI @ 110°C, hr[2]	5.15	10.75	4.15	30.05	6.35
Oxidized fatty acids, %[2]	1.6	0.9	2.2	1.2	1.4
Peroxide value, Meq/kg[2]	1.4	0.4	0.4	1.2	1.6
Polar compounds, %[2]	9.38	9.55	20.53	7.40	3.46
TBA value[2,4]	0.04	0.03	0.03	0.01	0.06

[1] Abbreviations: DCO, distillers corn oil, CWG, choice white grease; PO, palm oil; SO, soybean oil; Not detected or below detection limit.

[2] Analyzed by Barrow-Agee, Memphis, TN.

[3] No other FA were detected besides those listed. Not detected = ND.

[4] There are no units for anisidine value or TBA value.

[5] Analyzed by Kemin Industries, Des Moines, IA.

Table 2. Ingredient and calculated composition of basal diets, as-is basis

Item, %	Percent
Corn	60.82
Soybean meal	17.96
Whey, dried	10.00
Fish meal	5.00
Soy protein concentrate	2.50
Plasma protein, porcine	1.25
Calcium carbonate	1.00
Monocalcium phosphate	0.19
Sodium chloride	0.35
Vitamin mix[1]	0.20
Trace mineral mix[2]	0.20
L-Lys·HCl	0.34
DL-Met	0.11
L-Thr	0.08
TOTAL	100.00
Calculated composition, % unless otherwise noted	
ME, kcal/kg	3,335
CP	20.94
Lys, digestible	1.30
TSAA:Lys	0.550
Thr:Lys	0.585
Trp:Lys	0.165
Ile:Lys	0.515
Val:Lys	0.640
Calcium	0.75
Phosphorus[3]	0.36

[1]Provided the following per kilogram of diet: vitamin A, 6,125 IU; vitamin D_3, 700 IU; vitamin E, 50 IU; vitamin K, 30 mg; vitamin B_{12}, 0.05 mg; riboflavin, 11 mg; niacin, 56 mg; and pantothenic acid, 27 mg.

[2]Provided the following per kilogram of diet: Cu (as $CuSO_4$), 22 mg; Fe (as $FeSO_4$), 220 mg; I (as $Ca(IO_3)_2$), 0.4 mg; Mn (as $MnSO_4$), 52 mg; Zn (as $ZnSO_4$), 220 mg; and Se (Na_2SeO_3), 0.4 mg.

[3]Standardized total tract digestible.

Table 3**.** Analyzed composition of experimental diets, as-is basis

		Lipid treatment, %[1]				
Criterion	Basal	DCO-1	DCO-2	CWG	PO	SO
GE, kcal/kg	3,827	4,325	4,333	4,344	4,324	4,345
EE, %	3.35	12.50	12.87	12.74	12.14	11.81
DM, %	91.75	91.99	92.33	92.32	92.03	92.37

[1] Lipid treatment diets contained 90% basal and 10% added oil.
Abbreviations: DCO, distillers corn oil, CWG, choice white grease; PO, palm oil; SO, soybean oil.

Table 4. Energy and lipid digestibility, and energy values in nursery pigs fed different lipid sources, as-is basis

	Lipid treatment[1]					Statistics[3]	
Criterion	DCO-1	DCO-2	CWG	PO	SO	SE	P value
Final BW, kg[2]	10.88	10.56	11.76	11.35	10.61	0.41	0.19
GE, kcal/kg	9,392	9,395	9,365	9,419	9,419	-	-
DE, kcal/kg	8,001^{b}	8,052^{b}	8,531^{b}	8,293^{b}	9,388^{a}	223	0.01
DE, % of GE	85.19^{b}	85.70^{b}	91.10^{b}	88.04^{b}	99.68^{a}	2.38	0.01
EE digestibility, %	84.62^{b}	85.57^{a}	85.50^{a}	84.44^{b}	85.08^{ab}	0.23	0.01
ME, kcal/kg	7,921^{b}	7,955^{b}	8,535^{b}	8,350^{b}	9,408^{a}	255	0.01
ME, % of DE	98.75	98.77	100.09	100.63	100.22	0.85	0.36

[1]Abbreviations: DCO, distillers corn oil, CWG, choice white grease; PO, palm oil; SO, soybean oil

[2] Diets were imposed on pigs 1 wk post-weaning after which they were fed for 17 d prior to a 4-d total feces and urine collection period. There were 9 individually fed pigs per dietary treatment. Feed intake during the collection period did not differ among pigs fed the lipid treatments, averaging 405 g/head/d, provided in 2 equally spaced feedings. Pigs fed the basal diet consumed an average of 427 g/d with a final BW of 11.46 kg, with the basal diet analyzed to contain 3,827 kcal GE/kg, 3,355 kcal DE/kg, and 3,265 kcal ME/kg; with an apparent total tract digestibility of EE of 50.63%.

[3] Superscripts reflect corn oil treatment differences (abc, $P \leq 0.05$).

Table 5. Oxidative stress and gut integrity measures in nursery pigs fed different lipid sources

	Lipid treatment[1]					Statistics[3]	
Criterion	DCO-1	DCO-2	CWG	PO	SO	SE	P value
Serum[2]							
TBARS, μM/mL	9.68	8.26	8.64	9.74	9.29	0.74	0.54
Endotoxin, EU/ml	320	268	286	211	343	40.9	0.21
Urine[2]							
TBARS, μM	1,948^{xy}	2,133^{x}	1,564^{yz}	1,424^{z}	1,404^{z}	304	0.06
ISP, pg	77.0^{ab}	41.5^{c}	44.4^{bc}	104.6^{a}	34.0^{c}	11.58	0.01
L:M ratio	1.3	1.9	1.7	1.0	1.0	0.31	0.17

[1] Abbreviations: DCO, distillers corn oil, CWG, choice white grease; PO, palm oil; SO, soybean oil; TBARS, thiobarbituric acid reactive substances; IsoP, isoprostane; L:M, lactulose:mannitol.

[2] Serum obtained after a 17 h fast. Urine collected and quantitated for 5 h following a 12 h fast. Pigs fed the basal diet had a serum TBARS concentration of 6.57 μM/ml, serum endotoxin concentration of 242.4 endotoxin units (EU)/ml; excreted 1,988 μM of TBARS and 95.5 pg of isoprostane in the urine during the 5 h urine collection period, and a urinary lactulose:mannitol ratio of 1.66. There were 9 individually fed pigs per dietary treatment.

[3] Superscripts reflect corn oil treatment differences (abc, $P \leq 0.05$; xyz, $P \leq 0.10$).