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Effects of Feeding Distillers Grains Throughout Different Phases of Production on Shelf Life of Ground Beef

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EFFECTS OF FEEDING DISTILLERS GRAINS THROUGHOUT DIFFERENT PHASES OF PRODUCTION ON SHELF LIFE OF GROUND BEEF

By

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A THESIS

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These studies analyzed the effects of feeding distillers grains on the shelf life of cooked and raw ground beef, and how the addition of postmortem or dietary antioxidants impact shelf life.

For study one, cattle were assigned to backgrounding diets containing low or high concentrations of wet distillers grains (WDGS) and either corn gluten feed or modified wet distillers grains (MDGS) during finishing. For study two, cattle were fed one of five finishing diets; corn, wet distillers grains (WDGS), WDGS + vitamin E, WDGS + Ethoxyquin/TBHQ, or WDGS + vitamin E + Ethoxyquin/TBHQ.

Shoulder clods from each dietary treatment were ground and lean, fat, and composite samples were taken for fatty acid analysis. Patties were overwrapped for retail display and analyzed for lipid oxidation, discoloration and objective color. Cooked beef links were manufactured with salt and phosphate. Links from the first study contained different concentrations of rosemary and green tea extract. Beef was stuffed into links, cooked, and lipid oxidation was measured throughout refrigerated or frozen storage.
For study one, there were no differences in lipid oxidation of patties, yet ground beef from heifers finished with MDGS discolored faster than beef from cattle finished on corn gluten feed. Increased lipid oxidation in beef links occurred when cattle were fed distillers grains during backgrounding or finishing but antioxidants reduced lipid oxidation similarly regardless of concentration.

For study two, ground beef patties showed no dietary differences for instrumental color, only increased percent discoloration over time. All ground beef TBARS increased over time, and supplementation of vitamin E resulted in lower TBARS values than corn after 2 d of retail display. An increase in PUFA and C18:2 was observed in lean and composite fatty acids in WDGS versus corn-finished cattle.

Both dietary and postmortem antioxidants were effective at extending shelf life in ground beef from cattle fed distillers grains. Additionally, the effect of feeding distillers grains on shelf life varies depending on when distillers grains was supplemented.
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1. Introduction

The fermentation of grain has been available to the animal feed industry since the 19\textsuperscript{th} century, where dried distillers grains plus solubles were being fed to cattle (Klopfenstein et al., 2008). Approximately one third of each bushel of grain used for fuel ethanol production is made into distillers grains, of which 43\% is utilized as feed by the beef cattle sector (RFA, 2010). Some of the fat in distillers grains is protected from rumen biohydrogenation, resulting in an increased concentration of unsaturated fatty acids absorbed into the lean and fat tissues, particularly in the phospholipid membranes (Gill et al., 2008; Koger et al., 2010; Vander Pol et al., 2009). Due to the increase in polyunsaturated fatty acids, meat from animals fed distillers grains tend to have reduced shelf life, both from a lipid and myoglobin oxidation perspective (Gill et al., 2008). Further processing often has a detrimental impact on shelf life that could magnify the reduction in shelf life in beef from cattle fed distillers grains. For example, grinding of meat products disrupt the phospholipid membranes and allows increased exposure to oxygen, hence increasing the product susceptibility to lipid oxidation. Additionally, heating allows the phospholipids to become more exposed and vulnerable to oxygen and free radicals, and has been shown to increase thiobarbituric acid reactive substances (TBARS) values, a measure of lipid oxidation, up to four-fold (McCarthy et al., 2001).

Both dietary and postmortem antioxidants can be used to compensate for the negative effects of diet and processing. The phenolic compounds of natural plant extracts, such as rosemary and green tea, act as a hydrogen donor to stabilize free radicals, in a manner functionally identical to that of synthetic antioxidant, BHA/BHT (McCarthy et al., 2001). The effectiveness of the antioxidant depends on how the antioxidant itself was processed, what concentration is added, the environment it is in, and how the product is processed and stored.
Vitamin E is also known to be a primary antioxidant by releasing a hydrogen atom to quench free radicals (Berges, 1999). Govaris et al. (2004) determined that the antioxidant effect of direct addition of α-tocopherol was inferior compared to dietary supplementation. The antioxidant Ethoxyquin/TBHQ (Agrado Plus, Novus International, St. Louis, MO) is approved for usage in feed rations to reduce oxidation of ingredients with higher fat content, such as supplemented vitamin E (Maddock et al., 2003). Ethoxyquin/TBHQ supplementation does not increase serum Vitamin E values directly, but could indirectly increase vitamin E concentrations over time because Agrado Plus is able to stabilize vitamin E in the feed ration (Choat et al., 2002).

The purposes of these studies were to evaluate the effect of feeding distillers grains on the shelf life and fatty acid composition of both cooked and raw ground beef, as well as the effect of both dietary and postmortem antioxidants on shelf life.
2. **Review of Literature**

2.1 **Distillers Grains**

The fermentation of grain has been available to the animal feed industry since the 19th century, where dried distillers grains plus solubles were being fed to cattle (Klopfenstein et al., 2008). One third of each bushel of grain used for fuel ethanol production is returned to the animal feed industry as distillers grains (RFA, 2010). Several types of distillers grains exist, dependent on the processing technique. The initial step in ethanol production is to remove the starch from the grain and hydrolyze it to dextrin using the enzyme glucoamylase. This dextrin is converted to glucose by a glucoamylase sugar, where yeast converts the glucose to ethanol and CO$_2$ (Davis, 2001). The ethanol is removed and the remaining product is centrifuged. The coarser particles are wet distillers grains (WDG) or may be dried into dried distillers grains (DDG). The drying process allows the coarser particulates to pass through a rotary dryer. The remaining liquid (called thin stillage) goes through an evaporator to remove additional moisture, producing a syrup-consistency product called solubles. These solubles are often added back to DDG or WDG to form DDG/WDG plus solubles (DDGS/WDGS, respectively; Stock et al., 1999). Partially dried WDGS with a moisture percentage of approximately 50 percent are termed modified distillers grains plus solubles (MDGS). Other processes have been identified to remove fiber or oil content of the distillers grains (Berger & Singh, 2010).

In ethanol production, sulfuric acid is used to regulate pH in fermentation, and is added to the grain during steeping, thus contributing to a higher sulfur content to the byproduct. Wet distillers grains has approximately 0.79% sulfur, based on 1200 samples from various Nebraska ethanol plants (Buckner et al., 2008). An upper limit of sulfur in finishing diets at 0.4% is set by the National Research Council to reduce the risk of polioencephalomalacia, which in turn
typically limits the concentrations of DGS fed in the diet to no higher than 50%, depending on the percent sulfur in the batch being used.

Larson et al. (1993) showed an increase in average daily gain in both calves and yearlings fed distillers grains when compared to corn, likely due to a combination of factors, including increased energy utilization, reduced acidosis, and yeast products. This could also be attributed to more unsaturated fatty acids reaching the duodenum, increased fat digestibility, and more propionate production (Vander Pol et al., 2009). Vander Pol et al. (2006) also determined in an earlier study that feeding DGS at 0, 10, 20, 30, 40 and 50 resulted in a quadratic response in average daily gain and feed efficiency, and that feed efficiency was higher in WDGS diets than in corn diets.

2.1.1 Fatty Acid Composition and Lipid Oxidation

Meat from cattle fed distillers grains have increased polyunsaturated fatty acid (PUFA) concentrations when compared to corn based diets, making it more prone to lipid oxidation (Gill et al., 2008; Koger, et al., 2010). Biohydrogenation in the rumen conserves some of the fats, resulting in an increased concentration of unsaturated fatty acids in the small intestines which could later be absorbed into the lean tissue (Vander Pol et al., 2009). Unsaturated fatty acids are converted to saturated fatty acids through an isomerization process in the rumen that forms trans fatty acids intermediates, including conjugated linoleic acid (CLA) (Mello et al., 2012). Therefore, the increase in trans fats seen in beef from cattle fed MDGS may be attributed to the increased digestibility of MDGS fat increasing the initial hydrogenation process of linoleic acid. As the amount of distillers grains fed increased, the total polyunsaturated fatty acids (PUFA), linoleic acid (18:2) and CLA increased in tissue samples (Depenbusch et al., 2009; Mello et al., 2012). To complement this, several studies report a decrease in several monounsaturated fatty
acids (MUFA) and saturated fatty acids (SFA) in cattle supplemented DGS (Depenbusch et al., 2009; Koger et al., 2010; Mello et al., 2012).

Due to the shift in fatty acid composition, meat from animals fed distillers grains tend to have reduced a reduced shelf life, both from a lipid and myoglobin oxidation perspective (Gill et al., 2008). Depenbusch et al. (2009) observed a decrease in redness (a* values) of steaks in display over a 7 day period once the concentration of DGS exceeded 45% of the ration. Koger et al. (2010) saw a decrease in color stability in lower L* values and a* values in beef from cattle fed DGS versus steam flaked corn. Additionally, increased concentrations of DGS (20% to 40% DM) increased TBARS concentrations in ground beef (Koger et al., 2010).

2.2 Lipid Oxidation

Lipids are the most energy dense storage compound at 9 calories per gram (Wong et al., 1989). Therefore, lipids remain an imperative part of the human diet, and maintaining the stability of these lipids is necessary. Lipid oxidation deteriorates food quality and consequently is one of the most intensely researched areas (Ladikos & Lougovois, 1990; Wong et al., 1989). Lipid oxidation most often occurs in unsaturated fatty acids where the higher the degree of unsaturation, the more prone the fatty acid is to oxidation (Schultz & Sinnhuber, 1962). Therefore, greater amounts of polyunsaturated fatty acids tends to lead to less lipid stability. Phospholipid membranes, which only account for approximately 1% of tissue weight, contain large amounts of polyunsaturated fatty acids (Christie, 1978). The most common unsaturated fatty acids found in beef are oleic, linoleic, linolenic and arachidonic. Lipid degradation occurs by one of two ways, enzymatic lipolysis and oxidation. The enzymatic approach utilizes lipases to release free fatty acids from the triglycerides by hydrolyzing the ester bond that attaches the fatty acid to the glycerol backbone (Forrest et al., 2012). The oxidation of lipids is the reaction
with oxygen and unsaturated fatty acids, and is of greater concern than lipase activity in meat products (Wong et al., 1989; Forrest et al., 2012). Autooxidation and photo-sensitized oxidation are two different pathways to initiate the oxidative process (Wong, 1989). Autooxidation is a cyclic reaction involving development of free-radicals.

### 2.2.1 Steps of Lipid Oxidation

The three steps of autooxidation include: initiation, propagation and termination. The initiation stage is when labile hydrogen is abstracted to form a carbon centered alkyl radical in the presence of an initiator such as oxygen, heavy metals or light (Wong et al., 1989; Ladikos & Lougovois, 1990). The free radicals formed in initiation react with oxygen to form peroxy radicals, which react with a second unsaturated fatty acid to form a hydroperoxide and a alkyl radical on the second fatty acid. This is the propagation stage, and is a recurring, cyclical event until the lipid runs out of double bonds to react or outside compounds (such as antioxidants) terminate the process. The rate at which this process occurs is dependent on the degree of unsaturation of the lipid (Wong et al., 1989). During the autooxidation process, primary and secondary products are produced. Primary products are in the form of peroxides, whereas secondary products, primarily aldehydes, ketones, acids, alcohols, carbonyls, esters and hydrocarbons, are formed from the breakdown of primary oxidation products. Primary products of oxidation are reactive with functional groups of amino acids, such as sulfur or amines, and could be a contributor to nutritional loss in food products (Ladikos & Lougovois, 1990). Secondary products of oxidation promote oxidation after binding with the thiols of cysteine, which further allows nutritional loss (Ladikos & Lougovois, 1990).
2.2.2 Flavor and Lipid Oxidation

Hydroperoxides formed during lipid oxidation are unstable, and undergo cleavage at the carbon-carbon bond on either side of the alkoxy radical. These radicals react among others and form volatile compounds (alcohols, carbonyls, esters and hydrocarbons). Some secondary products are responsible for the oxidized flavor in foods (Wong et al., 1989). Hydroperoxides may convert to hydroxy and alkoxy radicals, where cleavage of adjacent fatty acid chains result in volatile compounds that contribute to flavor and aroma (Ladikos & Lougovois, 1990). Warmed over flavor is an off flavor due to secondary products of lipid oxidation that you see in cooked meats, and typically become apparent within 48 h at refrigerated temperatures (Forrest et al., 2012). Other flavors that are used to describe lipid oxidation are: rancid, cardboard flavor, dirty sock and musty (Sato & Hegarty, 1971; Pearson et al., 1977).

Despite this, not all oxidation processes are negative. In certain dry-cured country hams and fermented sausages, the desired end flavor is not achieved until a specific level of hydrolysis and oxidation of the fat occurs (Pearson et al., 1977). Additionally, lipid oxidation immediately prior to cooking may be a source of intermediates that react with other components to contribute to desirable cooked flavor (Enser et al., 1987)

2.2.3 Promoters of Lipid Oxidation

Several factors act as catalysts on the lipid oxidation process, both endogenously and exogenously. For endogenous factors, muscle type and specie differences exist due to the differing concentrations of polyunsaturated fatty acids (Govaris et al., 2004). Lipid concentrations, unsaturation of the fatty acids, and iron content are responsible for the variability between specie (Tang et al., 2001). Metal ions, such as iron, copper, cobalt and calcium cause autooxidation of the reductant (e.g. catalase, reduced glutathione, ascorbic acid) to produce $\text{O}_2^-$, a
superoxi
dec anion (Lawrence et al., 2003; Maestre et al., 2009; Wong et al., 1989). The metals increase the rate at which electron transfer occurs, resulting in increased free radical formation (Ingold, 1962). Furthermore, the oxidation state of the metal will determine the effect as a pro-
ioxidant. In the case of free iron, both ferrous and ferric states of iron can act as pro-oxidants, where ferrous iron would require an oxidant compound (such as oxygen) to initiate peroxidation, and ferric iron would require a reducing agent to initiate peroxidation (Braughler et al., 1986). The ferrous state has been shown to have more pro-oxidant activity than ferric, particularly since oxidants are more readily available than reductants to initiate the oxidation process (Pearson et al., 1977; Braughler et al., 1986).

For exogenous factors, simply eliminating exposure to oxygen through packaging techniques will reduce lipid oxidation (Tang et al., 2001). Furthermore, lipid oxidation is accelerated during cooking due to the release of free and heme iron from myoglobin, as well as disruption of cell structure and antioxidant enzymes (Min et al., 2008). Non-heme iron is considered most responsible for lipid oxidation in cooked meats, since a portion of the heme iron is destroyed during cooking (Han et al., 1993). Min et al. (2008) determined that free ionic iron and ferric iron reducing capacity were large contributors to lipid oxidation in cooked meats. Additionally, meats with increased concentrations of heme pigment (beef, for example), produce more hydrogen peroxide during myoglobin oxidation, where the secondary products of this reaction can initiate lipid oxidation (Min et al., 2008). Higher temperatures observed during cooking allow the phospholipids to become more exposed and vulnerable to oxygen and free radicals, and have been shown to increase thiobarbituric acid reactive substances (TBARS) values up to four-fold (McCarthy et al., 2001). However, Huang and Greene (1978) and Roux et al. (2014) found that meat heated at lower temperatures and/or short periods of time had higher
TBARS values than those cooked for a longer period of time at higher temperatures. They determined that this may have resulted from antioxidant substances with reducing properties that form from browning reactions. Grinding of meat products disrupt the phospholipid membranes and allows increased exposure to oxygen, thus increasing the rate of lipid oxidation significantly (Sato et al., 1971). Furthermore, salt contains metallic elements that would act as prooxidants (Wong, 1989).

2.2.4 Measurement of Lipid Oxidation in Meat

Measurement of lipid oxidation can be conducted by analyzing for primary or secondary oxidation products. This is dependent on several factors, including the predicted stage of oxidation, processing or storage conditions, or the correlation to sensory analysis. The 2-thiobarbituric acid (TBA) test are the most widely used in meat, since TBARS values, a measurement of malonaldehyde (secondary oxidation products) are more closely correlated to sensory differences than peroxide values, a measure of primary oxidation products (Ladikos & Lougovois, 1990). Malonaldehyde is a product of oxidation of polyunsaturated fats, particularly omega-6 fatty acids (Ayala et al., 2014). Muscles with high concentrations of polyunsaturated fats tend to have lower peroxide values since the conversion to secondary oxidation products occurs at a much faster rate (Gheisari et al., 2011). Additionally, presence of pro-oxidants, such as heme iron, will cause a faster conversion from primary to secondary lipid oxidation products (Min et al., 2008). The TBARS method utilizes spectrophotometric determination of the extracted malonaldehyde from the food product (Ahn et al., 1998). Thiobarbituric acid reactive substances measurements have been highly correlated to the sensory measures of lipid oxidation flavors, including warmed over flavor (Ahn et al., 1998). Other secondary products can be measured, such as formation of carbonyls and hydrocarbons, but may not have as strong of a
correlation to the sensory data (Ladikos & Lougovois, 1990). Hexanal, a volatile aldehyde that is a product of linoleic acid oxidation, is associated with cooked meats under refrigerated storage (St. Angelo et al., 1987). Primary changes, such as hydroperoxide formation, oxygen uptake and loss of polyunsaturated fatty acids are generally used to measure early stages of oxidation, where very little oxidation has occurred (Ladikos & Lougovois, 1990).

### 2.2.5 Health Implications of Lipid Oxidation Products

Approximately 90-95% of oxygen in the body is converted to water. The remaining 5-10% goes through a univalent and divalent reduction to produce reactive oxygen species, such as peroxides (Esterbauer, 1993). The body contains several enzymes and antioxidants to protect human cells from the toxic effects of the radicals, but certain circumstances suggest that these protective systems are overwhelmed by the quantities of radicals and the cells may still be damaged (Esterbauer, 1993). It is thought that the atherogenic potential of low-density lipoproteins increase if modified by lipid oxidation (Esterbauer et al., 1990; Steinberg et al., 1989). Several studies suggest that oral consumption of heavily oxidized oils are not acutely toxic, mostly because the di- and polymeric oxidation products are not easily absorbed by the small intestine and do not make it to the blood stream, or are detoxified in the harsh environments of the stomach (Esterbauer, 1993). Lower molecular weight oxidation products are more readily absorbed. However, it is highly unlikely that a human ingests the same magnitude of oxidative products as the animal models used in these studies. Endogenous lipid oxidation products are more likely to have a higher health risk. This is thought to be due to higher TBARS values of human serum during certain pathological conditions, such as diabetes (Janero, 1990). Different secondary oxidation products have varying toxicity levels. For
example, longer chain length aldehydes are more likely to have higher toxicity (Esterbauer, 1993).

2.3 Fresh Meat Color

2.3.1 Myoglobin in Fresh Meat

Myoglobin (Mb) is the primary protein pigment associated with meat color (Mancini & Hunt, 2005). Myoglobin delivers oxygen from the sarcolemma to the mitochondria. It is found in all muscle fiber types, but is present in greater concentrations in type I muscle fibers (Wittenberg & Wittenberg, 2003). Myoglobin is a water soluble, monomeric globular heme protein with 150 amino acid residues (Livingston & Brown, 1981) and contains 8 α-helices (A–H) linked by short nonhelical sections (Mancini & Hunt, 2005). The heme group consists of a planar porphyrin ring that is made up of four pyrrole rings attached to each other through methane bridges (Clydesdale & Francis, 1972). An iron atom which is centrally located in the ring has the ability to form six bonds. Two of these bonds are perpendicular to the plane with the 5th coordinating with the proximal histidine-93 of myoglobin and the sixth remaining open for reversible bonding with ligands (Mancini & Hunt, 2005). Meat color is a result of the ligand occupying the sixth position and the oxidation state of the iron atom. The other four bonds lie in the plane and are with pyrrole nitrogens.

Myoglobin is more prone to oxidation than hemoglobin due to myoglobin’s functional ability at lower oxygen pressure (George & Stratmann, 1952). A conformational change occurs in the prosthetic heme due to the presence of molecular oxygen that stabilizes the electronic structure of oxymyoglobin (Omb) and delays Mb oxidation (George & Stratmann, 1952). Myoglobin is more susceptible to oxidation after deoxygenation of the heme occurs because of
factors including decreased pH values, greater temperatures, and a decreased oxygen partial pressure (Mancini & Hunt, 2005; Renerre, 1990).

### 2.3.2 Forms of Myoglobin

![Myoglobin Forms Diagram]

**Rx 1 (Oxygenation):** DMb + O₂ → OMb

**Rx 2a (Oxidation):** OMb + [oxygen consumption or low O₂ partial pressure] – e⁻ → MMb

**Rx 2b (Oxidation):** [ DMb – hydroxyl ion – Hydrogen ion complex ]+ O² → MMb + O²⁻

**Rx 3 (Reduction):** MMb + Oxygen consumption + metmyoglobin reducing activity → DMb

*Figure 1. Practical depiction of the visual color and dynamics of myoglobin redox interconversions on the surface of meat. (modified from Mancini & Hunt, 2005).*

Oxymyoglobin (OMb), deoxymyoglobin (DMb) and metmyoglobin (MMb) are the three primary forms of myoglobin that contribute to fresh meat color. When no ligand is present at the sixth position and the heme iron is ferrous, a deoxygenated form (DMb) of Mb exits (Figure 1). Deoxymyoglobin is usually observed with vacuum packaged meat or muscle that has recently been cut. In beef, it appears purplish-red in color (Mancini & Hunt, 2005). In order to maintain meat in the DMb state, a very low oxygen tension (<1.4 mm Hg; Brooks, 1938) is required.
When Mb is exposed to oxygen and it occupies the sixth position, the formation of OMb occurs and the bright red desired pigment of fresh meat develops (Figure 1). Oxymyoglobin is associated with the “bloomed” color of meat. The iron is still in the ferrous state but when the distal histidine interacts with bound oxygen it alters myoglobin’s structure and stability. Meat temperature, oxygen partial pressure, pH, and competition for oxygen by endogenous systems can all affect the depth of oxygen penetration and thickness of the OMb section of the muscle tissue (Mancini & Hunt, 2005).

Browning (discoloration) of meat is a sign of the formation of MMb on the meat surface. This pigment formation results from the oxidation of the heme iron ferrous to ferric ion. Several factors affect the formation of MMb and influence the rate of this discoloration including low pH, high temperature, low metmyoglobin reducing ability, and very low oxygen partial pressure (Mancini & Hunt, 2005).

The oxygenation reaction is the process of DMb being exposed to oxygen and changing into the OMb form. Oxidation is the loss of an electron and the transformation of Fe$^{2+}$ to Fe$^{3+}$. Mancini and Hunt (2005) describe the redox conversion of OMb to DMb as an indirect, two-step process. The OMb visually appears to first convert to MMb as the muscle consumes oxygen, which creates a low oxygen partial pressure that auto-oxidizes the heme iron, which produces MMb. Then the MMb can be converted to DMb, depending upon the muscle's reducing capacity and the meat temperature (Mancini & Hunt, 2005). In conclusion, fresh meat Mb has three forms that are influenced by the heme iron state and the ligand occupying the sixth position.

2.3.3 Myoglobin Concentration

Fresh meat color is predominantly determined by myoglobin concentration, redox state, and bound ligand. Myoglobin typically is distributed uniformly within individual muscles but
varies by types of muscle, species, sex, breed, and age (Lawrie, 1998). In fresh meat, the meat color portrays the most abundant Mb redox form present, even though the redox states of Mb are continuously changing (Mancini & Hunt, 2005).

When comparing muscles within an animal, differences in Mb concentration have a significant impact not only on meat color but also color stability. Red (oxidative) muscles that appear darker contain more Mb than white (glycolytic) muscles (Seideman et al., 1984). Hunt and Hedrick (1977) reported that the myoglobin concentrations in beef M. longissimus (3.48 mg/g), M. gluteus medius (4.11 mg/g), and the inner (2.97 mg/g) and outer (1.95 mg/g) M. semitendinosus varied significantly. Additionally, myoglobin concentration varies between different animal species. For example, the Mb concentration ranges from 2.0-5.0 mg/g wet weight in beef (Hunt & Hedrick, 1977; Rickansrud & Henrickson, 1967), 4-7 mg/g in lamb (Ledward & Shorthose, 1971), and 2.5-7.0 mg/g in pork (Topel et al., 1966). Meat from older animals is darker due to the increase in Mb concentration as animals age. The American Meat Institute Foundation (1960) stated that animal age has reportedly influenced the myoglobin content in several species, as myoglobin content of muscle tissue in cattle was reported as 1 to 3 mg/g of wet tissue in veal, 4 to 10 mg/g in beef, and up to 16 to 20 mg/g in old beef. Overall, Mb content differs by muscle fiber type, species, and age.

### 2.3.4 Oxygen Consumption

Oxygen consumption is an inherent property of meat where a series of reactions, principally involving the Kreb cycle enzymes, scavenge oxygen in meat. Oxygen consumption is responsible for the deoxygenation of OMb and the further decrease of oxygen concentration to zero allowing the reduction of MMb to DMb (AMSA, 2014). Oxygen consumption rate, a measurement of the rate where oxygen consumption per unit time is calculated, is a major
contributor to meat color stability. The bright red color of postmortem muscle tissue is determined by the rate of DMb oxygenation and depth of oxygen penetration beneath the surface of meat. These are impacted by many factors including partial oxygen pressure at the meat surface, rate of oxygen diffusion, oxygen consumption by muscle enzymes, and the product temperature (O'Keefe & Hood, 1982).

Mitochondria are important subcellular organelles involved in energy metabolism. Mitochondrial enzymes such as cytochrome c oxidase continue to consume oxygen postmortem reducing the amount available to bind to myoglobin, which leads to deoxymyoglobin formation instead of oxymyoglobin (Tang et al., 2005). Furthermore, deoxymyoglobin is more susceptible to oxidation than is oxymyoglobin (Richards et al., 2002). Myoglobin’s role in muscle tissues is to transport oxygen to mitochondria in cells for energy production (Wittenberg et al., 1975). Muscles with weaker color stability have been linked with greater mitochondrial content (Tang et al., 2005). Faster rates of pH decline and lower final pH may inhibit the respiratory activity of mitochondria (Lanari & Cassens, 1991). Postmortem muscles with a lower pH were discovered to have a lower oxygen consumption rate leading to improved color stability (Lanari & Cassens, 1991; Tang et al., 2005) while increasing pH or temperature has been reported to increase tissue oxygen uptake (Urbin & Wilson, 1958).

The oxygen tension above the meat surface greatly influences where the sub-surface MMb layer forms between the outer surface OMb and interior DMb (Atkinson & Follet, 1973). The deeper the OMb layer, the longer it takes for the sub-surface MMb to move upward and impact the hue and discolor the meat. When the mitochondria have a greater oxygen consumption rate, the oxygen surrounding the meat will be used up and Mb will be susceptible to becoming the brown MMb pigment (Tang et al., 2005; Lanari & Cassens, 1991).
2.3.5 Metmyoglobin Reducing Activity

Metmyoglobin reducing activity (MRA) is essential for meat color life because the state of MMb on meat surface is not appealing to consumers (Mancini & Hunt, 2005). Metmyoglobin reducing activity is a property of meat where a series of reactions help reduce MMb to DMb. In addition, this trait is directly related to color stability where greater MRA results in more stable meat color (AMSA, 2014). Many factors, including the NADH (reduced form of nicotinamide adenine dinucleotide) pool, muscle’s oxygen scavenging enzymes, and reducing enzyme systems, help give the muscle the ability to go from the MMb form and return to DMb (Mancini & Hunt, 2005). Metmyoglobin reduction activity is the enzymatic pathway of reducing the ferric (Fe$^{3+}$) iron molecule in MMb back to the ferrous (Fe$^{2+}$) state in the presence of the NADH (Renerre, 1990). The major components required for the enzymatic reduction of MMb are the enzyme (NADH-cytochrome b5 MMb reductase), the intermediate (cytochrome b5) and the cofactor NADH (Bekhit & Fautsman, 2005).

Nicotinamide adenine dinucleotide (NAD) concentrations in post-mortem muscle varied with breed (Holstein had higher NADH than crossbred) and muscle type (M. longissimus dorsi were greater than M. gluteus medius) and NADH concentration decreased with storage time during storage at 4ºC (Faustman & Cassens, 1991). While NAD concentration was negatively correlated with MMb accumulation in the Holstein breed animals tested, no significant correlation was observed with crossbred animals. Sammel et al. (2002a and 2002b) reported that NAD and NADH concentrations were location dependent in the M. semimembranosus muscle (external vs. internal location) and attributed the location dependent effect to differences in relative chilling rate (i.e. the outer portion would cool faster and not result in rapid depletion of
metabolites). McKenna et al. (2005) studied the biochemical properties of 19 beef muscles and found that those with high color stability also had highest MRA.

Madhavi and Carpenter (1993) studied the effects of aging and processing on muscle color and MRA and found that surface MMb accumulation, MRA, and OCR were affected by muscle type, post-mortem aging and fabrication method. These authors also reported that color labile muscle (M. psoas major) appears to have greater MMb accumulation, less MRA, and greater OCR than color stable muscle (M. longissimus dorsi). In addition, the effect of temperature on MRA is pH dependent. Reddy and Carpenter (1991) reported that enzyme activity was greatest at pH 6.4 and 30°C, compared to pH 5.8 or 7.0 and 4°C.

2.3.6 Grinding and Color Stability

Sato et al. (1971) concluded that grinding meat disrupts the phospholipid membranes and allows increases exposure to oxygen and release of the intracellular reductant, NADH, thus increasing susceptibility to both lipid and myoglobin oxidation. The correlation between myoglobin and lipid oxidation thus decreases the overall color stability of ground meat when compared to intact steaks. Strange et al. (1974) observed TBARS values of ground products were two to three times greater than those of unground product under similar conditions, which would potentially result in a decrease in color stability as well. The same study concluded that a freshly prepared ground sample held at 25°C discolored to a brownish red color description within 15 minutes, and was completely brown after 2 hours. Thus, proper storage conditions and antioxidant control are useful in sustaining color stability in ground products.

2.3.7 Relationship of Lipid Oxidation and Meat Color

Lipid oxidation has a positive correlation with color instability (Faustman et al., 1989; Zakryś et al., 2008). The radicals formed from lipid oxidation could act directly or indirectly on
color pigments to influence color stability (Liu et al., 1995). Incubation of lipid oxidation products with oxymyoglobin increased metmyoglobin formation, indicating a direct effect on the pigments (Yin & Faustman, 1994). On the other hand, greater concentrations of iron and myoglobin are correlated to lipid oxidation (Faustman et al., 1991). The dissociation of iron from the heme of myoglobin could be a contributor to lipid oxidation (Faustman et al., 2010). Additionally, myoglobin with high heme affinity (in the oxymyoglobin state) was less effective as a prooxidant than myoglobin with a low heme affinity (metmyoglobin; Grunwald & Richards, 2006; Richards et al., 2002). O’Grady et al. (2001) suggested that lipid oxidation occurs after oxymyoglobin oxidation, yet pigment oxidation can be enhanced by lipid oxidation primary products. Secondary lipid oxidation products, such as 4-Hydroxy-2-nonenal (HNE) can bind directly to myoglobin and lower the redox stability (Alderton et al., 2003; Faustman et al., 2010; Suman at al., 2007).

2.4 Antioxidants

There are two different types of antioxidants: primary and secondary (Shahidi & Zhong, 1992). Primary antioxidants terminate the lipid oxidation process itself, whereas secondary antioxidants react with pro-oxidants or regenerate other antioxidant compounds. Primary antioxidants act by quenching free radicals, most often through phenolic compounds (Wong, 1989). Tert-Butylhydroquinon (TBHQ), Propyl Gallate (PG), Butylated hydroxyanisole and Butylated hydroxytoluene (BHA/BHT) are synthetic primary antioxidants available for food use (Shahidi and Zhong, 2005). Since these antioxidants have been shown to inflict cellular damage at higher concentrations, government regulations exist for the utilization of these antioxidants (Shahidi & Zhong, 2005). Secondary antioxidants help prevent lipid oxidation in ways other than direct impact on the chemical process itself. These antioxidants react with other
prooxidants, regenerate current antioxidants, or act as reducing agents. Ethylenediaminetetraacetic acid (EDTA) is an effective synthetic metal chelator, however the transition toward natural ingredients increase the need to find another naturally occurring compound with similar traits. Reducing sugars, such as whey products, form antioxidative substances during the Maillard reaction of cooking (Ranken, 1987, Bedinghaus & Ockerman, 1995). Moreover, casein peptides found in bovine milk are capable of chelating calcium, iron, copper and zinc (Baumy & Brule, 1999, Singh & Fox, 1995). The antioxidant activity is largely due to the concentration used, since the study showed that the peptide had a prooxidant effect at greater concentrations. Many metal chelators have prooxidant effects when the concentrations are lower than that of iron, which is still not clearly understood (Diaz & Decker, 2004). Vitamin C, ascorbic acid, is involved in the reduction of tocopheroxyl, causing a regenerative effect on tocopherol (Descalzo et al., 2008). Therefore, vitamin C and α-tocopherol can act synergistically. On the other hand, high concentrations of ascorbic acid in the presence of oxygen may form $\text{H}_2\text{O}_2$ and have detrimental effects on meat color (Descalzo et al., 2008).

If a maximum antioxidant effect is desired, a combination of a postmortem antioxidant and dietary antioxidants (Vitamin E) is the most effective (Formanek et al., 2001). The same study also concluded that the natural and dietary antioxidant combination was as effective as a synthetic and dietary antioxidant combination. Antioxidants may act differently in cooked products than raw products, where McCarthy et al. (2001) determined that mustard has antioxidant properties in raw products, but acts as a prooxidant in cooked products. Therefore, it cannot be assumed that antioxidants will act in the same manner throughout different processing techniques.
2.4.1 Impacts of Antioxidants on Meat Color

Because of the interrelationship of lipid and color oxidation, both dietary and postmortem antioxidants have been shown to increase color stability in meat products (Arnold et al., 1992; Bloomberg et al., 2011; Maddock et al., 2003). Lanari and others (1994) reported that vitamin E could stabilize the oxymyoglobin complex in muscles through increasing deoxymyoglobin oxygenation and decreasing oxymyoglobin oxidation. The supplementation of ethoxyquin in conjunction with vitamin E even further enhances a* values, but the same may not apply to supplementation of ethoxyquin alone (Maddock et al., 2003; Walenciak et al., 1999).

In reference to postmortem antioxidants, butylated hydroxyanisole and butylated hydroxytoluene (BHA/BHT) have been shown to be the most effective at retaining fresh meat color when compared to natural antioxidants. Despite this, in raw pork patties, fenugreek, tea catechins and whey protein had equal effectiveness in retaining redness as BHA/BHT (McCarthy et al., 2001). Rosemary extracts are found to improve color stability in turkey, beef and pork in ground and injected products (Yu et al., 2002; Formanek et al., 2003; Lawrence et al., 2004; McCarthy et al., 2001; Sebranek et al., 2005). In contradiction, not all antioxidants contribute to color stability. The use of trans-cinnamaldehyde, for example, was shown to lower TBARS values, but did not improve color scores (Naveena et al., 2014).

2.5 Natural Antioxidants

Concern about the safety of synthetic antioxidants, such as BHA, BHT and TBHQ, has opened up a large opportunity for the meat industry to research and utilize natural antioxidants. Much research has been conducted to evaluate the effectiveness of natural antioxidants in comparison to synthetic antioxidants, and the economic value of using such products. Many natural antioxidants exist, including but not limited to: rosemary, sage, ginger, fenugreek,
mustard, grape seed extract and tea catechins. The antioxidant activity of these substances is not newly recognized, as Chipault et al. (1952) reported strong antioxidant activity in the leaves of rosemary in 1952.

2.5.1 Rosemary as an Antioxidant

Rosemary, like many other plant-based antioxidants, gains its antioxidant activity through phenolic compounds. The phenolic compounds are different depending on the antioxidant, but in the case of rosemary it is primarily diterpenes such as carnosol and carnosic acid (Decker & Mei, 1996). These phenolic compounds act as hydrogen donors to stabilize free radicals. This process is identical to that of BHA/BHT (McCarthy et al., 2001). Rosemary extracts have been shown to provide greater (Wu et al., 1982) or lesser antioxidant activity in comparison to BHA/BHT (Ahn et al., 2002). The results are heavily dependent on processing technique and concentration of antioxidant used.

Efficacy of antioxidants depends on how the antioxidant itself was processed, what concentration is added, the environment it is in, and how the product is processed and stored. Application of rosemary extract to the trim prior to grinding has been shown to be more effective at extending color stability and reducing TBARS values than addition of antioxidant after the grinding process (Balentine et al., 2006). Murphy et al. (1998) discovered that the presence of rosemary in cooked, salt-added roast beef resulted in lower TBARS values than the control. Rojas and Brewer (2007) determined that grape seed extract, when used at the same amount of rosemary oleoresin, generated lower TBARS values than rosemary extract at 8 days. This is largely because greater concentrations of rosemary extract would be necessary to match the antioxidant levels of grape seed extract in this instance. This being said, using specific extracts at higher levels may begin to alter sensory characteristics of the product, and conducting sensory
research on plant-based extracts is imperative to determine if it is acceptable for use in food products.

**2.5.2 Tea Catechins as Antioxidants**

Like rosemary, tea catechins are primary antioxidants that use catechin, epicatechin gallate (ECG), epigallocatechin gallate (EGCG), and epicatechin to donate the phenolic hydrogen to quench free radicals (Tang et al., 2001). The phenolic compounds ECG and EGCG specifically have a high affinity for lipid bilayers and are more able to penetrate the lipid bilayer to interact with phospholipids susceptible to lipid oxidation (Hashimoto et al., 1999). Roedig-Penman and Gordon (1997) discovered that tea extract at 0.03% had equal antioxidant activity to that of BHA/BHT at 0.02% over a 40 day period in an oil-in-water emulsion. McCarthy et al. (2001) reported that tea catechins have similar TBARS values to those of BHA/BHT and of rosemary extract in raw pork patties. In cooked patties, tea catechins were the best at reducing TBARS values over a period of 9 days. Tea catechins are effective in all species, but have less effect in meat from species with higher concentrations of unsaturated fats and iron (Tang et al., 2001). Although tea catechins are acceptable to reduce lipid oxidation, they have shown to have a negative effect on a* and b* values of cooked beef and chicken patties possibly due to binding of the iron component of myoglobin (Mitumoto et al., 2005).

**2.6 Dietary Antioxidants**

**2.6.1 Tocopherols**

There are four tocopherols: α, β, γ, δ. (Sales & Koukolová, 2011). Alpha-tocopherol is significantly more effective as an antioxidant than other tocopherols and is the most common lipid soluble antioxidant in animal tissue. Vitamin E cannot be synthesized by animals and must be found in the diet (Sales & Koukolová, 2011). Vitamin E is known to be a primary antioxidant
by releasing a hydrogen atom to counteract free radicals (Berges, 1999). Absorption of vitamin E occurs in the lymphatic system. It is secreted into the liver and incorporated into very-low-density lipoproteins, and transported into the interior of cells inside low-density lipoproteins (Berges, 1999). Muscles with a higher exposure to blood flow contain higher concentrations of α-tocopherol than those with lower vascularity (Sheldon et al., 1997). Dietary supplementation with vitamin E in the form of α-tocopherol acetate delays lipid oxidation more effectively than direct addition because it allows the vitamin E to be incorporated into the phospholipid membranes rather than the neutral membranes (Ahn et al., 2002). Vitamin E is heat stable and still retains the ability to act as an antioxidant in cooked meat products (Faustman et al., 1989). Narciso Gaytán et al. (2010) determined that despite the rapid development of lipid oxidation in cooked chicken patties versus fresh samples, vitamin E fed at higher concentrations in the diet may combat the increased rate of oxidation. Postmortem addition of vitamin E can potentially act as a prooxidant in some cases, by reducing ferric iron to a more active ferrous form (Pokorny, 1991). Govaris et al. (2004) determined that the antioxidant effect of direct addition of α-tocopherol was inferior, as dietary α-tocopherol contained 90-fold more α-tocopherol in the membranes of turkey meat. Roux et al. (2014) detected that feed with six times more vitamin E than the control had nearly equivalent differences in the meat itself, with 5.4 times more vitamin E in the meat from chickens supplemented with vitamin E. Concentration of α-tocopherol varies between muscles, and is dependent on the capillary density and capillary-to-fiber ratio in turkey femoris muscle (Bartov et al., 1983). However, vitamin E supplementation will not likely alter the fatty acid composition of the tissue (Narciso-Gaytan et al., 2010).

Other compounds can be used synergistically with vitamin E to create a protective effect on α-tocopherol. Ascorbic acid has been used in conjunction with vitamin E since it serves as a
reducing agent on tocopherol (Descalzo et al., 2008). Oregano oil exerted protective in vivo effects on the concentrations of \( \alpha \)-tocopherol in turkey muscle, suggesting that it has reducing properties as well as phenolic compounds (Govaris et al., 2004).

2.6.2 Ethoxyquin/TBHQ

Ethoxyquin/TBHQ (Agrado Plus, Novus International, St. Louis, MO) is approved for usage in feed rations to reduce oxidation of ingredients with higher fat ratios (Maddock et al., 2003). It is important to note that this ingredient prevents oxidation of the feed itself and does not act as an antioxidant in the body. Choat et al. (2002) reported that Agrado supplementation did not increase serum Vitamin E values directly, but could increase vitamin E concentrations over time since Agrado is able to stabilize vitamin E in the feed ration.

Ethoxyquin/TBHQ has previously been shown to decrease TBARS values in beef (Walenciak et al., 1999, Krumsiek & Owens, 1998). Choat et al. (2002), however, did not see this decrease in TBARS values. This would likely be attributed to the absence of vitamin E supplementation in the diet of the cattle used in this study. The contribution of Agrado to color stability has contradictory results, where Krumsiek and Owens (1998) and Choat et al. (2002) had an increased time period before discoloration of steaks in Agrado supplemented cattle compared to the control. On the contrary, Walenciak et al. (1999) did not see any differences in color stability over time. This could be attributed to the supplementation time, where the Krumsiek and Ownes (1998) study had cattle supplemented with Agrado for 28 d prior to slaughter and the Walenciak et al. (1999) study used cattle supplemented for 123 d. Additionally, Krumsiek and Owens (1998) and Choat et al. (2002) studied color stability on steaks only, whereas Walenciak et al. (1999) utilized both ground and whole muscle beef. The
intensity of lipid oxidation occurring in ground products could have overwhelmed the antioxidant capabilities of vitamin E available in that study.

Agrado, specifically, was initially used in the dairy industry to reduce feed oxidation and is making its way into the beef sector. This being said, there are many research opportunities in Agrado supplementation, including feeding to different species, how it acts as an antioxidant, and the effect on meat products, specifically processed meats.

2.7 Conclusion

Much research has shown that the increased usage of ethanol co-products in cattle diets increases the PUFA concentration of muscle which decreases lipid and myoglobin stability in fresh meat. Furthermore, processing techniques can reduce color and lipid stability. Comminution increases susceptibility to lipid oxidation by disrupting the phospholipid membranes and further exposing them to oxygen which can in turn act on the muscle pigments to reduce color stability. Cooking further promotes lipid oxidation by the release of both free and heme iron from myoglobin. However, little work has been conducted on the impact of animal diet on raw and cooked ground beef characteristics. The use of antioxidants, whether dietary or postmortem, is essential to counterbalance the negative impacts of diet and processing technique on the shelf life. Therefore, the objectives of these studies are to determine the impact of feeding ethanol co-products to cattle and the effect of dietary and postmortem antioxidants on the shelf life of raw and cooked ground beef and to determine if dietary or postmortem antioxidants can counteract any negative impacts of feeding distillers grains on shelf life.
3. **Materials and Methods**

3.1 **Dietary Treatments and Product Collection**

In the first study, heifers (n=64) were randomly assigned to a 2 X 2 factorial dietary treatments that included 0.91 or 2.27 kg of wet distillers grains (WDGS) during the winter backgrounding phase and finished with a corn based diet with either corn gluten feed (Sweet Bran®; Cargill, Minneapolis, MN) or modified wet distillers grains (WDGS) included (40% dry matter basis; DM). All cattle were supplemented with modified wet distillers grains (MDGS) at a rate of 0.6% of BW during the summer months. After commercial harvest and grading, USDA Choice beef shoulder clods (IMPS # 114; NAMP, 2007) were vacuum-packed, transported to Loeffel Meat Laboratory at the University of Nebraska-Lincoln and stored at 2°C until processing. A total of 16 shoulder clods from the right side of carcasses were collected representing four shoulder clods from each dietary treatment group.

In the second study, cattle (n=100) were randomly assigned to one of five 106-day finishing diets; corn-based diet with no WDGS (control), wet distillers grains (WDGS, 30% dry matter basis), WDGS + 1000 IU/hd/d vitamin E, WDGS + 3 g/hd/d Agrado Plus, or WDGS + 500 IU/hd/d vitamin E + 3 g/hd/d Agrado Plus. At the conclusion of the finishing phase, cattle were harvested at commercial abattoir. Forty-eight h post-harvest, seven USDA Choice beef shoulder clods (IMPS # 114; NAMP, 2007) from each dietary treatment group were collected from the right side of carcasses, vacuum packaged, and shipped to the University of Nebraska-Lincoln Loeffel Meat Laboratory.
3.2 Beef Link Manufacture

On d 14 postmortem, subcutaneous fat and lean samples were taken from each shoulder clod on the ventral end from the triceps brachii muscle for fatty acid analysis. Each shoulder clod was independently ground through a 1.27 cm plate and then through a 0.476 cm plate (Model 4732, Hobart; Troy, OH), a composite sample taken for fatty acid analysis, and the remainder was weighed into 2.27 kg batches. All treatments contained 0.75% salt, 0.25% sodium phosphate (Brifisol 85 instant, BK Giuliani Corp., Simi Valley, CA). In the first study, three 2.27 kg batches were weighed and 0%, 0.13% or 0.20% rosemary and green tea natural plant extract (FORTIUM RGT12 Plus Dry Natural Plant Extract; Kemin, Des Moines, IA) was added in addition to salt and phosphate. Beef and non-meat ingredients were mixed for 1 min and the mixture was stuffed into 22 mm diameter skinless links using a hydraulic piston stuffer (Talsa H31P, Talsabell S.A., Valencia, Spain) and Colosimo press (model 200, Gale Colosimo Seasoning Co., South Salt Lake, UT). After cooking, links were placed in individual foil trays for each shoulder clod and cooked to an internal temperature of 71 °C. Links were placed in oxygen impermeable zip-top bags (Ziploc® Storage Bags, 1 Gallon, S. C. Johnson, Racine, Wisconsin) and placed in either dark refrigerated storage at 2°C or dark frozen storage at -20°C.

3.3 Beef Patty Manufacture and Simulated Retail Display

On d 14, each shoulder clod was independently ground, 113-g patties were formed using a hand operated hamburger press, placed on styrofoam trays, overwrapped with permeable oxygen polyvinyl chloride wrap (Prime Source Meat Film, Prime Source Sanitary Supply, St. George, UT) and placed under simulated retail display. Beef patties (first study - two full patties for color analysis, ten patties for TBARS, per sample; second study – two full patties for color
analysis, six, ½ patties for TBARS, per sample) were packaged on Styrofoam trays (13.3 × 25.6 × 1.4 cm, Styro-Tech, Denver, CO), overwrapped with oxygen permeable polyvinyl chloride film (Prime Source Meat Film, Prime Source Sanitary Supply, St. George, UT) and placed on a table in a cooler maintained at 0 to 2°C under continuous 1,000 to 1,800 Lux warm white fluorescence lighting (PHILIPS F32T8/TL741 ALTO 700 Series, 32 WATT B7, Royal Philips Electronics, Amsterdam, Netherlands) to provide simulated retail display conditions.

3.4 Proximate Analysis

Moisture and total fat of pulverized raw meat samples were determined. Two g of pulverized tissue in duplicate were used to quantify moisture and ash using a LECO thermogravimetric analyzer (LECO Corporation, model 604- 100- 400, St. Joseph, MI). Total fat was determined as outlined by AOAC (1990) using the Soxhlet extraction procedure.

3.5 Objective color evaluation

The patty color was measured with a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer and a D65 illuminant. The calibration plate was read through polyvinyl chloride overwrap film since patties were still within the packaging during the color measurement. The color of the two patties at three locations each was measured and the resulting measurements were averaged for each sample. Color values were collected for L*, a*, and b*. Color was measured on d 0, 0.5, 1, 2, 3, 5, and 6, and for the first study and on d 0, 1, 2, 3, 4, 5, 6, and 7 for the second study.
3.6 Subjective color evaluation

A five-person trained panel composed of graduate students of the Department of Animal Science at University of Nebraska-Lincoln subjectively evaluated the percentage discoloration every day. Discoloration was evaluated as percentage surface discoloration ranging from 0 to 100%. Subjective color was evaluated every 24 h for 7 d, with the exception of d 4 on the first study.

3.7 Lipid Oxidation

The 2-thiobarbuteric acid reactive substance assay (TBARS) described by Ahn et al. (1998), which was a modification of the TBARS assay developed by Beuge and Aust (1978), was used to measure the oxidation status of refrigerated and frozen cooked, ground, beef links and fresh ground beef patties over storage time. Refrigerated links TBARS measurements were taken every 3 d for 18 d, beginning at d 0. Frozen links TBARS measurements were taken every 28 d for 252 d, beginning at d 0.

Fourteen mL of deionized, distilled water and 1 mL of butylated hydroxyanisole solution (10% BHA in 90% ethanol) were added to 5 g of pulverized sample. After homogenizing for 15 s using a Polytron (POLYTRON® Kinimatica CH-6010, Switzerland), the homogenate was centrifuged for 2000 × g for 5 m. One mL of homogenate was mixed with 2 mL of 2-thiobarbituric acid and trichloroacetic acid mixture (15% TCA (w/v) and 20 nM TBA in deionized distilled H_{2}O) and vortexed for 5 sec. The sample mixture was incubated at 70°C in a water bath for 30 min to develop color. After samples were cooled in a cold-water bath for 10 m, the sample mixture was centrifuged at 2,000 × g for 15 m. Duplicate 200 μL aliquots of each sample were transferred into wells on a 96-well plate and the absorbance was read at 540 nm to
calculate the mg of malonaldehyde per kg of tissue using 1,1,3,3-tetraethoxypropane as the standard solution.

3.8 Fatty Acid Analysis

Total lipid was extracted following the chloroform-methanol procedure of Folch et al. (1957). After extraction, the lipids were converted to fatty acid methyl esters according to Morrison and Smith (1964) and Metcalfe et al. (1966). Following dicing, each frozen fat, composite, and lean portion of each shoulder clod was dipped in liquid nitrogen and macerated using a Waring commercial blender (Model 51BL32, Waring Commercial, Torrington, CT). Powdered samples were stored at -80°C until analysis. Each fatty acid was determined by gas chromatography (GC) after fat extraction from samples followed by formation of methyl esters of fatty acids. Powdered lean, composite, and fat samples (one gram for lean and composite, 0.1 g for fat) were dissolved in 5 mL of 2:1 chloroform:methanol (v/v) to extract the lipid fraction after vortexing for 5 s and letting them stand for 1 h at room temperature. The homogenate was filtered through Watman #2 filter paper into a 13 × 150 mm screw-cap tube and the final volume was brought up to 10 mL with 2:1 chloroform:methanol mix. Then, 2 mL of 0.74% KCl solution was added to the lipid extract and vortexed for 5 s to separate extracted proteins. Samples were centrifuged at 1000 × g for 5 m. Following centrifugation, the aqueous layer was aspirated and evaporated to dryness under nitrogen at 60°C (Folch et al., 1957). Then, 0.5 mL of 0.5 M NaOH in methanol was added, vortexed again for 5 s and heated for 5 m at 100°C under nitrogen for saponification. To methylate the fatty acids, 0.5 mL of boron trifluoride in 14% methanol was added and heated for 5 m at 100°C to methylate the fatty acids (Metcalfe et al., 1966). One mL of saturated salt solution and 1 mL of hexane were added and vortexed for 5 s. Following centrifugation at 1,000 × g for 5 min, the hexane layer was removed and placed in a gas
chromatography (GC) vial. Sample containing fatty acids in the GC vial was purged with nitrogen, capped and stored at -80°C until the sample was read on a Hewlett-Packard Gas Chromatograph (Agilent Technologies, model 5890A series, Santa Clara, CA) attached to a Hewlett-Packard Autosampler (Agilent Technologies, model 6890A series, Santa Clara, CA). Total fatty acids converted to methyl esters were separated on a fused silica column (Chrompack CP-Sil 88; 0.25 mm × 100 m, Santa Clara, CA), which was placed in an oven programmed from 140°C for 10 m to 220°C at a rate of 2°C/m and held at 220°C for 20 m. Total run time was 70 m. The injector and detector were programmed to work at 270°C and 300°C, respectively. Each lipid extract was separated into fatty acids by using helium as the carrier gas at a flow rate of 1 mL/m. Individual fatty acids of each sample were determined by comparison of retention times with known standards. Each standard contained known fatty acids that were ran through gas chromatography at the same time as the samples in order to provide a reference point to identify fatty acids in the sample. Each fatty acid was expressed as weight percentage value, which were relative proportions of all peaks observed by gas chromatography.

3.9 Statistical Analysis

For the first study, data for cooked link TBARS were analyzed as a 2 × 2 × 3 factorial (backgrounding diet × finishing diet × antioxidant) using the PROC GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC USA) with repeated measures (day) where applicable. Cooked link TBARS were analyzed as a 2 × 2 factorial using the PROC GLIMMIX procedure of SAS with repeated measures to specifically observe effects of diet on TBARS. Data for raw patty TBARS proximate analysis, objective color and percent discoloration were analyzed as a 2 × 2 factorial using the PROC GLIMMIX procedure of SAS with repeated measures (day) where
applicable. Fatty acid data analysis was conducted as a 2 × 2 factorial using the PROC GLIMMIX procedure of SAS.

For the second study, data for TBARS (refrigerated, frozen and raw patty), fatty acid analysis, proximate analysis, objective color and percent discoloration were analyzed for treatment (dietary antioxidant or corn control) main effects using the PROC GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC USA) with day as repeated measures when traits were measured over time.

All means were separated utilizing the LSMEANS and PDIFF option when level of significance indicated by ANOVA was $P < 0.05$. 
4. Literature Cited


5. Manuscripts
Effect of feeding distillers grains during different phases of production and addition of postmortem antioxidants on shelf life of ground beef

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ABSTRACT

The objective of this study was to evaluate the impact of feeding distillers grains and added antioxidants on the shelf life of ground beef products. Heifers were assigned to diets containing low or high concentrations of wet distillers grains (WDGS) during backgrounding and either corn gluten feed or modified wet distillers grains (MDGS) during finishing. Four beef shoulder clods from each dietary group were ground. Raw patties were overwrapped for retail display and analyzed for lipid oxidation, percent discoloration and objective color. Cooked beef links were manufactured with salt, phosphate, and a blend of rosemary and green tea extract, stuffed into links, cooked and lipid oxidation was measured throughout storage. There were no differences in lipid oxidation of raw patties regardless of diet, yet beef from heifers finished with MDGS discolored at a greater rate. Increased lipid oxidation in cooked beef links occurred when cattle were fed WDGS during backgrounding or finishing but antioxidants reduced lipid oxidation regardless of dietary treatment.

Keywords: ground beef, antioxidant, TBARS, distillers grains, fatty acid

1. Introduction

In 2013 in the United States, 35.5 million metric tons of distillers grains (DGS) were produced as by-products of the fuel ethanol industry and has increased by nearly 30 million metric tons annually over the past ten years (RFA, 2014). Each 25.4 kg bushel of corn used in dry-mill ethanol production generates about 7.89 kg of DGS available for livestock feed (USDA Economic Research Service, 2012) and beef cattle account for nearly half of DGS consumption (RFA, 2014). As a result of the rapid growth of the ethanol industry, many cattle producers include ethanol by-products in cattle diets. Previous research has shown that steaks from cattle fed WDGS have an increase in PUFA, and have decreased oxidative stability (Depenbush,
Coleman, Higgins, & Drouillard, 2009; Jenschke et al., 2008). Furthermore, higher concentrations of DGS (20% to 40% DM) increased lipid oxidation in ground beef (Koger et al., 2010).

Lipid oxidation occurs most commonly in polyunsaturated fatty acids (PUFA) that more readily undergo free-radical chain reactions resulting in deterioration of the lipid. Lipid oxidation has a positive correlation with color instability (Faustman et al., 1989; Zakrys, Hogan, O’Sullivan, Allen, & Kerry, 2008). The radicals formed from lipid oxidation could act directly or indirectly on color pigments to influence color stability (Liu, Lanari, & Schaefer, 1995) and likewise, pigment oxidation can promote lipid oxidation (Faustman, Sun, Mancini, & Suman, 2010). It is hypothesized that products of unsaturated fatty acid oxidation can bind directly to myoglobin and lower the redox stability (Faustman et al., 2010). In addition, comminution of meat products disrupts the phospholipid membranes and allows increased exposure to oxygen, thus increasing the rate of lipid oxidation (Sato & Hegarty, 1971). Cooking meat increases lipid oxidation by release of free and heme iron from myoglobin (Greene & Cumuze, 1982). These processing and cooking techniques reduce shelf life and decrease overall desirability of the product by increasing "warmed over" or "rancid" flavors. Warmed over flavor is an off flavor due to secondary products of lipid oxidation found in cooked meats, and typically become apparent within 48 h at refrigeration temperatures (Forrest, Aberle, Gerrard, Mills, 2012). Prior to this study, Dierks et al. (2014) were the only ones to look at the impact of distillers grains on cooked beef, where they observed that supplementation of DGS during backgrounding increased lipid oxidation compared to cooked beef from non-supplemented cattle.

The use of plant extracts, such as rosemary or green tea, is becoming increasingly popular in meat processing as natural antioxidants to increase shelf life of cooked meat products. This
becomes particularly beneficial for companies seeking use "natural" labeling claims for their product. Both rosemary and green tea have phenolic compounds that donate the phenolic hydrogen to quench free radicals formed during lipid oxidation (Tang, Kerry, Sheehan, Buckley, & Morrissey, 2001).

Therefore, the objectives of this trial were to evaluate the effect of feeding different concentrations of WDGS during winter backgrounding and either MDGS or corn gluten feed (Sweet Bran, Cargill®, Blair, NE) during the finishing phase on raw and cooked ground beef and to evaluate the effectiveness of natural rosemary and green tea extract in the cooked beef samples.

2. Materials and methods

2.1 Dietary Treatments and Product Collection

Heifers (n=64) were randomly assigned to a 2 X 2 factorial dietary treatments that included 0.91 or 2.27 kg of wet distillers grains (WDGS) per d during the winter backgrounding phase and finished on a corn-based diet with either corn gluten feed (40% dry matter basis; DM) or modified wet distillers grains (WDGS) included (40% DM; Figure 1). All cattle were supplemented with modified wet distillers grains (MDGS) at a rate of 0.6% of BW during the summer months. After commercial harvest and grading, untrimmed beef shoulder clods (IMPS # 114; NAMP, 2007) were vacuum-packed, transported to Loeffel Meat Laboratory at the University of Nebraska-Lincoln and stored at 2°C until processing on d 14. A total of 16 shoulder clods from the right side of carcasses were collected representing four shoulder clods from each dietary treatment group.
2.2 Beef Link Manufacture

On d 14 postmortem, subcutaneous fat and lean samples were taken from the triceps brachii muscle on the ventral of each shoulder clod for fatty acid analysis. Each shoulder clod was independently ground through a 1.27 cm plate and then through a 0.476 cm plate (Model 4732, Hobart; Troy, OH), a sample taken for fatty acid analysis, and the remainder was divided into three 2.27 kg batches. All treatments contained 0.75% salt, 0.25% sodium phosphate (Brifisol 85 instant, BK Giulini Corp., Simi Valley, CA) and either 0%, 0.13% or 0.20% rosemary and green tea natural plant extract (FORTIUM RGT12 Plus Dry Natural Plant Extract; Kemin, Des Moines, IA). Beef and non-meat ingredients were mixed for 1 min and the mixture was stuffed into 22 mm diameter skinless links using a hydraulic piston stuffer (Talsa H31P, Talsabell S.A., Valencia, Spain) and Colosimo press (model 200, Gale Colosimo Seasoning Co., South Salt Lake, UT). Links were placed in individual foil trays for each shoulder clod and cooked to an internal temperature of 71 °C. Links were placed in oxygen impermeable zip-top bags (Ziploc® Storage Bags, 1 Gallon, S. C. Johnson, Racine, Wisconsin) and kept in either dark refrigerated storage at 2°C or dark frozen storage at -20°C until appropriate sampling days.

2.3 Beef Patty Manufacture and Simulated Retail Display

On d 14 from each ground shoulder clod, twelve, 113-g patties were formed using a 11 cm diameter hand operated hamburger press, placed on styrofoam trays, overwrapped with permeable oxygen polyvinyl chloride wrap (Prime Source Meat Film, Prime Source Sanitary Supply, St. George, UT) and placed under simulated retail display for 7 d. Beef patties (two full patties for color analysis, ten patties for TBARS, per shoulder clod) were packaged on Styrofoam trays (13.3 × 25.6 × 1.4 cm, Styro-Tech, Denver, CO), overwrapped with oxygen permeable
polyvinyl chloride film and placed on a table in a cooler maintained at 0 to 2°C under continuous
1,000 to 1,800 Lux warm white fluorescence lighting (PHILIPS F32T8/TL741 ALTO 700
Series, 32 WATT B7, Royal Philips Electronics, Amsterdam, Netherlands) to provide simulated
retail display conditions.

2.4 Proximate Analysis

Moisture and total fat of pulverized raw meat samples were determined for lean and
composite samples. Two g of pulverized tissue in duplicate were used to quantify moisture and
ash using a LECO thermo-gravimetric analyzer (LECO Corporation, model 604-100-400, St.
Joseph, MI). Total fat was determined as outlined by AOAC (1990) using the Soxhlet extraction
procedure.

2.5 Fatty Acid Analysis

Total lipid was extracted following the chloroform-methanol procedure of Folch et al.
(1957). After extraction, the lipids were converted to fatty acid methyl esters according to
Morrison and Smith (1964) and Metcalfe et al. (1966). Following dicing, each frozen lean,
composite and subcutaneous fat, samples from each shoulder clod was frozen in liquid nitrogen
and powdered to ensure a homogenized sample using a Waring commercial blender (Model
51BL32, Waring Commercial, Torrington, CT). Powdered samples were stored at -80°C until
analysis. Fatty acid composition was determined by gas chromatography (GC) after fat
extraction from samples followed by formation of methyl esters of fatty acids. Powdered lean,
composite, and subcutaneous fat samples (1 gram for lean and composite, 0.1 g for fat) were
dissolved in 5 mL of 2:1 chloroform:methanol (v/v) to extract the lipid fraction after vortexing
for 5 s and letting them stand for 1 h at room temperature. The homogenate was filtered through
Watman #2 filter paper into a 13 × 150 mm screw-cap tube and the final volume was brought up to 10 mL with 2:1 chloroform:methanol mix. Then, 2 mL of 0.74% KCl solution was added to the lipid extract and vortexed for 5 s to separate extracted proteins. Samples were centrifuged at 1,000 × g for 5 m. Following centrifugation, the aqueous layer was aspirated and evaporated to dryness under nitrogen at 60°C (Folch et al., 1957). Then, 0.5 mL of 0.5 M NaOH in methanol was added, vortexed again for 5 s and heated for 5 m at 100°C under nitrogen for saponification. To methylate the fatty acids, 0.5 mL of boron triflouride in 14% methanol was added and heated for 5 m at 100°C to methylate the fatty acids (Metcalfe et al., 1966). One mL of saturated salt solution and 1 mL of hexane were added and vortexed for 5 s. Following centrifugation at 1,000 × g for 5 m, the hexane layer was removed and placed in a GC sampling vial. Sample containing fatty acids in the GC vial was purged with nitrogen, capped and stored at -80°C until the sample was read on a Hewlett-Packard Gas Chromatograph (Agilent Technologies, model 5890A series, Santa Clara, CA) attached to a Hewlett-Packard Autosampler (Agilent Technologies, model 6890A series, Santa Clara, CA). Fatty acid methyl esters were separated on a fused silica column (Chrompack CP-Sil 88; 0.25 mm × 100 m, Santa Clara, CA), which was placed in an oven programmed from 140°C for 10 m to 220°C at a rate of 2°C/m and held at 220°C for 20 m. Total run time was 70 m. The injector and detector were programmed to operate at 270°C and 300°C, respectively. Each lipid extract was separated into fatty acids by using helium as the carrier gas at a flow rate of 1 mL/m. Individual fatty acids of each sample were determined by comparison of retention times with known standards. Each standard contained known fatty acids that were ran through gas chromatography at the same time as the samples in order to provide a reference point to identify fatty acids in the sample. Each fatty acid was expressed as weight percentage value, which were relative proportions of all peaks observed by gas chromatography.
2.6 Objective color evaluation

The patty color was measured with a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer and a D65 illuminant. The calibration plate was read through polyvinyl chloride overwrap film since patties were still within the packaging during the color measurement. The color of the two patties at three locations each was measured and the resulting measurements were averaged for each sample. Color values were collected for L*, a*, and b*. Color was measured on d 0, 0.5, 1, 2, 3, 5, and 6.

2.7 Subjective color evaluation

A five-person trained panel composed of graduate students of the Department of Animal Science at University of Nebraska-Lincoln subjectively evaluated the percentage discoloration every day. Discoloration was evaluated as percentage surface discoloration ranging from 0 to 100%. Subjective color was evaluated on d 0, 1, 2, 3, 4, 5, and 6.

2.8 Lipid Oxidation

The 2-thiobarbuteric acid reactive substance assay (TBARS) described by Ahn et al. (1998), which was a modification of the TBARS assay developed by Beuge and Aust (1978), was used to measure the oxidation status of refrigerated and frozen cooked, ground, beef links and fresh ground beef patties over storage time. Refrigerated links TBARS measurements were taken every 3 d for 18 d, beginning at d 0. Frozen links TBARS measurements were taken every 28 d for 252 d, beginning at d 0.

Fourteen mL of deionized, distilled water and 1 mL of butylated hydroxyanisole solution (10% BHA in 90% ethanol) were added to 5 g of sample. After homogenizing for 15 s using a
Polytron (POLYTRON® Kinimatica CH-6010, Switzerland), the homogenate was centrifuged for 2,000 × g for 5 m. One mL of homogenate was mixed with 2 mL of 2-thiobarbituric acid and trichloroacetic acid mixture (15% TCA (w/v) and 20 nM TBA in double distilled H₂O and vortexed for 5 s. The sample mixture was incubated at 70°C in a water bath for 30 m to develop color. After samples were cooled in a cold-water bath for 10 min, the sample mixture was centrifuged at 2,000 × g for 15 m. Duplicate 200 μL aliquots of each sample were transferred into wells on a 96-well plate and the absorbance was read at 540 nm to calculate the mg of malonaldehyde per kg of tissue using 1,1,3,3-tetraethoxypropane as the standard solution.

2.9 Statistical Analysis

Data for raw patty TBARS, proximate analysis, objective color and percent discoloration were analyzed as a 2 × 2 factorial using the PROC GLIMMIX procedure of SAS with repeated measures (day) where applicable. Fatty acid data analysis was conducted as a 2 × 2 factorial using the PROC GLIMMIX procedure of SAS. Data for cooked link TBARS were analyzed as a 2 × 2 × 3 factorial (backgrounding diet × finishing diet × antioxidant) using the PROC GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC USA) with repeated measures (day) where applicable. Additional analysis of cooked link TBARS were analyzed as a 2 × 2 factorial using the PROC GLIMMIX procedure of SAS with repeated measures (day) to specifically observe effects of diet on TBARS. All means were separated utilizing the LSMEANS and PDIFF option when level of significance indicated by ANOVA was \( P < 0.05 \).
3. Results and Discussion

3.1 Proximate Composition

No dietary effects were found for fat or moisture for the composite or lean samples \((P > 0.39\), data not shown\). The average fat percentage for composite and lean samples was 22.8 \% and 7.8 \%, respectively. The average percent moisture for composite and lean samples was 60 \% and 74 \%, respectively.

3.2 Fatty Acid Composition

For the lean, subcutaneous fat, and composite portion fatty acid analyses, a finishing effect was observed where beef from cattle finished on MDGS had greater amounts of C18:2 and total PUFA \((P \leq 0.0283\), Table 1\). The composite sample also had a finishing effect where cattle finished on MDGS had greater amounts of C16:1 \((P = .0425)\) and lesser amounts of C17:0 and C17:1 \((P = .0018\) and .0064, respectively\). These changes in PUFA concentration are likely directly related to changes in lipid oxidation related to finishing diet since you would expect a decrease in lipid stability with the increase in PUFA. The subcutaneous fat portion had a backgrounding effect where there was a greater amount of UFA, lower concentrations of C18:0, and a lower UFA:SFA \((P = .0045, .0060,\) and .0139 respectively, Table 2\) in beef from cattle supplemented with greater amounts of MDGS while no significant backgrounding diet effects \((P > 0.05)\) were found for the lean or composite samples. The backgrounding diet impact on fatty acid composition does not fully explain differences in oxidation due to backgounding diet in cooked beef links. Dierks et al. (2014) also found limited differences in fatty acid profile that did not fully explain differences in oxidation when only ground composite samples from the shoulder clod were evaluated. Thus, this study utilized three different fat depots for fatty acid analysis to see the impact of type of fat storage. Domenech et al. (2014) also saw the increase of
total PUFA and 18:2 of cattle fed wet distillers grains in raw beef strip steaks. This also agrees with the research of Gill and others (2008), where there was an increase in all types of 18:2, total PUFA and total conjugated linoleic acid in both corn and sorghum distillers grains when compared to steam flaked corn.

The expected increase of PUFA and 18:2 was seen in the composite, lean, and fat samples from the shoulder clod from cattle fed distillers grains during the finishing phase. The significant increase in C16:1 and total unsaturated fatty acids (UFA) during backgrounding of cattle supplemented with higher concentrations of distillers grains suggests that higher supplementation has an effect on fatty acid composition but not to the extent of the finishing diet. The lack of significant differences in PUFA during backgrounding may suggest that feeding distillers grains causes incorporation of polyunsaturated fatty acids into the phospholipid membranes at similar amounts, regardless of concentration fed during backgrounding. This could be that even the slight increase in PUFA, even though not significant, seen in high supplementation of distillers grains during backgrounding was enough to increase oxidation susceptibility.

### 3.3 Lipid Oxidation—Raw Patties

As expected, there was an increase (P<0.001) over simulated retail display time for TBARS concentrations (Table 3) but backgrounding nor finishing diet did not impact lipid oxidation of ground beef links (P = 0.53 and 0.47, respectively, data not shown). This agrees with a companion study conducted on strip loin steaks from the same group of cattle where there were no dietary effects on lipid oxidation (Sudbeck, Varnold, MacDonald, Calkins, & Erickson, 2014). In contrast, Koger et al. (2010) observed increased TBARS values on d 7 of display in
ground beef from cattle finished on both DDGS and WDGS at 40% inclusion. Grinding of meat products disrupt the phospholipid membranes and allows increased exposure to oxygen, thus increasing the rate of lipid oxidation when compared to intact steaks (Sato et al., 1971). It may be that no dietary differences were observed for lipid oxidation in raw patties as the oxygen environment post grinding provided an optimal environment for lipid oxidation.

3.4 Objective Color and Subjective Color – Raw Patties

For objective color, both a* and b* values decreased (P < 0.0001) over time regardless of treatment (Table 4). Both finishing diet and day had an impact (P < 0.03) on L* values. Patties from heifers fed MDGS had lower (P=0.03) L* values compared to heifers fed corn gluten feed during the finishing phase (Table 4) and L* increased (P<0.001) as days of simulated retail display increased independent from diet (Table 4). Koger et al. (2010) did not see any differences in objective color values due to diet. The differences observed in our study could be attributed to the extended period of time that DGS was fed.

There was a finishing diet by day interaction (P<0.001) for percent discoloration of ground beef patties, where patties from heifers fed MDGS were observed to have a greater (P<0.02) discoloration on d 3, 5, and 6 when compared to patties from heifers finished with corn gluten feed (all other days were similar; P>0.19; Table 5). The main effects of backgrounding and finishing diet were not significant (P>0.65). Sudbeck et al. (2014) showed a lack of main dietary effects on discoloration of strip loins from the same cattle used in this experiment for both backgrounding and finishing. Additionally, Koger et al. (2010) did not observe a dietary difference during finishing on discoloration of ground beef from cattle fed DGS compared to corn, however discoloration in their study was only analyzed on day 0, 2 and 7 and cattle were
not supplemented DGS during any other phase of production. The daily discoloration evaluation may have helped to detect dietary differences observed in this study. The extended supplementation during summer grazing of DGS seems to have contributed to discoloration.

Despite the lack of dietary effects on TBARS concentrations, finishing diet showed an increase in L* values of beef from cattle fed corn gluten feed compared to MDGS. The increase in PUFA concentration in the composite sample of cattle finished on MDGS compared to corn gluten feed may explain decreased brightness observed, since an increase in PUFA has been linked to oxidation of both myoglobin and lipid. All samples became darker and less red over time, regardless of dietary treatment. Beef patties discolored at a faster rate than strip steaks from the same dietary treatments in a companion study (Sudbeck et al., 2014). This is expected, since grinding increases availability to oxygen, creating an atmosphere more prone to lipid oxidation. The results of this trial suggest that while there were no differences in lipid oxidation regardless of dietary treatment, ground beef from heifers finished with MDGS discolored at a greater rate compared to ground beef from heifers finished with corn gluten feed.

3.5 Lipid Oxidation – Cooked Links

An antioxidant concentration by day interaction (P<0.05) for refrigerated cooked link oxidation was observed, however no significant dietary treatment interactions or main effects were observed in the full factorial analysis. The lack of dietary effects (P >0.16) is likely due to the addition of antioxidants masking differences. Therefore, subsequent analyses using only samples without added antioxidant was conducted to independently determine dietary impacts. In the full analysis, links with no added antioxidants were more oxidized at d 9, 12, 15 and 18 (P ≤ 0.0001 for all) than any treatments containing antioxidants on any day (Figure 2). There were no (P>0.05) differences in lipid oxidation between any day when using 0.13 or 0.20%
concentrations of antioxidants. The sensory perception threshold of TBARS been reported to be between 1 and 2 mg malonaldehyde/ kg of sample (Campo et al., 2006, Greene & Cumuze, 1981, Tarladgis, Watts, Younathan, Dugan Jr, 1960). Ahn, Grün, and Mustapha (2007) determined that cooked ground beef with no added antioxidants was already above the general perception of the upper threshold of 2 mg/malonaldehyde at day 0, and was significantly higher than ground beef with grape seed extract, pine bark extract or oleoresin of rosemary, at all time periods. Additionally, grape seed extract, pine bark extract and rosemary oleoresin were below the threshold through 9 d of refrigerated storage. Our results indicate that cooked ground beef links with the addition of antioxidant at 0.13% or 0.20% were below this threshold for up to 18 d of storage.

When only 0%-added antioxidant samples were analyzed as a 2 × 2 factorial diet treatment, significant winter backgrounding diet by day (P=0.008) and finishing diet by day (P=0.02) interactions were identified. During winter backgrounding, there were no differences (P>0.05) in lipid oxidation between cattle fed 0.91 kg and 2.27 kg of MDGS on days 0, 3 and 6 of refrigerated storage. However, cattle fed 2.27 kg of MDGS during backgrounding had greater lipid oxidation than cattle fed 0.91 kg of distillers grains for days 9, 12 and 18 (P < 0.02; Figure 3). This suggests that feeding MDGS at any point in production can increase lipid oxidation but greater backgrounding supplementation may have a greater impact. For the finishing diet by day interaction, there was an increase in lipid oxidation for days 0, 3, 6, 9 and 12 for cattle fed MDGS whereas cattle finished on corn gluten feed had little increase in oxidation until day 12. However only on day 9 was the oxidation of beef from cattle fed MDGS greater than those fed corn gluten feed (P = 0.02; Figure 4). Additionally, ready-to-eat beef links from cattle fed 2.27 kg/hd/day of MDGS during backgrounding had greater TBARS concentration with extended
storage than those from cattle fed 0.91 kg/hd/day. This agrees with the work of Dierks et al. (2014), where they found an increase in lipid oxidation on both refrigerated and frozen cooked links from cattle supplemented DGS during backgrounding. Gunn et al. (2009) did not see a finishing dietary effect on raw top round samples, however the samples were only aged for 7 d in a vacuum package, then frozen until TBARS analysis, so no exposure to oxygen nor cooking occurred. Comminution and cooking likely intensifies the susceptibility of beef from cattle supplemented distillers grains, hence the increase in TBARS values of beef from cattle supplemented distillers grains during finishing earlier in storage (Sato & Hegarty, 1971). This concept also applies to beef from cattle supplemented higher concentrations of DGS during backgrounding. Despite this, all cooked beef links without antioxidants regardless of diet exceeded the 2 mg/malonaldehyde threshold after day 6 of refrigerated storage, hence highlighting the further benefits of antioxidants.

For beef links in frozen storage, there was a time effect ($P<.0001$) where 0 d was less oxidized ($P<.0001$) than the other days. Additionally, the control samples had greater TBARS concentrations for day 28, 56, 112, 140 and 168 than samples with either amount of added antioxidant (Figure 5). This is in agreement with the results from beef links in refrigerated storage where no differences in lipid oxidation were observed among 0.13 and 0.20% antioxidant concentrations (Figure 5). In frozen cooked ground beef, diet or antioxidant concentration (0.13% and 0.20%) did not have any effect on oxidation. All cooked link samples with added antioxidant were below the 2 mg malonaldehyde/ kg of sample threshold. Very little increase in lipid oxidation was seen beyond day 0, indicating that frozen storage can slow the rate of lipid oxidation.
As with refrigerated cooked beef links, a subsequent analysis was conducted on samples without added antioxidants to determine the dietary impact on lipid oxidation in frozen beef links. Dietary by time interactions were observed for backgrounding diet by day and finishing diet by day interactions ($P = 0.02$ and $0.01$, respectively). In backgrounding, both supplementation concentrations increased from day 0 to 28 of frozen storage. Oxidation from the higher supplementation amount of DGS continued to increase with time whereas the low supplementation cattle plateaued but was only greater on day 168 ($P = 0.04$; Table 6). For finishing, cattle finished on MDGS had higher TBARS values on day 0 and 56 of storage ($P = 0.04$ and $0.001$, respectively; Table 6).

Regardless of dietary treatment, the addition of rosemary and green tree extract (0.13% and 0.20%) was effective limiting lipid oxidation in refrigerated or frozen storage. The lack of dietary effects when antioxidants were included in the TBARS analysis is likely due to effectiveness of the antioxidant in limiting oxidation. Analysis of only samples without added antioxidants indicate a dietary effect where high supplementation of distillers grains during backgrounding had higher TBARS values than low supplementation on days 9, 12, and 18 of storage. This agrees with the research conducted by Dierks et al. (2014), which determined that supplementation of distillers grains during backgrounding increased lipid oxidation of cooked beef samples, regardless of finishing diet. During finishing, feeding MDGS showed higher TBARS values for day 9. This is in contrast to Dierks et al. (2014) who reported a trend that a MDGS finishing diet had less oxidation than a corn finishing diet.
Conclusion

Feeding distillers grain during backgrounding and finishing phases of production impacted cooked and raw ground beef quality. Feeding distillers grains during the finishing phase increased the PUFA concentration in composite, lean and subcutaneous fat samples while backgrounding diet only increased UFA and UFA:SFA ratio in subcutaneous fat samples. Retail shelf life of raw ground beef fed distillers grains during finishing is shortened due visual discoloration, while the shelf life in cooked beef is reduced due to lipid oxidation by feeding distillers products at any phase of production. Feeding distillers grains in the backgrounding phase has a significant effect on the finished product, which is then masked when WDGS are fed in the finishing phase. Cooking magnifies dietary effects on TBARS concentrations. However, the addition of antioxidant compounds has the ability to counteract any dietary impact of feeding distillers grains in both frozen and refrigerated cooked beef links. These findings highlight impacts of feeding ethanol co-products on raw and cooked ground beef and demonstrate the need to use added antioxidants in value-added beef products.
4. Tables and Figures

Table 1. Effect of finishing diet on fatty acid composition (mg fatty acid /100g raw sample) of beef shoulder clod composite, lean and subcutaneous fat samples

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<tr>
<th>Composite†</th>
<th>Corn Gluten Feed</th>
<th>Modified Distiller’s Grain</th>
<th>P-Value</th>
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<tr>
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<td>5372</td>
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<td>58878</td>
<td>0.381</td>
</tr>
</tbody>
</table>

Means in the same row with different superscripts are significantly different (P ≤ 0.05).

¹Saturated Fatty Acids: C14:0, C15:0, C16:0, C17:0, C18:0.
²Polyunsaturated Fatty Acids: C18:2.
³Monounsaturated Fatty Acids: C14:1, C16:1, C17:1, C18:1T, C18:1, C18:1V, C20:1.
†Ground composite, lean, and subcutaneous fat samples from the beef shoulder clods.
Table 2. Effect of supplementation concentration (0.91 or 2.27 kg/head/day) of wet distillers grains during backgrounding on fatty acid composition (mg/100g raw sample) of beef shoulder clod composite, lean and subcutaneous fat samples

<table>
<thead>
<tr>
<th>Backgrounding Diet</th>
<th>Composite†</th>
<th>Low</th>
<th>High</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0 (mg/100g)</td>
<td>5052</td>
<td>5091</td>
<td>0.909</td>
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</tr>
<tr>
<td>C16:1 (mg/100g)</td>
<td>652.14</td>
<td>685.76</td>
<td>0.596</td>
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</tr>
<tr>
<td>C17:0 (mg/100g)</td>
<td>324.34</td>
<td>326.04</td>
<td>0.945</td>
<td></td>
</tr>
<tr>
<td>C17:1 (mg/100g)</td>
<td>281.42</td>
<td>297.11</td>
<td>0.630</td>
<td></td>
</tr>
<tr>
<td>C18:0 (mg/100g)</td>
<td>3527</td>
<td>3172</td>
<td>0.223</td>
<td></td>
</tr>
<tr>
<td>C18:1 (mg/100g)</td>
<td>10670</td>
<td>10664</td>
<td>0.994</td>
<td></td>
</tr>
<tr>
<td>SFA¹ (mg/100g)</td>
<td>9553</td>
<td>9255</td>
<td>0.636</td>
<td></td>
</tr>
<tr>
<td>PUFA² (mg/100g)</td>
<td>695.61</td>
<td>740.20</td>
<td>0.503</td>
<td></td>
</tr>
<tr>
<td>MUFA³ (mg/100g)</td>
<td>12163</td>
<td>12236</td>
<td>0.926</td>
<td></td>
</tr>
<tr>
<td>UFA⁴ (mg/100g)</td>
<td>2262</td>
<td>2239</td>
<td>0.860</td>
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<table>
<thead>
<tr>
<th>Lean†</th>
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</thead>
<tbody>
<tr>
<td>C16:0 (mg/100g)</td>
<td>1614</td>
<td>1745</td>
<td>0.567</td>
</tr>
<tr>
<td>C16:1 (mg/100g)</td>
<td>317.74</td>
<td>330.96</td>
<td>0.838</td>
</tr>
<tr>
<td>C17:0 (mg/100g)</td>
<td>113.48</td>
<td>116.66</td>
<td>0.914</td>
</tr>
<tr>
<td>C17:1 (mg/100g)</td>
<td>97.46</td>
<td>104.08</td>
<td>0.914</td>
</tr>
<tr>
<td>C18:0 (mg/100g)</td>
<td>937.37</td>
<td>975.71</td>
<td>0.783</td>
</tr>
<tr>
<td>C18:1 (mg/100g)</td>
<td>3574</td>
<td>3699</td>
<td>0.800</td>
</tr>
<tr>
<td>SFA¹ (mg/100g)</td>
<td>2849</td>
<td>3042</td>
<td>0.628</td>
</tr>
<tr>
<td>PUFA² (mg/100g)</td>
<td>421.44</td>
<td>416.98</td>
<td>0.944</td>
</tr>
<tr>
<td>MUFA³ (mg/100g)</td>
<td>4190</td>
<td>4548</td>
<td>0.551</td>
</tr>
<tr>
<td>UFA⁴ (mg/100g)</td>
<td>1038</td>
<td>1266</td>
<td>0.300</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fat†</th>
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<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0 (mg/100g)</td>
<td>22879</td>
<td>23653</td>
<td>0.215</td>
</tr>
<tr>
<td>C16:1 (mg/100g)</td>
<td>4273b</td>
<td>5743a</td>
<td>0.020</td>
</tr>
<tr>
<td>C17:0 (mg/100g)</td>
<td>1295</td>
<td>1280</td>
<td>0.842</td>
</tr>
<tr>
<td>C17:1 (mg/100g)</td>
<td>1240</td>
<td>1497</td>
<td>0.842</td>
</tr>
<tr>
<td>C18:0 (mg/100g)</td>
<td>11894a</td>
<td>8939b</td>
<td>0.005</td>
</tr>
<tr>
<td>C18:1 (mg/100g)</td>
<td>51242</td>
<td>51340</td>
<td>0.924</td>
</tr>
<tr>
<td>SFA¹ (mg/100g)</td>
<td>39203</td>
<td>36911</td>
<td>0.075</td>
</tr>
<tr>
<td>PUFA² (mg/100g)</td>
<td>1974</td>
<td>1990</td>
<td>0.890</td>
</tr>
<tr>
<td>MUFA³ (mg/100g)</td>
<td>58279</td>
<td>60552</td>
<td>0.079</td>
</tr>
<tr>
<td>UFA⁴ (mg/100g)</td>
<td>9011b</td>
<td>11201a</td>
<td>0.006</td>
</tr>
</tbody>
</table>

ab Means in the same row with different superscripts are significantly different (P ≤ 0.05).

1Saturated Fatty Acids: C14:0, C15:0, C16:0, C17:0, C18:0.
2Polyunsaturated Fatty Acids: C18:2, C20:4.
3Monounsaturated Fatty Acids: C14:1, C16:1, C17:1, C18:1T, C18:1, C18:1V, C20:1.
4Unsaturated Fatty Acids: C14:1, C16:1, C17:1, C18:1T, C18:1, C18:1V, C18:2, C20:1, C20:4.
†Subcutaneous fat, lean and composite samples from beef shoulder clods.
Table 3. Effect of day of simulated retail display on lipid oxidation in raw ground beef patties

<table>
<thead>
<tr>
<th>Day</th>
<th>mg of malonaldehyde/kg of tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.38&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.5</td>
<td>1.64&lt;sup&gt;ed&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>2.38&lt;sup&gt;dc&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>3.11&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>6.24&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>10.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-e</sup> Means in the same column with different superscripts are significantly different ($P \leq 0.05$).
Table 4. Effect of dietary treatment and time of simulated retail display on objective color on ground beef patties

<table>
<thead>
<tr>
<th>Diet</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
<th>Day</th>
<th>SB†</th>
<th>MDGS†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>53.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.69&lt;sup&gt;dc&lt;/sup&gt;</td>
<td>52.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>51.54&lt;sup&gt;dc&lt;/sup&gt;</td>
<td>52.43&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15.07</td>
<td>14.91</td>
<td>23.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.35&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.80&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.27&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10.49</td>
<td>10.42</td>
<td>12.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.86&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9.59&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-g</sup> Means in the same row with different superscripts are significantly different (P ≤ 0.05).

†SB= Corn Gluten Feed (Sweet Bran); MDGS =Modified Distillers Grains plus Solubles.
Table 5. Finishing diet by day of simulated retail display interaction for percent discoloration of ground beef patties

<table>
<thead>
<tr>
<th>Day</th>
<th>SB†</th>
<th>MDGS†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.35&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.83&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>2.08&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>2.65&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>5.18&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>9.00&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>10.43&lt;sup&gt;e&lt;/sup&gt;</td>
<td>24.88&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>47.98&lt;sup&gt;c&lt;/sup&gt;</td>
<td>74.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>73.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>91.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.40&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a–f</sup> Means within a table with different superscripts are significantly different (<i>P</i> ≤ 0.05).

†SB - Corn Gluten Feed (Sweet Bran); MDGS – Modified Distillers Grains plus Solubles; % Dis – percent discoloration of surface on ground beef.
Table 6. Effect of backgrounding and finishing diets on lipid oxidation\(^1\) in frozen cooked beef links

<table>
<thead>
<tr>
<th>Day</th>
<th>Backgrounding</th>
<th>Finishing</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High(^\dagger)</td>
<td>Low(^\dagger)</td>
<td>MDGS(^\dagger)</td>
<td>SB(^\dagger)</td>
</tr>
<tr>
<td>0</td>
<td>0.43</td>
<td>0.4</td>
<td>0.71(^a)</td>
<td>0.11(^b)</td>
</tr>
<tr>
<td>28</td>
<td>1.7</td>
<td>1.68</td>
<td>1.6</td>
<td>1.78</td>
</tr>
<tr>
<td>56</td>
<td>1.66</td>
<td>1.71</td>
<td>2.13(^a)</td>
<td>1.25(^b)</td>
</tr>
<tr>
<td>112</td>
<td>2.18</td>
<td>1.75</td>
<td>2.08</td>
<td>1.85</td>
</tr>
<tr>
<td>140</td>
<td>2.27</td>
<td>1.74</td>
<td>2.05</td>
<td>1.96</td>
</tr>
<tr>
<td>168</td>
<td>2.65(^a)</td>
<td>1.72(^b)</td>
<td>2.22</td>
<td>2.15</td>
</tr>
</tbody>
</table>

\(^{a,b}\) Means in the same row within a feeding period with different superscripts are significantly different (P ≤ 0.05).

\(^1\) TBARS, mg of malonaldehyde/kg of sample

\(^\dagger\) High – 2.27 kg/hd/d supplementation of WDGS; Low – 0.91 kg/hd/d supplementation of WDGS; SB - Corn Gluten Feed (Sweet Bran®); MDGS – Modified Distillers Grains plus Solubles.
Received 0.91 kg DM (Low) or 2.27 kg DM (High) concentration of distillers grains supplementation during winter corn stalk grazing period

Received a distillers grains supplement at 0.6% of body weight during summer grazing period

Received a finishing diet consisting of either 40% Sweet Bran (SB) or 40% distillers grains (DGS)

Figure 1. Treatments for heifers fed distillers grains throughout a yearling beef production system
Figure 2. Effect of adding no, low, or high concentrations (0%, 0.13%, 0.20%) of rosemary and green tea natural plant extract on the lipid oxidation (mg of malonaldehyde/kg of sample) in cooked beef links during refrigerated storage.
Figure 3. Effect of supplementation concentration of wet distillers grains (0.91 or 2.27 kg/head/day DM basis) during backgrounding on lipid oxidation (mg of malonaldehyde/kg of sample) in cooked beef links during refrigerated storage.
Figure 4. Effect of feeding corn gluten feed (Sweet Bran®, Bran) or modified wet distillers grains during finishing on lipid oxidation (mg of malonaldehyde/kg of sample) in cooked beef links during refrigerated storage.
Figure 5. Effect of adding no, low, or high concentrations (0%, 0.13%, 0.20%) of rosemary and green tea natural plant extract on the lipid oxidation (mg of malonaldehyde/kg of sample) in frozen cooked beef links.
LITERATURE CITED


Effect of feeding distillers grains and supplementing with dietary antioxidants on shelf life of ground beef

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A contribution to the University of Nebraska Agriculture Research Division, Lincoln, NE 68583

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Prepared using the guide for authors for the journal Meat Science
ABSTRACT
The objective was to evaluate the effect of feeding distillers grains and dietary antioxidants on shelf life and quality of ground beef. Cattle were assigned to one of five finishing diets; corn, wet distillers grains (WDGS), WDGS + vitamin E, WDGS + Agrado Plus, or WDGS + vitamin E + Agrado Plus. Seven beef shoulder clods from each diet were ground and patties were manufactured for simulated retail display, where percent discoloration, objective color and lipid oxidation were analyzed. Cooked beef links were manufactured with salt and phosphate, placed in refrigerated or frozen storage to measure lipid oxidation. In raw ground beef no dietary treatment effects on color. All ground beef TBARS (cooked or raw) increased over time and supplementation of vitamin E sustained lower TBARS values in raw patties than corn after 2 d of simulated retail display. Diet did not impact cooked beef oxidation. An increase in PUFA and C18:2 was observed in lean and composite fatty acids in WDGS versus corn finished cattle.

Keywords: ground beef, distillers grains, vitamin E, Agrado, color, TBARS

1. Introduction

Cattle fed distillers grains have increased polyunsaturated fatty acid (PUFA) concentrations when compared to corn based diets, making them more prone to lipid oxidation (Gill, VanOverbeke, Depenbush, Drouillard, & DiCostanzo, 2008, Koger, et al., 2010). Some fats in distillers grains are protected from biohydrogenation, resulting in an increased concentration of unsaturated fatty acids in the duodenum which could later be absorbed into the tissue (Vander Pol, Luebbe, Crawford, Erickson, & Klopfenstein, 2009). Since greater unsaturation makes lipids more prone to oxidation (Schultz & Sinnhuber, 1962), polyunsaturated fatty acids tend to have less lipid stability. Polyunsaturated fatty acids are the primary lipid in phospholipid membranes, which is often the site of lipid oxidation initiation (Christie, 1978).
Grinding of meat products disrupts the phospholipid membranes and allows increases exposure to oxygen, thus increasing the rate of lipid oxidation significantly (Sato & Hegarty, 1971). Furthermore, higher temperatures observed during cooking allow the phospholipids to become more exposed and vulnerable to oxygen and free radicals, and cooking beef has been shown to increase TBARS values up to four-fold in comparison to their raw counterpart (McCarthy, Kerry, Kerry, Lynch, & Buckley, 2001).

Vitamin E cannot be synthesized by animals and must be included in the diet (Sales & Koukolová, 2010). It can function as a primary antioxidant by donating a hydrogen atom to quench free radicals (Berges, 1999). Absorption of vitamin E occurs in the lymphatic system, where it is secreted into the liver and incorporated into very-low-density lipoproteins, and transported into the interior of cells inside low-density lipoproteins (Berges, 1999). Dietary supplementation with vitamin E in the form of α-tocopherol acetate delays lipid oxidation more effectively than direct addition to meat because it allows the vitamin E to be incorporated into the phospholipid membranes rather than the neutral membranes (Ahn, Grun, & Fernando, 2002). Vitamin E is heat stable and still retains the ability to act as an antioxidant in cooked meat products (Faustman et al., 1989).

Ethoxyquin/TBHQ (Agrado Plus, Novus International, St. Louis, MO) is approved for usage in feed rations to reduce oxidation of ingredients in diets high in unsaturated fat (Maddock, Wulf, & McKenna, 2003). It is important to note that this ingredient prevents oxidation of the feed itself and does not act as an antioxidant in the body. Choat et al. (2002) reported that Agrado supplementation did not increase serum Vitamin E values directly, but could increase vitamin E concentrations over time since Agrado is able to stabilize vitamin E in the feed ration. Ethoxyquin/TBHQ has previously been shown to decrease lipid oxidation in beef (Walenciak,
Gardner, Dolezal, & Owens, 1999; Krumsiek & Owens, 1998). The contribution of Agrado to color stability has contradictory results, where Krumsiek and Owens (1998) and Choat et al. (2002) had an increased time period before discoloration of steaks in Agrado supplemented cattle compared to the control. On the contrary, Walenciak et al. (1999) did not see any differences in color stability over time. This could be contributed to the supplementation time, where Krumsiek and Owens (1998) supplemented cattle with Agrado for 28d prior to slaughter and Walenciak et al. (1999) supplemented cattle for 123d. Additionally, Krumsiek and Owens (1998) and Choat et al. (2002) studied color stability on steaks only, whereas Walenciak et al. (1999) utilized both ground and whole muscle beef. The intensity of lipid oxidation occurring in ground products could have overwhelmed the antioxidant capabilities of vitamin E available in that study.

Cleveland et al. (2014) determined that feeding distillers grains during the finishing decreased lipid stability in cooked ground beef patties. No research has been conducted to evaluating combination of feeding distillers grains and dietary antioxidants on raw or cooked ground beef products. Therefore, the objectives of this trial were to evaluate the effect of feeding distillers grains during the finishing phase and the addition of dietary antioxidants on ground beef color and lipid oxidation during simulated retail display, lipid oxidation in ready-to-eat beef links, and fatty acid composition.

2. **Materials and Methods**

2.1 **Dietary Treatments and Product Collection**

On d 14 postmortem, subcutaneous fat and lean samples were taken from each shoulder clod on the ventral side from the triceps brachii muscle for fatty acid analysis. Each shoulder clod was independently ground through a 1.27 cm plate and then through a 0.476 cm plate
(Model 4732, Hobart; Troy, OH), a composite sample taken for fatty acid analysis, and the remainder was weighed into three 2.27 kg batches. All treatments contained 0.75% salt, 0.25% sodium phosphate (Brifisol 85 instant, BK Giulini Corp., Simi Valley, CA). Beef and non-meat ingredients were mixed for 1 min and the mixture was stuffed into 2.2 cm diameter skinless links using a hydraulic piston stuffer (Talsa H31P, Talsabell S.A., Valencia, Spain) and Colosimo press (model 200, Gale Colosimo Seasoning Co., South Salt Lake, UT). After cooking, links were placed in individual foil trays for each shoulder clod and cooked to an internal temperature of 71 °C. Links were placed in oxygen impermeable zip-top bags (Ziploc® Storage Bags, 1 Gallon, S. C. Johnson, Racine, Wisconsin) and placed in either dark refrigerated storage at 2°C or dark frozen storage at -20°C until appropriate day of analysis.

2.2 Beef Link Manufacture

On d 14 postmortem, subcutaneous fat and lean samples were taken from each shoulder clod on the ventral side from the triceps brachii muscle for fatty acid analysis. Each shoulder clod was independently ground through a 1.27 cm plate and then through a 0.476 cm plate (Model 4732, Hobart; Troy, OH), a composite sample taken for fatty acid analysis. All treatments of cooked beef links contained 0.75% salt, 0.25% sodium phosphate (Brifisol 85 instant, BK Giulini Corp., Simi Valley, CA). Beef and non-meat ingredients were mixed for 1 min and the mixture was stuffed into 22 mm diameter skinless links using a hydraulic piston stuffer (Talsa H31P, Talsabell S.A., Valencia, Spain) and Colosimo press (model 200, Gale Colosimo Seasoning Co., South Salt Lake, UT). After cooking, links were placed in individual foil trays for each shoulder clod and cooked to an internal temperature of 71 °C. Links were placed in oxygen impermeable zip-top bags (Ziploc® Storage Bags, 1 Gallon, S. C. Johnson,
Racine, Wisconsin) and placed in either dark refrigerated storage at 2°C or dark frozen storage at -20°C until appropriate day of analysis.

2.3 Beef Patty Manufacture and Simulated Retail Display

On d 14, six, 113-g patties from each shoulder clod were formed using a manual, single-patty press. Patties from each shoulder clod were placed under simulated retail display for 7 d at 2.8°C. Beef patties (Two for color analysis, six half patties for TBARS, per sample) were packaged on Styrofoam trays (13.3 × 25.6 × 1.4 cm, Styro-Tech, Denver, CO), overwrapped with oxygen permeable polyvinyl chloride film (Prime Source Meat Film, Prime Source Sanitary Supply, St. George, UT) and placed on a table in a cooler maintained at 0 to 2°C under continuous 1,000 to 1,800 Lux warm white fluorescence lighting (PHILIPS F32T8/TL741 ALTO 700 Series, 32 WATT B7, Royal Philips Electronics, Amsterdam, Netherlands) to provide simulated retail display conditions.

2.4 Proximate Analysis

Moisture and total fat of pulverized meat samples (lean tissue and ground composite from each shoulder clod) were determined. Two g of pulverized tissue in duplicate were used to quantify moisture using a LECO thermo-gravimetric analyzer (LECO Corporation, model 604-100-400, St. Joseph, MI). Total fat was determined as outlined by AOAC (1990) using the Soxhlet extraction procedure.

2.5 Fatty Acid Analysis

Total lipid was extracted following the chloroform-methanol procedure of Folch et al. (1957). After extraction, the lipids were converted to fatty acid methyl esters according to
Morrison and Smith (1964) and Metcalfe et al. (1966). Following dicing, each frozen lean, composite and subcutaneous fat, samples from each shoulder clod was frozen in liquid nitrogen and powdered to ensure a homogenized sample using a Waring commercial blender (Model 51BL32, Waring Commercial, Torrington, CT). Subcutaneous fat samples were prepared using a 5 mm diameter core punch. Powdered samples and punched fats were stored at -80°C until analysis. Fatty acid composition was determined by gas chromatography (GC) after fat extraction from samples followed by formation of methyl esters of fatty acids. Powdered lean, composite, and subcutaneous fat samples (1 gram for lean and composite, 0.1 g for fat) were dissolved in 5 mL of 2:1 chloroform:methanol (v/v) to extract the lipid fraction after vortexing for 5 s and letting them stand for 1 h at room temperature. The homogenate was filtered through Watman #2 filter paper into a 13 × 150 mm screw-cap tube and the final volume was brought up to 10 mL with 2:1 chloroform:methanol mix. Then, 2 mL of 0.74% KCl solution was added to the lipid extract and vortexed for 5 s to separate extracted proteins. Samples were centrifuges at 1,000 × g for 5 m. Following centrifugation, the aqueous layer was aspirated and evaporated to dryness under nitrogen at 60°C (Folch et al., 1957). Then, 0.5 mL of 0.5 M NaOH in methanol was added, vortexed again for 5 s and heated for 5 m at 100°C under nitrogen for saponification. To methylate the fatty acids, 0.5 mL of boron trifluoride in 14% methanol was added and heated for 5 m at 100°C to methylate the fatty acids (Metcalfe et al., 1966). One mL of saturated salt solution and 1 mL of hexane were added and vortexed for 5 s. Following centrifugation at 1,000 × g for 5 m, the hexane layer was removed and placed in a GC sampling vial. Sample containing fatty acids in the GC vial was purged with nitrogen, capped and stored at -80°C until the sample was read on a Hewlett-Packard Gas Chromatograph (Agilent Technologies, model 5890A series, Santa Clara, CA) attached to a Hewlett-Packard Autosampler (Agilent Technologies, model
Fatty acid methyl esters were separated on a fused silica column (Chrompack CP-Sil 88; 0.25 mm × 100 m, Santa Clara, CA), which was placed in an oven programmed from 140°C for 10 m to 220°C at a rate of 2°C/m and held at 220°C for 20 m. Total run time was 70 m. The injector and detector were programmed to operate at 270°C and 300°C, respectively. Each lipid extract was separated into fatty acids by using helium as the carrier gas at a flow rate of 1 mL/m. Individual fatty acids of each sample were determined by comparison of retention times with known standards. Each standard contained known fatty acids that were ran through gas chromatography at the same time as the samples in order to provide a reference point to identify fatty acids in the sample. Each fatty acid was expressed as weight percentage value, which were relative proportions of all peaks observed by gas chromatography.

2.6 Objective Color Evaluation of Raw Beef Patties

The patty color was measured with a colorimeter (Chroma Meter CR-400; Konica Minolta Sensing Americas, Inc., Ramsey, NJ) using a 2° standard observer and a D65 illuminant. The calibration plate was read through polyvinyl chloride overwrap film used above since patties were still within the packaging during the color measurement. The color of three locations on each of two patties was measured, and the resulting measurements were averaged to calculate the color values for each treatment. The same two patties were evaluated throughout display time. Color was measured on days 0, 1, 2, 3, 4, 5, 6, and 7.

2.7 Subjective color evaluation

A five-person trained panel composed of graduate students of the Department of Animal Science at University of Nebraska-Lincoln subjectively evaluated the percentage discoloration. Discoloration was evaluated as percentage surface discoloration ranging from 0 to 100%. The
same two patties were evaluated throughout display time. Subjective color was evaluated on
days 0, 1, 2, 3, 4, 5, 6, and 7.

2.8 Lipid Oxidation

The 2-thiobarbituric acid reactive substance assay (TBARS) described by Ahn et al.
(1998), which was a modification of the TBARS assay developed by Beuge and Aust (1978),
was used to measure the oxidation status of refrigerated and frozen cooked, ground, beef links
and fresh ground beef patties over storage time. Refrigerated links TBARS measurements were
taken every 3 d for 18 d, beginning at d 0. Frozen links TBARS measurements were taken every
28 d for 252 d, beginning at d 0.

Fourteen mL of deionized, distilled water and 1 mL of butylated hydroxyanisole solution
(10% BHA in 90% ethanol) were added to 5 g of pulverized sample. After homogenizing for 15
s using a Polytron (POLYTRON® Kinimatica CH-6010, Switzerland), the homogenate was
centrifuged for 2,000 × g for 5 m. One mL of homogenate was mixed with 2 mL of 2-
thiobarbituric acid and trichloroacetic acid mixture (15% TCA (w/v) and 20 nM TBA in ddH2O)
and vortexed for 5 s. The sample mixture was incubated at 70°C in a water bath for 30 min to
develop color. After samples were cooled in a cold-water bath for 10 m, the sample mixture was
centrifuged at 2,000 × g for 15 m. Duplicate 200 μL aliquots of each sample were transferred
into wells on a 96-well plate and the absorbance was read at 540 nm to calculate the mg of
malonaldehyde per kg of tissue using 1,1,3,3-tetraethoxypropane as the standard solution.

2.9 Statistical Analysis

Data for TBARS (refrigerated, frozen and raw patty), fatty acid analysis, proximate
analysis, objective color and percent discoloration were analyzed for dietary treatment (dietary
antioxidant or corn control) main effects using the PROC GLIMMIX procedure of SAS (SAS Inst. Inc., Cary, NC USA) with day included as repeated measures when traits were evaluated over time. All means were separated utilizing the LSMEANS and PDIF option when level of significance indicated by ANOVA was $P < 0.05$.

**Results and Discussion**

3.1 Proximate Composition

For composite samples, there were no dietary treatment effects on moisture or fat composition ($P = 0.33$ and $P = 0.48$, respectively). However, the lean samples showed a dietary treatment effect for both moisture and fat ($P = 0.01$ and $P = 0.02$, respectively). For lean muscle samples moisture, beef from cattle on the control diet had the greatest moisture content, and cattle fed WDGSA showed the lowest moisture content. Additionally, lean portion from cattle on the control diet had the lowest fat percentage, where lean from cattle fed WDGSA had the greatest percent fat (Table 1).

3.2 Fatty Acid Composition

All fatty acid composition data can be found in Table 2. The lean portions showed treatment effects for C10:0, C17:0, C17:1, C18:1, C18:2 and PUFA ($P \leq 0.032$). With the exception of C10:0, all the fatty acids with a dietary effect listed above showed highest concentrations in sample from cattle fed MDGS, with the lowest concentrations in samples from cattle finished on the corn control diet.

For the subcutaneous fat samples, a treatment effect was observed for C15:0, C16:0, C17:0, C17:1 and C18:2 ($P \leq 0.013$). Fat from cattle finished on corn displayed lower
concentrations of C15:0 and C17:1 compared to all other dietary treatments. For C16:0, corn had greater concentrations than diets with any supplementation of vitamin E (WDGSE and WDGSAE), with WDGSA and WDGS having intermediate values. Corn-finished cattle had lower concentrations of C17:0 than all other diets except WDGSA. WDGS and WDGSE-finished cattle had greater concentrations of C18:2 than corn-finished cattle, with WDGSA and WDGSAE similar to all treatments.

For the composite (ground) samples, a treatment effect was observed for C15:0, C16:1, C17:0, C17:1, C18:0, C18:1T, C18:2, C20:3\(\omega_6\), UFA, SFA:UFA and PUFA \((P \leq 0.027)\). Fatty acid C15:0 and SFA:UFA in ground beef from a corn finishing diet were lower than all other dietary treatments. Corn finished cattle also had lower concentrations of C17:0, C20:3\(\omega_6\) and PUFA than WDGS finished cattle, with all other diets similar to all treatments. For C17:1, C18:1T and UFA, corn finished cattle had lower concentrations than WDGS and WDGSE, with cattle supplemented Agrado having intermediate values. WDGSAE had lower concentrations of C18:0 than WDGS, with WDGSA, WDGSE and corn having intermediate values. In C18:2, corn had the lowest concentrations than all dietary treatments, followed by WDGSAE. WDGS had the highest C18:2 concentrations. Fatty acid C16:1 was the only fatty acid where higher concentrations were observed in corn cattle than WDGS and WDGSE, with Agrado supplemented cattle having intermediate values.

It would be expected that the shift in PUFA and C18:2 concentrations in MDGS would decrease lipid stability, which did not hold true in our raw beef study. It is likely that the dietary antioxidant counteracted the fatty acid composition differences in lipid oxidation that would be expected with increased PUFA content. The differences between fat, lean, and composite samples suggest that different fat depots have different fatty acid profiles, where lean sample
would be indicative of intramuscular fat, fat sample would represent subcutaneous fat, and the
composite sample is homogenous representation of the whole shoulder clod.

3.3 Lipid Oxidation – Raw Patties

A day by dietary treatment effect was observed for oxidation of raw patties \( P = 0.03 \). On days 2 through 7 of simulated retail display, the corn control had higher TBARS values than the patties from cattle with supplementation of vitamin E (WDGSE and WDGSAE). On days 3 and 5, the corn diet was more oxidized than all other dietary treatments. On the day 7 of simulated retail display, beef from cattle fed WDGS displayed greater TBARS values than WDGSAE (Table 3). All diets, with the exception of WDGSAE, displayed a significant increase in TBARS values from day 5 to day 7 (Table 3). The inclusion of vitamin E resulted in less lipid oxidation than the corn control on all days beyond day 0 (Table 3). In contrast, Koger et al. (2010) determined that cattle fed distillers grains (40% DM) had higher TBARS values than ground beef from cattle fed corn on d 2 and d 7 of display. The results of this study agreed with those of Chao, Domenech-Perez, Voegele, Kunze, and Calkins (2015), where intact strip steaks from the same cattle from this study showed greater lipid oxidation in the corn control than those fed WDGS. Similar results were also seen by Domenech et al. (2014) in strip steaks, where the corn control diet had higher mean TBARS values than any percentage of de-oiled distillers grains (35, 50 and 65%). This is unexpected since the increase in PUFA in MDGS fed cattle would typically result in lipid instability. However, Song et al. (2013) determined that the increased sulfur content observed in DGS increased the concentrations of sulfur-containing amino acids in beef. These amino acids are known to display antioxidant properties by acting a hydrogen donor, which could explain why our corn control was more oxidized than WDGS, despite the increase in PUFA in WDGS samples (Song et al., 2013).
3.4 Simulated Retail Display Color – Raw Patties

A time effect was observed for percent discoloration, where patties became more discolored over time in simulated retail display ($P < 0.0001$, Table 4). No dietary effects were observed ($P = 0.60$). This does not coincide with lipid oxidation, since the corn control diet had greater oxidation than all vitamin E diets but no difference in color stability. A time effect was observed for $L^*$, $a^*$ and $b^*$ values ($P < 0.0001$) (Table 4) but no dietary effects were observed for instrumental color measurements ($P = 0.39$, 0.07 and 0.60 for $L^*$, $a^*$, and $b^*$, respectively). Dietary treatment differences in TBARS concentrations observed in raw patties did not correlate to color stability in this study. In a previous study completed on ground beef stability from cattle fed DGS, percent discoloration was higher and $L^*$ values were lower for cattle supplement MDGS versus corn gluten feed in the finishing phase (Buntyn et al., 2014). The increase in time on DGS and concentration could heighten the dietary effects on color stability.

3.5 Lipid Oxidation– Cooked Beef Links

An expected a day of storage effect was observed for lipid oxidation in cooked beef links in refrigerated storage where TBARS concentrations increased over time ($P = 0.01$; Table 5). A trend for dietary effect was observed, where beef links from WDGS + Agrado cattle tended to have higher TBARS values than the other dietary treatments ($P = 0.10$). Since this effect was not observed in raw patties from the same shoulder clod, it may suggest that Ethoxyquin (EQ) or TBHQ is heat sensitive and gains pro-oxidative activities after cooking. Phenolic antioxidants under the right conditions may be converted to phenoxy radicals with prooxidant activity (Błaszczyk, Augustyniak, & Skolimowski, 2013). Therefore, ethoxyquin nitroxide, which is
produced by EQ oxidation, may show prooxidative properties when exposed to oxidative stress (Błaszczyk et al., 2013).

As with beef links in refrigerated storage, frozen beef links had greater oxidation with increased storage. \((P = <0.0001; \text{Figure 1})\). No dietary treatment effects were found \((P = 0.13)\). As dietary treatment effects on lipid oxidation of beef links during refrigerated storage was only a trend, this may not indicate major changes.

3 Conclusion

Raw ground beef patties discolored over time, and no dietary treatment effects were observed. Conversely, the TBARS concentrations showed that patties from cattle fed the corn control diet during finishing were among the most oxidized. A trend for refrigerated, cooked beef links suggests that cattle fed WDGSA had greater lipid oxidation than other dietary treatments and may be due to heating causing EQ to become oxidized, and acting as a pro-oxidant. These dietary effects did not carry through to the frozen links, however, suggesting that frozen storage may reduce the pro-oxidant effect. As expected, an increase in C18:2 (linoleic acid) was seen for all samples from cattle finished on MDGS, as well as an increase in PUFA in lean and composite samples. The lack of relationships between fatty acid composition, raw patty color stability, and lipid oxidation in raw and cooked ground beef may be related to the effectiveness of vitamin E as an antioxidant in raw and cooked ground beef.
4 Tables and Figures

Table 1. Effect of dietary treatment on percent moisture and fat of lean muscle samples from beef shoulder clod

<table>
<thead>
<tr>
<th>Diet</th>
<th>Percent Fat</th>
<th>Percent Moisture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control&lt;sup&gt;1&lt;/sup&gt;</td>
<td>3.96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.48&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WDGS&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>73.04&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>WDGSE&lt;sup&gt;3&lt;/sup&gt;</td>
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<td>WDGSA&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>WDGSAE&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.60&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>74.47&lt;sup&gt;abc&lt;/sup&gt;</td>
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</table>

<sup>a-c</sup> Means in the same row with different superscripts are significantly different ($P \leq 0.05$).

<sup>1</sup> Corn control finishing diet.
<sup>2</sup> Wet distillers grains at 30% DM inclusion.
<sup>3</sup> Wet distillers grains + 1000 IU/hd/d vitamin E.
<sup>4</sup> Wet distillers grains + 3 g/hd/d Agrado Plus.
<sup>5</sup> Wet distillers grains + 500 IU/hd/d vitamin E + 3 g/hd/d Agrado Plus.
Table 2. Effect of finishing diet on fatty acid composition (mg/100g raw sample) of beef shoulder clod composite, lean tissue and subcutaneous fat samples

<table>
<thead>
<tr>
<th>Composite</th>
<th>Corn</th>
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<th>WDGSAE</th>
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<td>PUFA (mg/100g)</td>
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<td>1973ab</td>
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ab Means in the same row with different superscripts are significantly different (P ≤ 0.05)

-- Indicates specific fatty acid was not recovered from the sample.

1 Unsaturated Fatty Acids: C14:1, C15:1, C16:1, C17:1, C18:1T, C18:1, C18:1V, C18:2TT, C18:2, C18:3o3, C18:3o6, C20:1, C20:3, C20:3o6, C20:4, C20:3, C22:1, C22:4, C22:5.

2 Saturated Fatty Acid to Unsaturated Fatty Acid Ratio.

3 Polyunsaturated Fatty Acids: C18:2TT, C18:2, C18:3o3, C18:3o6, C20:1, C20:3, C20:3o6, C20:4, C20:3, C22:1, C22:4, C22:5.

4 Corn control finishing diet.

5 Wet distillers grains at 30% DM inclusion.

6 Wet distillers grains + 1000 IU/hd/d vitamin E.

7 Wet distillers grains + 150 ppm/hd/d Agrado Plus.

8 Wet distillers grains + 500 IU/hd/d vitamin E + 150 ppm/hd/d Agrado Plus.
Table 3. Effect of day of simulated retail display by dietary treatment interaction ($P = 0.03$) on lipid oxidation$^\dagger$ in raw ground beef patties

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<td>$3.21^{cde}$</td>
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$^a$ Means in the same table with different superscripts are significantly different ($P \leq 0.05$).

1 Corn control finishing diet.

2 Wet distillers grains at 30% DM inclusion.

3 Wet distillers grains + 1000 IU/hd/d vitamin E.

4 Wet distillers grains + 3 g/hd/d Agrado Plus.

5 Wet distillers grains + 500 IU/hd/d vitamin E + 3 g/hd/d Agrado Plus.

$^\dagger$ All means for lipid oxidation presented as mg of malonaldehyde/kg of sample.
Table 4. Effect of day of simulated retail display on percent discoloration objective color of raw ground beef patties

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<th>a*</th>
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<sup>a-h</sup>Means in the same row with different superscripts are significantly different ($P \leq 0.05$).
Table 5. Effect of days of refrigerated storage on lipid oxidation in cooked beef links

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<td>18</td>
<td>4.88$^a$</td>
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$^a$: Means in the same column with different superscripts are significantly different ($P \leq 0.05$)

$^1$: mg of malonaldehyde/kg of sample
Figure 1. Effect of display time on lipid oxidation (mg of malonaldehyde/kg of product) in frozen cooked beef links
*Means in the same row with different superscripts are significantly different ($P \leq 0.05$).
Literature Cited


5. **Recommendations for Future Research**

These studies showed the effectiveness of natural plant extract and dietary vitamin E on increasing shelf life of both cooked and raw ground beef. Continuing to evaluate the effect of distillers grains with different processing methods or grain source, wheat distillers, for example, would be a good way to compare and contrast the different types of distillers and grain source. Additionally, a study conducted using a combination of the both of the antioxidant approaches would be helpful to determine if there are any synergistic impacts on shelf life. Further research on the stability and effect of Agrado in tissues would be necessary, particularly in the realm of the effect of heat treatment on antioxidant capabilities of Agrado. Guidelines could be established for heat tolerance of different types of antioxidants to determine if certain antioxidants gain pro-oxidative activity or lose their antioxidant abilities with different levels of heat. Finally, an economic perspective can be evaluated on the antioxidants, in terms of cost analysis for large scale producers and to what extent will the consumer pay for products with natural antioxidants added.
6. Appendices
Thiobarbituric Acid Assay:
Buege and Aust (1978), Modified by Ahn et al. 1998

TEP Solution (1,1,3,3-Tetraethoxypropane) (Make new weekly)
Stock Solution: Dilute 99 μl TEP (97%) bring volume to 100 mL ddH₂O
Working Solution: Dilute stock solution to 1:3 (TEP Solution:ddH₂O) (1x10⁻³M)

TBA/TCA (2-Thiobarbituric Acid/Trichloroacetic Acid) Stock Solution: 1L
15% TCA (w/v) and 20 mM (MW 144.5) reagent in ddH₂O.
Dissolve 2.88 g TBA in warm ddH₂O first, then add TCA (150g) and ddH₂O to 1L

BHA (ButylatedHydroxyAnisole) Stock Solution
Make 10% stock solution by dissolving in 90% ethanol
10g BHA dissolved in 90 mL ethanol (90%) + 5mL ddH₂O

Standards: In duplicate
Blank: 1 mL ddH₂O  Moles of TEP
Standard 5: 100 μL working TEP + 1.90 mL ddH₂O       (5x10⁻⁵M)
Standard 4: 1 mL Std. 5 + 1 mL ddH₂O                   (2.5x10⁻⁵M)
Standard 3: 1 mL Std. 4 + 1 mL ddH₂O                   (1.25x10⁻⁵M)
Standard 2: 1 mL Std. 3 + 1 mL ddH₂O                   (.625x10⁻⁵M)
Standard 1: 1 mL Std. 2 + 1 mL ddH₂O                   (.3125x10⁻⁵M)
Remove 1 mL of Standard 1 and discard it, leaving 1 mL behind.

Procedure
- Mix all reagents and standards before beginning
- Transfer 5 g of powdered sample into a 50 mL conical tube, add 14 mL of ddH₂O and 1.0 mL of BHA (Butylated hydroxyanisole)
- Homogenize for 15 sec with a polytron
- Centrifuge for 2000xg for 5 minutes
- Transfer 1 mL of homogenate or standard to 15 mL conical tube
- Add 2 mL of TBA/TCA solution, vortex
- Incubate in a 70°C water bath for 30 min to develop color
- Cool samples in a cold water bath for 10 min
- Centrifuge tubes at 2000xg for 15 min
- Transfer duplicate aliquots of 200 μL from each tube into wells on a 96 well plate
- Read absorbance at 540nm

Calculations: mgs of malonaldehyde/kg of tissue
K(extraction) = (S/A) x MW x (10⁶/E) x 100
Where  S = Standard concentration (1x10⁻⁸ moles 1,1,3,3-tetraethoxypropane)/5mL
        A = Absorbance of standard  MW = MW of malonaldehyde (72.063 g/mole)
E = sample equivalent (1) P = Percent recovery

**Final calculation:** \(0.012 \times \text{concentration} \times 72.063 \times 10^{-6} = \text{mgs of Malonaldehyde/kg of tissue}\)

Reagents (Sigma): TBA- T5500; TCA- T9159; TEP- T9889; BHA- B1253

**References:**


FATTY ACID DETERMINATION

Weigh out 1 g of pulverized muscle tissue. If extracting subcutaneous fat, weigh out 0.1 g of pulverized subcutaneous fat into centrifuge tube.

1. Add 5 mL of 2:1 choloform:methanol (v/v) for muscle tissue or 3 mL for subcutaneous fat.
2. Vortex for 5 s and let stand for 1 h at room temperature.
3. Filter homogenate through Whatman #2 filter paper into 13 x 150 mm screw cap tube bringing the final volume with choloform:methanol to 10 mL for muscle lipid and 5 mL for subcutaneous fat extract. If stopping at this point, purge test tube with nitrogen, cap tube, and store at -80°C.
4. Add 2 mL of a 0.74% KCl solution for muscle lipid extract or 1 mL for subcutaneous fat tissue extract and vortex for 5 s. If stopping at this point, purge test tube with nitrogen, cap tube, and store at -80°C.
5. Centrifuge samples at 1000 x g for 5 min. Following centrifugation, aspirate off the aqueous phase (top layer). If stopping at this point, purge test tube with nitrogen, cap tube, and store at -80°C.
6. Evaporate to dryness under nitrogen at 60°C.
7. Add 0.5 mL of a 0.5 M NaOH in methanol. Vortex for 5 sec. Heat for 5 min at 100°C
8. Add 0.5 mL of boron triflouride in 14% methanol. Vortex for 5 sec. Heat for 5 min at 100°C
9. Add 1 mL of saturated salt solution and 1 mL of hexane. Vortex for 5 sec.
10. Centrifuge samples at 1000 x g for 5 min. Following centrifugation, remove hexane layer (top layer) making sure not to disrupt the aqueous phase (lower layer) and place in GC vial. Purge GC vial with nitrogen, cap and crimp cap, and store at -80°C until sample is ready to be read on the GC.

GC Settings
Column- Chrompack CP-Sil 88 (0.25 mm x 100 m)
Injector Temp- 270°C
Detector Temp- 300°C
Head Pressure- 40 psi
Flow rate- 1.0 mL/min

Temperature Program- Start at 140°C and hold for 10 min. Following 10 min, raise temperature 2°C/min until temperature reaches 220°C. At 220°C, hold for 20 min.
References:


### P-Values for all Main Effects and Interactions for Manuscript 1

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