

EFFECTS OF PACKAGING AND TEMPERATURE
ON METMYOGLOBIN REDUCING ACTIVITY
OF COOKED GROUND BEEF PATTIES

By

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Title of Study: EFFECTS OF PACKAGING AND TEMPERATURE ON
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Abstract: Premature browning (PMB) is a condition wherein ground beef will have a well-done appearance before reaching the USDA recommended 71.1 °C to render meat safe for consumption. It has been estimated that 47% of ground beef is susceptible to PMB. This is a major safety concern. The mechanism of PMB is not fully understood. Therefore, the objectives of the study were to determine the role of metmyoglobin reducing activity of cooked ground beef in PMB and the effects of temperature and pH on the thermal stability of NADH-dependent reductase (NDR), lactate dehydrogenase (LDH), and oxymyoglobin (OxyMb) in vitro. Ground beef patties were packaged in high oxygen modified atmosphere (HiOX-MAP) or vacuum (VP), and cooked to 65.5 or 71.1 °C. Reducing activity was measured in both raw and cooked ground beef patties. Lactate dehydrogenase, NDR, and OxyMb were prepared at pH 5.6 and 6.4 and heated at different endpoint temperatures. The results indicated that both reducing activity of raw and cooked ground beef were dependent on temperature and packaging ($P < 0.05$). Thermal stability of oxymyoglobin, NDR, and LDH were different and pH dependent ($P < 0.05$). The NADH-dependent reductase was the least heat stable ($P < 0.05$) and showed no activity at 65 °C ($45 > 50 > 55$, $P < 0.05$). Lactate dehydrogenase was able to generate NADH even at 84 °C. Percentage of OxyMb denaturation increased along the temperature ($65 < 71 < 77 = 84$, $P < 0.05$). The proteins were more stable at pH 6.4 compared to pH 5.6 ($P < 0.05$). The results indicate that enzymes can generate NADH at cooking temperature. Hence, strategies that can increase reducing activity have the potential to limit incidence of PMB.

Key words: cooking, enzymes, metmyoglobin reducing activity, premature browning, thermal stability.

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CHAPTER I

INTRODUCTION

Cooked color is primarily due to the Maillard reaction and myoglobin denaturation. More specifically, the presence of either ferro- or ferrihemochrome will impart brown and red cooked color, respectively (Warren et al., 1996b; Lytras, Geilesky, King, & Ledward, 1999). Most of the consumers determine degree of doneness by checking the internal color of beef patties (USDA-ARS/FSIS, 1998; USDA FSIS, 1998; Killinger et al., 2000; Ralston et al., 2002). This can be a food safety concern as premature browning (PMB) is a condition in which ground beef patties will exhibit an internal brown and well-done appearance at temperatures lower than the USDA recommended 71.1°C to eliminate pathogenic bacteria (Hague et al., 1994; Hunt, Sørheim, & Slinde, 1999; USDA FSIS, 1998). A previous study indicated that approximately 47% of ground beef sold in the US is susceptible to PMB (Killinger et al., 2000). Although the mechanism of PMB is not clear, research indicated that the myoglobin form present within the interior of patties can influence cooked color (Hunt, Sørheim, & Slinde, 1999).

Introduction of centralized case ready facilities has allowed meat purveyors to use modified atmospheric packages such as high-oxygen (HiOX-MAP) to stabilize oxymyoglobin. Greater levels of oxygen within packages allow oxymyoglobin to penetrate further into the interior and can increase the incidence of PMB in comparison to the traditional polyvinyl chloride (PVC) overwrap film and vacuum package (Seyfert et al., 2004; Jayasingh et al., 2002). Previous research indicated that addition of antioxidants or reducing agents such as sodium hydrosulfite

can limit PMB by the formation of deoxymyoglobin (Suman et al., 2011). This indicates that if meat has greater reducing capacity, there is potential to limit PMB.

Metmyoglobin reducing activity (MRA) is the ability of the postmortem muscle to limit the formation of metmyoglobin by converting metmyoglobin to deoxymyoglobin (Bekhit & Faustman, 2005). This can occur by both enzymatic and non-enzymatic pathways (Bekhit & Faustman, 2005). An important factor that influences MRA is NADH. Kim et al. (2006) reported that NADH can be regenerated by lactic dehydrogenase activity. Similarly, among the various dehydrogenase systems characterized, NADH-dependent reductase has received special attention and has been shown to reduce metmyoglobin to deoxy- or oxymyoglobin (Hagler, Coppes, & Herman, 1979). This indicates that if meat has enough MRA, it has the potential to limit PMB by regenerating deoxymyoglobin. However, limited research has determined MRA of cooked ground beef patties. Similarly, limited studies have compared the thermal stabilities of LDH and NADH-dependent reductase. Therefore, the objectives of this study were to determine 1) the effects of temperature and packaging on metmyoglobin reducing activity of cooked ground beef patties, and 2) the effects of temperature and pH on thermal stability of LDH, NADH dependent reductase, and oxymyoglobin in-vitro.

CHAPTER II

LITERATURE REVIEW

MEAT COLOR

In today's market, the acceptability of whole muscle and ground beef is influenced by consumers' visual perception. Consumers use color as a benchmark to evaluate freshness and wholesomeness of fresh whole and minced beef (Mancini & Hunt, 2005; Faustman, Sun, Mancini, & Suman, 2010; Suman & Joseph, 2013). A bright, cherry-red color is preferred and associated with freshness and wholesomeness. On the other hand, discoloration of the cherry-red to brown or grayish color leads to rejection or reluctance to buy the product. Hood and Riordan (1973) showed that consumers' rejection was linearly correlated with the amount of surface discoloration in meat. A surface discoloration of 20% was associated with 1 in 2 probability that consumers deem the product unappealing and reject it (Hood & Riordan, 1973). It has been estimated that discoloration costs the US meat industry approximately \$1 billion (Smith et al., 2000).

MYOGLOBIN CHEMISTRY

The color of postmortem muscles of well bled animals is attributable to myoglobin and to a lesser extent hemoglobin and cytochromes (Suman and Joseph, 2013). Myoglobin (Mb) is a water soluble monomeric hemoprotein comprised of a globin and a prosthetic protoporphyrin IX ring moiety (Richards, 2013). The ring moiety, or heme group, is located in the hydrophobic core of the protein. The heme group contains an iron in the center. The iron has six coordination sites.

Of the six coordination sites, four are associated with nitrogen pyrrole rings of the ring group. The fifth coordination site is occupied by the proximal histidine -93. The sixth site is available to bind with biological molecules such as oxygen, carbon monoxide, nitrite oxide (Suman & Joseph, 2014). This ability confers to cardiac and skeletal muscles the function of transport and storage of oxygen in live animals (Kooyman & Ponganis, 1998; Ordway & Garry, 2004). An additional histidine, called distal, is placed in such a way that the prosthetic heme group is between the two histidine. This stabilizes the heme group in the heme crevice. The primary structure of myoglobin consists of 153 amino acids and has a molecular weight of 17.5 Kilodalton (KD). The globin moiety is a single polypeptide chain that is folded and wraps around the central heme iron and prevents from oxidation. The globin chain is composed of eight α -helices, namely, A through H, and is responsible for the water solubility of the prosthetic heme group. The presence of conjugated double bonds in the protoporphyrin ring confers myoglobin its light absorption properties broadly used to study the protein (Richards, 2013; Ordway & Garry, 2004; Suman & Joseph, 2013; Suman & Joseph, 2014).

Depending on the valence state of iron and type of ligand, myoglobin exists in three forms. Myoglobin is in a reduced form when the central iron is in ferrous state (Fe^{++}). For example, both deoxymyoglobin (DeoxyMb) and oxymyoglobin (OxyMb) are in reduced form. Deoxymyoglobin typically exists in freshly cut surfaces or under anaerobic conditions. Oxymyoglobin forms when meat is exposed to oxygen. This phenomenon is termed blooming. Oxymyoglobin provides consumer preferred cherry-red color. This form is present in oxygen permeable or HiOX-MAP products (Suman & Joseph, 2014). Oxidation of oxymyoglobin form results in formation of metmyoglobin. Metmyoglobin (MetMb) is responsible for surface discoloration and consumers' rejection (Ledward, 1970; Ledward, 1985; Hood & Riordan, 1973).

MYOGLOBIN AND LIPID OXIDATION

Several studies have investigated the relationship between lipid and myoglobin oxidation (Greene, Hsin, & Zipser, 1971; Mitsumoto et al., 1991; Chan et al., 1997; O'Grady, Monahan, & Brunton, 2001). Greene, Hsin, and Zipser (1971) reported a simultaneous increase in myoglobin and lipid oxidation, one favoring the other. Myoglobin oxidation generates free radicals that initiate and favor lipid oxidation, which in turn enhances myoglobin oxidation.

Hemoproteins in the ferric state are responsible for catalyzing lipid oxidation of meats. More specifically, released heme iron and hydrogen peroxide (H_2O_2) from myoglobin oxidation facilitate oxidative reactions and subsequent lipid oxidation. Lipid oxidation products are highly reactive and promote myoglobin oxidation by causing heme decomposition and subsequent color loss (Greene, 1969; Rhee, Ziprin, & Ordonez, 1987). Lipid oxidation results in organoleptic (off-flavor and off-odors) and nutritional deterioration (Ladikos & Lougovois, 1990). Presence of unsaturated fatty acids, oxygen, and prooxidants in meats is critical for lipid oxidation (Faustman et al., 2010). Lipid composition and the degree of unsaturation play a determinant role in lipid oxidation (Bhattacharya, Hanna, & Mandigo, 1988). In particular, phospholipids have been identified as primarily responsible for lipid oxidation (Greene, 1969; Greene, Hsin, & Zipser, 1971; Bhattacharya, Hanna, & Mandigo, 1988; Love & Pearson, 1971).

The contribution of triglycerides to lipid oxidation is time-dependent. Lipid oxidation increases as storage time prolongs and intervenes following oxidative rancidity of phospholipids (Bhattacharya, Hanna, & Mandigo, 1988). Interactions of lipid products with other food components such as pigments, proteins, carbohydrates, and vitamins result in organoleptic and nutritional deterioration of meats (Love & Pearson, 1971; Baron & Andersen, 2002; George & Stratmann, 1954). Products generated by oxidative reactions include, but are not limited to aldehydes, ketones, alcohols, hydrocarbons, furans, lactones, and esters (Frankel, 1984). Ground

beef is more prone to lipid oxidation compared to whole muscle meats as grinding increases surface area, mobility of reagents, and muscle disruption (Jayasingh et al., 2002). The different steps in lipid oxidation are summarized as follows:

Initiation $RH \rightarrow R\bullet$

$R^1-CH=CH-R'' + O_2 \rightarrow ROOH$

Propagation $R\bullet + O_2 \rightarrow ROO\bullet +$

$ROO\bullet + RH \rightarrow ROOH + R\bullet$

$ROOH \rightarrow RO\bullet + OH\bullet$

Termination $R\bullet + R\bullet \rightarrow$ Nonradical products

$2ROO\bullet \rightarrow R_2 - CO - R_2 + R_2 - CHOH - R_2 + O_2$

Lipid oxidation product such as malonaldehyde (MDA) and 4-hydroxy-2-nonenal have been shown to decrease activity of enzymes related to meat color (Ramanathan et al., 2014).

CASE-READY PACKAGING

In an effort to meet consumers' preferences for shelf and color stable meats, the meat industry has adopted case-ready packaging (Balev et al., 2011; Han, 2005). Buffo and Holley reported that centralized packaging systems reduce time-consuming labor required in a retail store. Further, meat fabrication is more homogeneous in a centralized operation. In the traditional meat distribution system, primal and subprimal cuts are transported in vacuum packages to retail stores where they are further processed into consumer-size cuts. Consumer-size cuts are packaged in retail trays and overwrapped with oxygen-permeable polyvinyl chloride (PVC) films (Buffo & Holley, 2005). Even though blooming is achieved, the display shelf life of the bright, cherry-red color is shorter than that achieved with modified atmospheric packaging (MAP). However,

research has shown that MAP has been associated with premature browning (Seyfert et al., 2004).

Under traditional oxygen permeable PVC films, oxymyoglobin forms mainly on the surface of meat cuts or ground beef patties. Deoxymyoglobin is predominantly present in the interior. However, under high-oxygen modified conditions, oxymyoglobin can penetrate further into the interior. Consequently, high percentage of oxymyoglobin in the interior makes meat susceptible to PMB (Hunt, Sørheim, & Slinde, 1999; Seyfert et al., 2004). High-oxygen modified atmosphere packaging has been shown to increase lipid oxidation with subsequent sensory quality defect (Resconi, et al., 2012). High concentrations of oxygen in MAP negatively affect color stability by the means of lipid oxidation. Resconi et al. (2012) reported greater TBARS values in high oxygen modified atmosphere packaged steaks as compared with vacuum packaged steaks.

COOKED COLOR AND PREMATURE BROWNING

Cooked color changes

Cooking is a traditional method to render food in general and meat in particular suitable for human consumption. It induces desirable traits that are particular to cooked products. Consumers have different preferences in regard of organoleptic characteristics of cooked meats (King & Whyte, 2006). Heat-induced denaturation of myoglobin and Maillard reaction are responsible for the development of the dull, brown color characteristic of cooked meat product (Ledward, 1971; King & Whyte, 2006; Bernofsky, Fox, & Schweigert, 1959).

During cooking, denaturation of the globin moiety causes myoglobin to unfold and expose the heme group to external environment. Denatured pigments coagulate and precipitate (Meersman, Smeller, & Heremans, 2002; Tornberg, 2005). Depending on the chemical state of myoglobin, a grey-brown to dull, brown ferrihemochrome under aerobic conditions and a red ferrohemochrome under anaerobic conditions are formed (King & Whyte, 2006; Bejerholm, Tørngren, & Aaslyng, 2014; Cornforth & Jayasingh, 2004). Also, larger molecular weight

compounds can attach to the iron and generate different hemochromes. Some of these hemochromes are responsible for persistent pinking in cooked pork or chicken meats (Cornforth, Calkins, Faustman, 1991). Persistent pink has been described the presence of undenatured pink hemochromes in meats cooked to a well-done degree of doneness (Cornforth, Calkins, & Faustman, 1991). This can mislead consumers who may overcook the product in order to eliminate the pink color. Persistent pinking is a quality defect in cooked products.

Conversely to pork and chicken, some ground beef appears fully cooked even though the internal temperature has not reached safe regulatory limits. This is a safety issue as likelihood of bacterial survival is higher (Hunt, Sørheim, & Slinde, 1999; King & Whyte, 2006; Cornforth, Calkins, & Faustman, 1991). Premature browning and persistent pink have led researchers to conclude that cooked color is not a reliable indicator of doneness (Hague et al., 1994; Hunt, Sørheim, & Slinde, 1999; Bigner-Georges & Berry, 2000).

Premature browning

Cooked color has become a concern because of its direct relationship to foodborne illnesses. Previous studies have described premature browning in injection-enhanced whole muscle (Seyfert et al., 2004) and ground beef (Hague et al., 1994; Warren et al., 1996a; Hunt, Sørheim, & Slinde 1999) as a condition where internal cooked color development indicates a well-done appearance at temperatures lower than the USDA recommended 71.1°C. At low temperatures pathogenic bacteria can survive. This represents a food safety risk for consumers who read degree of doneness by internal cooked color (Hunt et al, 1999). The first documented outbreaks of *Escherichia coli* O157:H7 in 1982 and 1993 were attributed to undercooked hamburgers (Davis, 1993; USDA, 1997; Phang & Bruhn, 2011).

Growing evidence has led the USDA to discourage the use of internal cooked color as an indicator of degree of doneness. Instead, thermometer use was recommended as a reliable method

to check degree of doneness (USDA-ARS/FSIS, 1998). Many researchers documented an inconsistency of cooked color in predicting the internal temperature of ground beef (Marksberry, 1990; Hague et al., 1994; Warren et al., 1996b; Hunt, Sørheim, & Slinde, 1999). Eating undercooked ground beef is a major vehicle for foodborne illnesses in the US (Rangel et al., 2005). Rangel et al. (2005) reported that *E. coli* was responsible for 33% of food related outbreaks in the US from 1982 to 2002, 41% of which were linked to ground beef. More than 26 billion pounds of ground beef are consumed in the US in a yearly basis (USDA, 2013; Beef Checkoff, 2016). Given 47% of ground beef purchased from local purveyors has been found susceptible to PMB, this represents a serious food safety challenge (Killinger et al., 2000). A survey indicated that 18% of respondents reported eating undercooked ground beef at home within 7 previous days (Taylor et al., 2012). Killinger et al. (2000) suggested that PMB may be a more serious problem at homes than in restaurants where endpoint temperatures are monitored.

Moreover, research indicated that the use of thermometer to assess degree of doneness is not widespread in homes. Consumers most likely cut open ground beef patty to visually appraise internal color (Anderson et al., 2004). Phang and Bruhn (2011) video recorded and interviewed 199 households from Northern California and reported that only 4% actually used thermometers to check doneness of their burgers.

Furthermore, 25% of foodborne diseases related to eating undercooked ground beef occurred at home (Rangel, et al., 2005; Hadler, et al., 2011). Non-O157 Shiga toxin-producing *E. coli* (STEC) infections were also linked to eating undercooked hamburger mainly prepared at home (Hadler, et al., 2011). Undercooked ground beef has also been identified as a vehicle for Salmonella and Salmonella multi-drug resistant strains infections (Centers for Disease Control and Prevention, 2013; Laufer et al., 2015). It is admitted that Salmonella and *E. coli* O157:H7 foodborne illnesses can be avoided by a thorough cooking of products to an internal temperature of 71 °C (Phang & Bruhn, 2011). Many factors have been shown to influence PMB. The valence

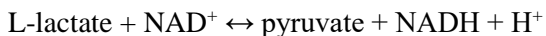
state and pH of meat or patties at the time of cooking contribute to premature browning (Warren, et al., 1996a; Warren, et al., 1996b; Hunt, Sørheim, & Slinde, 1999; Seyfert et al., 2004). Hunt, Sørheim, and Slinde (1999) reported that oxymyoglobin and metmyoglobin are more prone to PMB than deoxymyoglobin. They also reported that high pH myoglobin solutions were more heat-stable and less prone to PMB than low pH counterparts. Furthermore, Ballard (2004) classified myoglobin forms according to their thermal stabilities as follows: metmyoglobin < oxymyoglobin < deoxymyoglobin < carboxymyoglobin.

Killinger et al. (2000) reported that location within a package as well as time of purchase influence PMB. Seyfert et al. (2004) noted that PMB was dependent on packaging. Steaks packaged in HiOX-MAP were more susceptible to PMB than their vacuum and carbon monoxide packaged counterparts (Seyfert et al., 2004). Due to its high affinity to carbon monoxide, myoglobin forms a more stable bright red color.

Premature browning is also affected by reducing activity of ground beef. Patties made from cuts or muscles not aged for long time (greater reducing activity) may be less predisposed to PMB than aged meat (Madhavi & Carpenter, 1993). Greater reducing activity results in more deoxymyoglobin, which is more heat-stable. In support, Sepe et al. (2005) used reducing agents to improve presence of deoxymyoglobin and limit PMB. Mancini et al. (2011) and Phillips et al. (2001) determined the effects of succinate and erythorbic acid respectively on PMB and found that succinate increased pH and reducing activity and, subsequently, reduced occurrence of PMB. Antioxidants and substrates have also been utilized to prevent PMB (Suman et al., 2011; O'Keeffe & Hood, 1982; Phillips et al., 2001; Suman et al., 2005; Ramanathan et al, 2012).

LACTATE DEHYDROGENASE (LDH) BIOCHEMISTRY AND FUNCTION

Lactate dehydrogenase (LDH) is a tetrameric enzyme that carries out the reversible interconversion of pyruvate to lactate. The reaction is NAD/NADH dependent (Kim et al., 2006):



Lactate dehydrogenase transfers hydrogen while NAD^+ is the hydrogen acceptor in this reaction. The equilibrium of the reaction is more favorable to the reduction of pyruvate to L-lactate (Drent et al., 1996). Lactate dehydrogenase has been described as a regulator of anaerobic glycolytic pathway.

It has been shown that LDH exists in two forms, one mainly found in heart tissues (H or B) and the other in muscle tissues (M or A) (Pesce et al., 1967; Javed et al., 1997; Drent et al., 1996). Each subunit or form is under separate and independent genetic control and composed of two polypeptide chains. The random pattern of association of the four peptide chains originates five isozymes namely A_4 , A_3B , A_2B_2 , AB_3 , and B_4 . Even though the isozymes catalyze the same reaction, they differ in chemical, physical, and immunological properties as well as molecular structure (Drent et al., 1996; Javed et al., 1997; Karamanos, 2014).

The isozymes differ in the reactivity to substrates, sensitivity to inhibitors, to temperature (cold lability and heat resistance), electrophoretic mobility, and charges (Drent et al., 1996). The subunit A_4 is negatively charged at neutral pH and preferably oxidizes pyruvate to lactate. It is highly expressed in glycolytic white skeletal muscles. The subunit B_4 favors lactate conversion to pyruvate and formation of NADH. It is predominant in oxidative cardiac muscles. Bovine heart LDH has a molecular weight of 36,000/subunit or $136,700 \pm 2,100$ /tetramer (Fosmire & Timasheff, 1972; Huston, Fish, Mann, & Tanford, 1972). In postmortem muscle, lactate-LDH is believed to regenerate NADH, which is the ultimate electron donor for metmyoglobin reduction, by reducing NAD^+ and oxidizing L-lactate to pyruvate (Kim et al., 2006; Kim et al., 2009;

Ramanathan, Mancini, & Maheswarappa, 2010).

METMYOGLOBIN REDUCTASE ACTIVITY (MRA)

Metmyoglobin reducing activity is the important intrinsic property that helps maintain color stability in fresh meat (Ledward, 1985; Faustman, Cassens, & Greaser, 1988; Watts et al., 1966). The oxidation of oxymyoglobin to metmyoglobin is an undesirable post-mortem biological process that negatively affects the marketability and profitability of fresh meats. Metmyoglobin reducing activity regenerates deoxymyoglobin (Bekhit & Faustman, 2005) which can then be oxygenated to form oxymyoglobin.

The observation that metmyoglobin does not significantly accumulate in healthy living muscles led researchers to conclude to the existence of a reducing activity (Fox Jr., 1966). Stewart et al. (1965) showed that MRA is dependent on pH, temperature, and pigment concentration. Nicotinamide adenine dinucleotide-hydrogen dependent reductase is one of the enzymes responsible for enzymatic metmyoglobin reduction. Hagler et al. (1979) were the first to purify and characterize bovine metmyoglobin reductase from bovine heart muscle. They demonstrated that the enzyme was NADH-dependent and had more affinity for the substrate than nonspecific and non-enzymatic systems. Livingston et al. (1985) and Arihara et al. (1989a, 1989b) further identified NADH-dependent reductase as a NADH-cytochrome b5 reductase that requires cytochrome b5 as an electron transfer mediator. Also, they suggested that the enzyme may not utilize an artificial activator of electron transfer such as ferrocyanide or methylene blue in vivo to reduce metmyoglobin as suggested in early works. Arihara et al. (1995) localized NADH-cytochrome b5 reductase predominantly in the mitochondrial fraction and to a lesser extent in microsomal fraction.

However, studies that investigate the role of cooked reducing activity of ground beef on PMB are lacking in the literature. Thermal stability of NADH-dependent reductase may

determine its implication in cooked reducing activity. Nonetheless, previous investigations have not shed light on the activity of the enzyme at cooking temperatures in regard of cooked color development and PMB. Therefore, this study has been undertaken to investigate metmyoglobin reducing activity in cooked ground beef patties and the thermal stability of MRA enzymes as related to PMB.

CHAPTER III

METHODOLOGY

OBJECTIVE I: EFFECTS OF PACKAGING AND TEMPERATURE ON METMYOGLOBIN REDUCING ACTIVITY OF COOKED GROUND BEEF

Patties preparation and packaging

Fresh coarse ground chubs (85% lean, n = 6 chubs) were obtained from a local purveyor on the day of preparation. Coarse ground beef was fine ground using a LEM meat grinder (LEM Big Bite Grinder, LEM Products, West Chester, OH, USA). Nine patties (1 cm thickness x 10.5 cm diameter; 113 g) were prepared using a patty press (Adjust-A-Burger, Boyd's Equipment Inc., Amarillo, TX, USA) from each chub. Three of the nine patties were randomly allotted to vacuum packaging (VP, Prime Source Vacuum Pouches, 4 mil, KOCH Supplies Inc., Kansas City, MO, USA) and three for high-oxygen modified atmospheric packaging (HiOX-MAP, 80% oxygen and 20% carbon dioxide). The HiOX-MAP patties were placed in Rock-Tenn DuraFresh™ rigid trays with absorbent pads and sealed with a clear, multi-layer barrier film (LID 1050 film, cryovac sealed air, Duncan, SC, USA) using a semi-automatic Mondini tray-sealing machine (Model CV/VG-5, G. Mondini S.P.A. Cologne, Italy) and certified gas blends (Stillwater Steel & Welding Supply, Stillwater, OK, USA). Packaged patties were stored in the dark at 0 ± 2 °C for 48 h. The remaining three patties were used to characterize day 0 (no packaging) raw color and MRA. Of the three patties assigned to each packaging, one patty was allotted to end-point cooking temperature groups (65.5 °C and 71.1 °C). The third patty was utilized to measure

proximate and pH analyses, lipid oxidation, and metmyoglobin reducing activity (MRA).

Analysis of pH

The pH of ground beef patties was determined by inserting at two different locations within a patty an Accumet combination glass electrode connected to an Accumet 50 pH meter (Fisher Scientific, Fairlawn, NJ). The readings were averaged for statistical analysis.

Raw surface color measurement

Raw surface color was recorded on d 0 (2 h) and d 2 (48 h) following packaging. For both VP and HiOX-MAP patties, values of the Commission Internationale de l'Eclairage (CIE) L^* , a^* and b^* , and reflectance from 700 to 400 nm were recorded at two random locations of each patty using a Hunterlab MiniScan EZ 45°/0° color spectrophotometer (Hunterlab Associates, Reston, VA, USA). The a^* and b^* values were used to calculate chroma (C) = $\sqrt{a^{*2} + b^{*2}}$ (AMSA, 2012). Reflectance from 700 to 400 nm was used to determine K/S values according to the 2012 AMSA guidelines.

Raw internal color measurements

At the completion of 48 h storage (d 2), one patty from each packaging type (HiOX-MAP and VP) assigned for surface color was also used for interior color measurement. Upon removal from the package, raw surface color was measured. Then, patties were sliced parallel to the surface through the geometric center. The freshly cut interior of one half patty was placed in vacuum pouch (Prime Source Vacuum Pouches, 4 mil, KOCH Supplies Inc., Kansas City, MO) and immediately scanned for CIE L^* , a^* , b^* and reflectance from 700 to 400 nm at two random locations with a Hunterlab MiniScan EZ 45°/0° color spectrophotometer (Hunterlab Associates, Reston, VA, USA). Readings were averaged for statistical analysis. K/S values were

determined by utilizing the reflectance values according to the procedures described in the 2012 AMSA guidelines. The second half was used to measure MRA.

Lipid oxidation

Thiobarbituric acid reactive substances (TBARS) were determined by the method of Witte, Krause, and Bailey (1970). Five grams (5 g) of ground beef d 0 and d 2 that contained surface and interior was mixed with 25 mL trichloroacetic acid (TCA) solution (11% w/v, Sigma Aldrich, MO), blended with a Waring table top blender (Dynamics Corp. of America, New Hartford, CT) for 30 s. The homogenate was filtrated through a Whatman (No. 1) filter paper (Fisher Scientific, Bohemia, NY). The filtrate (1 mL) was mixed with 20 mM thiobarbituric acid (TBA) and incubated in boiling (100°C) water in a water bath (Isotemp, Fisher Scientific, Bohemia, NY, USA) for 10 min. The absorbance at 532 nm was measured using a Shimadzu UV-2600 UV-VIS spectrophotometer (Shimadzu Inc., Columbia, MD). The blank consisted of a mixture of TCA and TBA solutions in 1:1(v/v).

Metmyoglobin reducing activity (MRA)

Metmyoglobin reducing activity was determined on d 0 (2 h) and d 2 (48 h) according to a modified methodology described by Sammel et al. (2002). Briefly, patty halves were immersed in 0.3% sodium nitrite solution (Sigma Aldrich, St. Louis, MO, USA) for 20 min to allow metmyoglobin to form at the surface. They were removed, blotted dry, vacuum packaged (Prime Source Vacuum Pouches, 4 mil, KOCH Supplies Inc., Kansas City, MO), and immediately scanned for pre-incubation metmyoglobin a* and reflectance values from 700 to 400 nm with a Shimadzu UV-2600 UV-VIS spectrophotometer (Shimadzu Inc., Columbia, MD). Metmyoglobin reduction was completed by incubating the samples at 30°C for 4 h. Samples removed from the incubator were rescanned for post-incubation metmyoglobin a* and reflectance values. Metmyoglobin reducing activity was estimated by the change in the difference in absorbance at

at 630 nm and 580 nm according to the 2012 AMSA guidelines.

Cooked ground beef

Cooking

Patties were cooked to an internal endpoint temperature of 65.5 °C or 71.1 °C on a George Foreman grill (Salton Inc., Columbia, MO). The internal temperature was monitored by inserting horizontally to the surface into the geometric center a handheld ATKINS AquaTuff 351 thermometer probe (Atkins, Gainesville, FL). The temperature at the surface of the upper and lower grill surfaces was maintained at 170 – 180 °C. Patties were then placed into vacuum pouches (Prime Source Vacuum Pouches, 4 mil, KOCH Supplies Inc., Kansas City, MO) and held in ice to avoid post-cooking temperature rise.

Internal cooked color measurement and MRA

Cooked patties were cut in the edges and sliced parallel to the surface through the geometric center.

OBJECTIVE II: EFFECTS OF pH AND PACKAGING ON THERMAL STABILITY OF ENZYMES

Thermal stability of Lactate Dehydrogenase (LDH)

Purification and heat treatment

Lactate dehydrogenase from bovine heart muscle (Sigma-Aldrich, St. Louis, MO) was dialyzed against 50 mM phosphate buffer pH 5.6 (represents normal meat pH) or 6.4 (indicates dark cutting beef pH) for an hour with a buffer change at 30 min. The dialyzed LDH (9.45mg/mL) was transferred to test tubes and incubated in a water bath in a continuous heat increment of 3.5 °C/min. At endpoint temperatures (65, 71, 77, and 84°C), test tubes containing LDH solutions were withdrawn.

Thermal stability of LDH

Lactate dehydrogenase activity was determined by measuring the generation of NADH as described by in the following equation: L-Lactate + NAD⁺ ↔ Pyruvate + NADH + H⁺

Briefly, to initiate the reaction, 20 μL LDH (0.2 mM) was added in polystyrene disposable cuvette (1.5 mL semi-micro fisher scientific, USA) containing 700 μL of 50 mM citrate buffer (pH 7.4), 50 μL NAD⁺ (0.2 mM) NAD⁺ and 50 μL L-Lactate (50 mM). Nicotinamide adenine dinucleotide-hydrogen formation was monitored via increased absorbance at 340 nm recorded for 120 s using a Shimadzu UV-2600 UV-VIS spectrophotometer (Shimadzu Inc., Columbia, MD). An increase in absorbance was utilized to calculate LDH activity (expressed as μM/min/mg). Protein concentration was determined by bicinchoninic acid (BCA) method.

Thermal stability NADH-dependent reductase

Isolation of NADH-dependent reductase (NDR)

Reductase was isolated by the method of Hagler et al. (1979) and Faustman et al. (1988). Briefly, 200 g bovine longissimus lumborum muscle (pH 5.6, 7 d postmortem) devoid of visible fat and connective tissue was homogenized in cold distilled water for 1 min using a Waring

blender. The pH of the homogenate was adjusted to 7.5 with 2 N NH_4OH . The homogenate was centrifuged at 13,700 g for 20 min and filtrated through a double-layer cheese cloth. The supernatant was brought to 40% $(\text{NH}_4)_2\text{SO}_4$ saturation and centrifuged at 13,700 g for 20 min and filtrated through a double-layer cheese cloth. The resulting supernatant was made 70% $(\text{NH}_4)_2\text{SO}_4$ saturation and centrifuged at 13,700 g for 20 min. The pellet was re-suspended in distilled water and dialyzed against 20 mM sodium phosphate buffer (pH 6.0) with 3 changes of 10 volumes each. The solution was centrifuged at 26,000 g for 30 min and resulting supernatant was applied to CM- cellulose ion exchange column (4 x 5 cm, GE Healthcare BS, Sweden) previously equilibrated with 20 mM sodium phosphate buffer (pH 6.0). The isolated NADH-dependent reductase was stored at -80°C until further analysis.

NADH-dependent reductase (NDR) activity

The isolated NADH-dependent reductase was thawed at room temperature and the pH was adjusted by dialyzing against 50 mM phosphate buffer pH 5.6 or 6.4. Reductase activity was measured according to the methodology of Reddy & Carpenter (1991) in polystyrene cuvettes at 37°C . The reaction mixture contained 0.15 mM equine metmyoglobin, 5 mM EDTA, 3 mM potassium ferrocyanide, 1 mM NADH, and one of the following: purified NADH-dependent reductase heated to 37, 45, 50, 55, or 65°C . The heat treatment was performed as described previously for LDH. The final reaction volume was 1 mL. Nicotinamide adenine dinucleotide hydrogen was added last to initiate the reaction. The enzymatic reaction was monitored at 582 nm for 120 s using a Shimadzu UV-2600 UV-VIS spectrophotometer (Shimadzu Inc., Columbia, MD). Reductase activity was expressed as nM/min/mg.

Thermal stability of oxymyoglobin (OxyMb)

Oxymyoglobin preparation

Oxymyoglobin was prepared using the method described by Brown & Mebine (1969).

Oxymyoglobin solutions were prepared via sodium hydrosulfite (0.01 mg/1 mL) mediated reduction of equine metmyoglobin solutions followed by 10 min oxygenation. Equine myoglobin shares 88.2% homology with bovine counterpart (Elroy et al., 2015). To ensure rapid oxygenation, a gentle swirling and air bubbling as described by Allen and Cornforth (2006) was performed twice. Excess of the reducing agent was removed using a pre-calibrated PD-10 Desalting column (GE Healthcare BS, Sweden) primarily eluted with citrate buffer (pH 5.6 and 6.4). The desalting column is a chromatographic gel filtration tool containing a Sephadex G-25 medium to purify proteins based on molecular weight. This methodology has been previously used in oxymyoglobin preparation (Joseph et al., 2010).

Heat treatment and measurement

Oxymyoglobin solutions were incubated in water bath Isotemp 2223 (Fisher Scientific, Marietta, USA). Samples were heated in a continuous heat increment of 3.6°C/min, and withdrawn at given endpoint temperatures (65, 71, 77, and 84°C). Heat treated solution were then transferred to Eppendorf tubes and centrifuged at 15,000 x g for 5 min. The supernatant was centrifuged for a second time to remove additional turbidity. The resulting supernatant solutions were transferred into a disposable polypropylene spectrophotometer cuvettes (1 cm path length; Shimadzu Inc. Columbia, MD) and absorbance at 525 nm was recorded using the photometric mode. Percentage of myoglobin denaturation was estimated by the following equation:

$$\text{Percentage Mb denaturation} = [(A_{525} (\text{raw OxyMb}) - A_{525} (\text{heated OxyMb})) / A_{525} (\text{raw OxyMb})] \times 100.$$

STATISTICAL ANALYSES

For the objective 1, experimental design was a completely randomized block design. Each chub served as a block. For the objective 2, experimental design was a completely randomized design. The data were analyzed using the Mixed Procedure of SAS 9.4 (2012). In experiment 1,

least squares means were generated to assess the effects of packaging, packaging x time, and packaging x temperature on raw surface and internal color, lipid oxidation and raw internal MRA, and cooked internal color and internal MRA respectively. For experiment 2, least squares means were generated to assess the effect of temperature and pH and their interaction on thermal stability of lactate dehydrogenase, NADH-dependent reductase and oxymyoglobin. For all analyses, protected F-test was used to separate least squares means using the *PDIFF* option of SAS ($P < 0.05$).

CHAPTER IV

RESULTS

EXPERIMENT 1: EFFECTS OF PACKAGING AND TEMPERATURE ON METMYOGLOBIN REDUCING ACTIVITY OF COOKED GROUND BEEF

pH and proximate composition

The average pH of ground beef was 5.73 ± 0.02 (Mean \pm SEM). The proximate composition is summarized in the Table 1. Protein and fat content in percentage were 18.61 ± 0.21 and 16.88 ± 0.73 , respectively.

Effects of packaging on raw surface and internal color

There was a significant effect of packaging and storage time on surface redness (Table 2, $P < 0.05$). HiOX-MAP ground beef had greater a^* and more intense red color (chroma, $P < 0.05$) compared with VP ground beef patties at both 2 h and 48 h. Vacuum packaged ground beef patties had a lower ($P < 0.05$) $K/S474 \div K/S525$ (indicating more deoxymyoglobin) than HiOX-MAP at 2 and 48 h. HiOX-MAP ground beef patties had a lower ($P < 0.05$) $K/S610 \div K/S525$ ratios, indicating more oxymyoglobin on the surface than VP beef patties.

To the best of our knowledge, we are not aware of a methodology that accurately records internal color of ground beef patties. In the current study, internal color was reported by cutting open a patty parallel to the surface and internal color was recorded two minutes after slicing. Patties packaged in VP had a greater redness than HiOX-MAP patties. This is probably due to

conversion of deoxymyoglobin to oxymyoglobin upon exposure to oxygen.

Lipid oxidation

There was a significant packaging x time interaction observed for lipid oxidation (Figure 1). HiOX-MAP patties had a greater ($P < 0.05$) TBARS values compared with VP patties. Storage time significantly increased TBARS values in HiOX-MAP patties ($P < 0.05$) but did not affect VP patties.

Effects of packaging and temperature on internal cooked color

There was a significant packaging x temperature interaction resulted for cooked internal color (a^* , chroma) of ground beef patties. Beef patties packaged in high oxygen had lower ($P < 0.05$) a^* and chroma values than VP patties irrespective of the temperature (Figure 2). There was no difference in internal cooked color (a^*) between HiOX-MAP ground beef patties at 65.5 and 71.1°C ($P > 0.05$). The intensity of the internal color (chroma; Figure 3) of ground beef patties followed the same pattern as that of internal a^* values. Vacuum packaged ground beef patties retained a more pinkish red color ($P < 0.05$) at 65.5°C than at 71.1°C as indicated by greater a^* and chroma values. Lower internal a^* and chroma values ($P < 0.05$) of HiOX-MAP patties were indicative of a browner and more well-done appearance.

Effects of packaging and temperature on MRA of raw and cooked ground beef

Packaging affected MRA of both raw and cooked ground beef (Figure 4 & 5, $P < 0.05$). Ground beef patties packaged in HiOX-MAP had lower raw and cooked MRA compared to VP beef patties ($P < 0.05$) at the conclusion of 48 h storage. There was no difference in cooked patty MRA for HiOX-MAP between 65.5 and 71.1°C. Cooked patty MRA decreased as temperature increased for VP patties ($P < 0.05$).

EXPERIMENT 2: EFFECTS OF pH AND TEMPERATURE ON THERMAL STABILITY OF ENZYMES

Thermal stability of Lactate Dehydrogenase (LDH)

There was a significant pH x temperature interaction for LDH activity ($P < 0.05$; Figure 6). Up to 77°C, LDH activity at pH 6.4 was almost two fold higher than that of LDH at pH 5.6 (6.6 vs 3.38 $\mu\text{M}/\text{min}/\text{mg}$, 12.16 vs 6.28 $\mu\text{M}/\text{min}/\text{mg}$, and 18.58 vs 8.52 $\mu\text{M}/\text{min}/\text{mg}$ respectively; $P < 0.05$). LDH activity at both pH increased concomitantly to temperature increase up to 77°C, and dropped at 84°C. The activity of LDH at 84°C was similar for both pH. Nevertheless, current study indicates LDH was able to regenerate NADH at temperatures up to 77°C.

Thermal stability of NADH-dependent reductase (NDR)

There was a pH x temperature interaction for NDR activity (Figure 7, $P < 0.05$). A greater temperature decreased NDR activity. The NADH-dependent reductase was more stable ($P < 0.05$) at pH 6.4 than pH 5.4. This was shown by a greater enzyme activity exhibited across the range of temperatures at 6.4 compared to that at pH 5.6. Maximum activity was recorded at 45°C compared to other endpoint temperatures. At 65°C, NDR did not show any activity at both pH.

Effects of pH and temperature on oxymyoglobin denaturation

The interaction of pH x temperature was significant for oxymyoglobin denaturation (Figure 8, $P < 0.05$). For both pH, the increase in temperature resulted in an increase in percentage of myoglobin denaturation ($P < 0.05$). Most of the pigment denaturation occurred by 77°C at both pH (89% at pH 5.6 versus 57% at pH 6.4, respectively). Oxymyoglobin denaturation was pH dependent. Oxymyoglobin denaturation was significantly greater ($P < 0.05$) at pH 6.4 compared to pH 5.6.

CHAPTER V

DISCUSSION

Packaging has a significant effect on cooked color development. For example, high-oxygen modified atmosphere packaging (HiOX-MAP) makes patties very susceptible to PMB compared with PVC and VP. This is primarily due to difference in myoglobin forms present within the interior of patties before cooking (Hunt, Sørheim, & Slinde, 1999; Seyfert et al., 2004; John et al., 2004; John et al., 2005). In the current study, patties packaged in HiOX-MAP had a lower internal color compared with VP. Suman et al. (2009) noted that the interior of steaks packaged in HiOX-MAP were more brown than that of VP steaks due to greater pigment oxidation. However, limited studies have determined the role of MRA of cooked patties in cooked color development.

Red color in cooked patties is due to undenatured myoglobin (Cornforth, Calkins, and Faustman, 1991). Further, Van Laack, Berry, and Solomon (1996) noted that incomplete denaturation of Mb was responsible for pink cooked color in ground beef patties cooked to 71°C. Recently, researchers noted that ohmically cooked meats had greater instrumental redness (a^* values) compared to meats cooked by conventional methods (Shirsat et al., 2004; Tian et al., 2016). Their results suggested that a fast rate cooking method such as ohmic cooking resulted in greater redness possibly due to undenatured myoglobin. A fast rate cooking is believed to protect against pronounced denaturation (Tian et al., 2016). In the current study, we determined nitric oxide metmyoglobin reducing activity of patties packaged in HiOX-MAP and VP at different

temperatures. Patties packaged in VP had greater reducing activity than those in HiOX-MAP. This indicates that enzymes related to metmyoglobin reducing activity retain activity at higher temperatures.

In support, Osborn et al. (2003) observed that heated metmyoglobin was reduced to a red pigment that had a similar spectrum as that of oxymyoglobin. They also noticed that reacting reducing or oxidizing agents with the heated myoglobin yielded characteristic spectra of deoxymyoglobin or metmyoglobin respectively. The reduced heated pigment was able to oxygenate and form a spectrum with characteristic peaks at 542 nm and 582 nm, identical to that of raw oxymyoglobin. They concluded that the red cooked pigment was identical to nature oxymyoglobin in raw meats. They further implied that a reducing system capable of reducing metmyoglobin extract at cooking temperatures was responsible for the red color.

Using cooked meat extracts and NADH, Osborn et al. (2003) were able to confirm the postulate that heat-induced metmyoglobin formation is reversible provided the presence of NADH and muscle protein. They proposed that either a modified NADH-dependent reductase or a new protein system with a ferrylmyoglobin (ferrylMb) component was responsible for the reduction observed under heating conditions. Ferrylmyoglobin has been known to undergo a 2-electron reduction by NADH to form oxymyoglobin.

Bernofsky, Fox, and Schweigert (1959) determined that 95% of a cooked ground beef extract was constituted by oxymyoglobin. This means that reduction of cooked pigments does occur in cooked meats. High temperature has been shown to cause metmyoglobin formation (Brooks, 1935) due to release of oxygen from the oxygenated pigment and oxidation (Lanier, Carpenter, & Toledo, 1977; Yin & Faustman, 1993). However, presence of oxymyoglobin as undenatured pigment in the interior of cooked patties lays a support for reduction occurring at cooking temperatures. This study may provide further evidence for the existence of a reducing

system in cooked ground beef in agreement with aforementioned research.

In this study, greater reducing activity in both raw and cooked ground beef was observed in VP patties. HiOX-MAP decreased MRA in both raw and cooked patties. Osborn et al. (2003) and Van Laack et al. (1996) indicated that factors influencing raw reducing activity of meats seemingly affected cooked reducing activity. While VP has been shown to promote enzymatic reduction of metmyoglobin and prevent lipid oxidation (Stewart et al., 1965), high oxygen conditions stimulate lipid oxidation (Ismail et al., 2009; Kang et al., 2014). Lipid oxidation products have been shown to exert damaging influence on enzyme activity (Ramanathan, et al., 2014). Thus, it may be responsible for the decrease in MRA (Bekhit & Faustman, 2005; Liu et al., 2015).

To counteract the damaging effects of lipid oxidation products, different antioxidants and chelators have been used in meats (Shahidi, Rubin, & Wood, 1987; Suman, et al., 2005; Suman, et al., 2011). Suman et al. (2005) used erythorbate to limit lipid oxidation. They observed that total reducing activity increased in patties incorporated with the antioxidant compared to the control patties. They further showed that PMB was also reduced. Similarly, diverse reducing ingredients and substrates have also demonstrated positive influence on cooked color development (Mancini, et al., 2010; Zhu, Liu, Li, & Dai, 2009; Sorheim & Hoy, 2013). Hence, increased reducing activity may play a significant role in limiting PMB.

To further investigate the cooked reducing system, thermal stability of NADH-dependent reductase, lactate dehydrogenase, and oxymyoglobin were determined. NADH-dependent reductase had the lowest thermal stability when compared with lactate dehydrogenase. In agreement with previous studies (Reddy & Carpenter, 1991; Bekhit et al., 2001), activity of reductase was pH and temperature dependent. The pH/temperature combination that gave optimal reductase activity in this study was pH 6.4/45°C. Irrespective of pH, there was no NADH-

dependent reductase activity at 65°C. Other investigators have reported that reductase activity was optimal at pH 6.5 or 7.0 and 37°C and lost at temperatures higher than 50°C (Echevarne, Renerre, & Labas, 1990; Bekhit et al., 2001). This suggests that NADH-dependent reductase may be destroyed at cooking temperatures and may not be involved in the cooking reducing activity (Osborn et al., 2003).

However, there exists other enzymatic and non-enzymatic reduction systems that are capable of reducing metmyoglobin (Echevarne, Renerre, & Labas, 1990; Bekhit et al., 2001; Osborn et al., 2003; Bekhit & Faustman, 2005; Elroy et al., 2015). Osborn et al. (2003) suggested that either other systems or partially denatured NADH-dependent reductase may be responsible for the cooked reducing activity. Based on current study and previous findings (Echevarne, Renerre, & Labas, 1990), it is less likely that denatured NADH-dependent reductase is responsible of cooked reduction. However, in a complex system, interaction among different meat compounds may protect NADH-dependent reductase from a complete denaturation, thus allowing reduction. Further study is needed to elucidate the mechanism of cooked reduction.

Lactate dehydrogenase has been shown to regenerate NADH, which is further utilized to reduce metmyoglobin (Kim et al., 2006; Kim et al., 2009; Ramanathan, Mancini, & Konda, 2009; Ramanathan et al., 2010; Rodriguez et al., 2011). Kim et al. (2009) postulated that a lactate-LDH system, through NADH regeneration can reduce metmyoglobin. Osborn et al. (2003) demonstrated that cooked reducing activity of heated meat homogenates was enhanced by addition of NADH. Also, it has been shown previously that LDH was stable at cooking temperatures. Vesell & Yielding (1966) demonstrated that LDH was stable at 63°C. In agreement, this study suggests that a lactate NADH-dependent reducing system may be functional at cooking temperatures and involved in cooked reducing activity. This system may represent one of the reducing systems involved in post cooking pigment reduction.

The effects of temperature on LDH, NDR, and oxymyoglobin denaturation indicated that myoglobin and LDH were stable proteins conversely to NADH-dependent reductase. Function and structural conformation of proteins dictate their thermal stability. Proteins function is determined by environmental conditions. Alteration of physiological conditions in general and temperature in particular leads to loss of structural conformation and subsequent functional integrity (Argos et al., 1979). Differences in amino acid sequences, secondary, tertiary, and quaternary structures affect the stability against temperature. Further, different forces have been proposed to play a significant role in stabilizing proteins against thermolability (Yang & Honig, 1993). These factors include, but are not limited to disulfide linkages, salt bridges, increased hydrophobicity, increased hydrogen bonding, electrostatic interactions, packing, increased helical content, increased polar surface area, and side chains hydrogen bonds (Argos et al., 1979; Kumar, Tsai, & Nussinov, 2000; Pace, Shirley, & Thomson, 1990; Pace et al., 2011; Pace, Scholtz, & Grimsley, 2014). Lactate dehydrogenase is tetramer protein containing about 30 to 48% alpha helices and 19 to 23% beta sheets (Chen, Yang, & Martinez, 1972; Robbins, 2016; Proteopedia, 2016) whereas NADH-cytochrome b5 reductase contain 24.7% alpha helices and beta sheets (Sivan, Filo, & Siegelmann, 2007). Myoglobin has been shown to be a helical monomer. Argos et al. (1979) and Kumar et al. (2000) reported that more than beta sheets, greater proportion of helical structure can increase protein stability. This may explain the greater thermal stability of LDH and Mb compared to NDR.

Based on instability index (Bioinformatics, 2016), these three proteins can be classified as follows: NDR > LDH > OxyMb. Greater instability index is indicator of greater sensitivity to temperature. Myoglobin has the lowest index (15.13). Lactate dehydrogenase has an intermediary instability index (27.60) which is much lower than that of NADH-cytochrome b5 reductase estimated to be 48.02. The latter is classified as an unstable protein (Bioinformatics, 2016).

As expected, high pH has a protective effect on protein denaturation. Less denaturation was reported at pH 6.4 in comparison to pH 5.6. These results confirmed previous findings on the effect of pH on myoglobin denaturation (Hunt, Sørheim, & Slinde, 1999), LDH activity (Mendiola & De Costa, 1990; Tian et al., 2016) and NDR activity (Bekhit & Faustman, 2005; Bekhit et al., 2001).

Table 1: Means (n = 6) for pH and proximate composition of ground beef patties

	PROTEIN	FAT	MOISTURE	PH
MEAN	18.61	16.88	62.87	5.73
SE	0.21	0.73	0.88	0.02

Table 2: Least squares means (n = 6) for a*, chroma, k/s474 ÷ k/s525, and k/s610 ÷ k/s525 for surface color of ground beef patties packaged in high-oxygen modified atmosphere packaging (HiOX-MAP) and vacuum packaging (VP)

		a*	chroma	k/s474 ÷ k/s525	k/s610 ÷ k/s525
d 0¹	HiOX-MAP	31.45 ^d	39.69 ^d	0.87 ^c	0.17 ^a
	VP	19.09 ^a	24.25 ^a	0.64 ^b	0.37 ^d
	SE ³	0.34	0.41	0.00	0.00
d 2²	HiOX-MAP	26.33 ^c	33.78 ^c	0.96 ^d	0.19 ^b
	VP	22.24 ^b	26.08 ^b	0.54 ^a	0.34 ^c
	SE ³	0.34	0.41	0.00	0.00

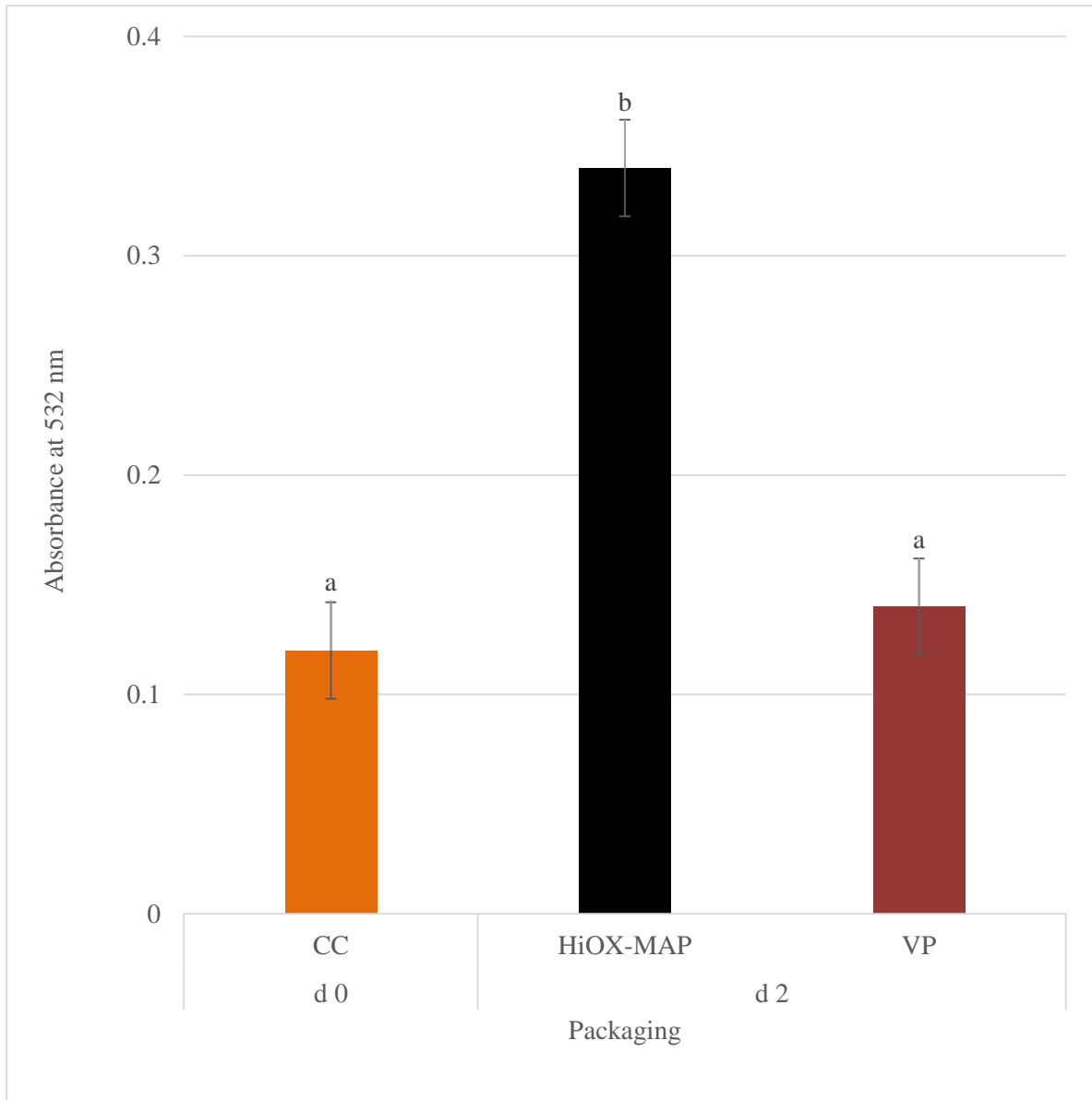
^{a-d} Least squares means in columns with different superscripts are significantly different ($P < 0.05$)

¹ d 0: patties packaged and stored in the dark for 2 h.

² d 2: patties packaged and stored in the dark for 48 h.

³ SE: Standard error.

Figure 1: Effects of packaging and storage time (d 0 vs d 2) on thiobarbituric acid reactive substances (TBARS) values of raw ground beef patties (n = 6) represented by absorbance at 532 nm

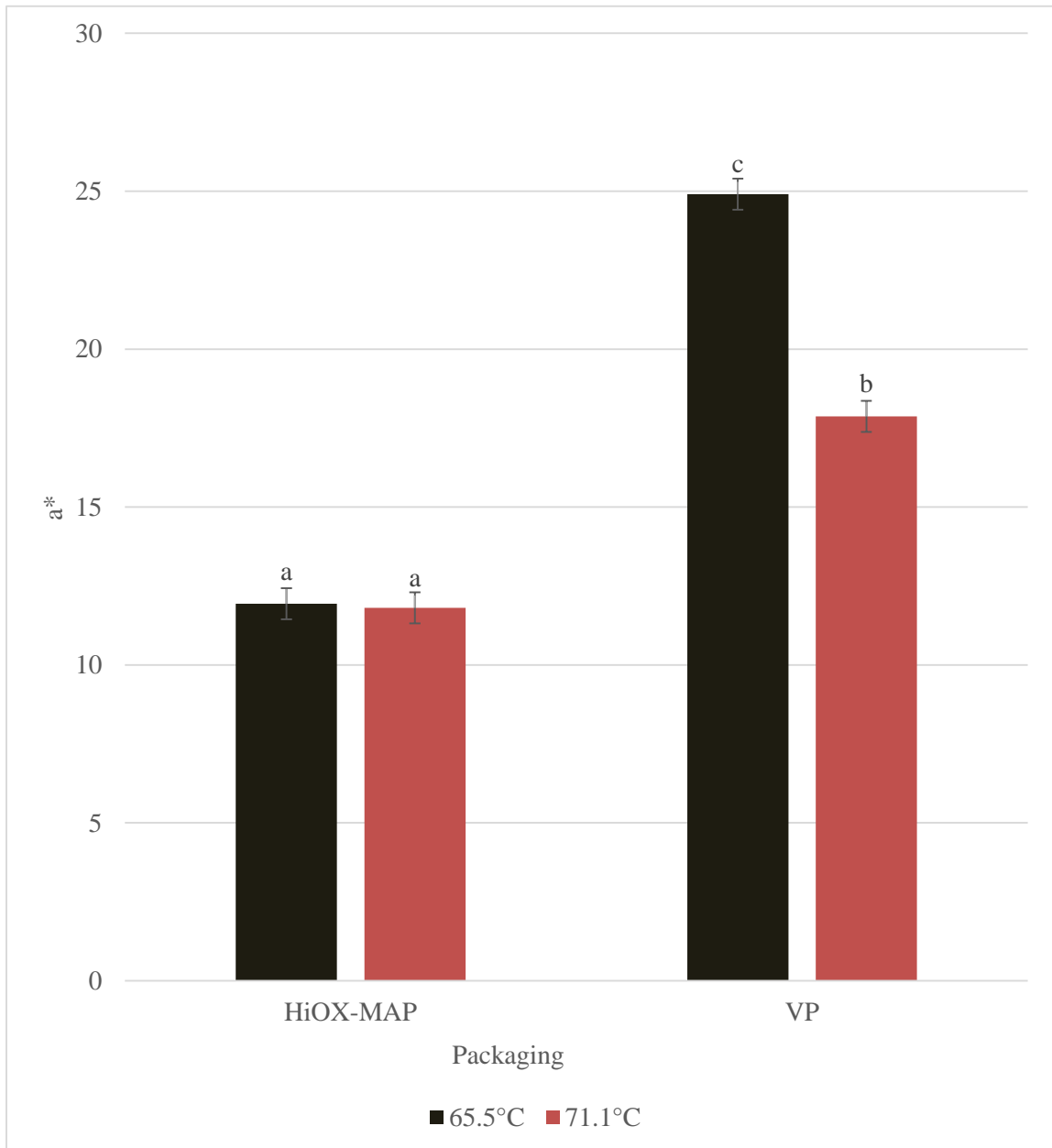


d 0: Patties labelled as control (CC), stored for 2 h in the dark.

d 2: Patties packaged in high oxygen modified atmosphere (HiOX-MAP) or in vacuum (VP) and stored in the dark for 48 h.

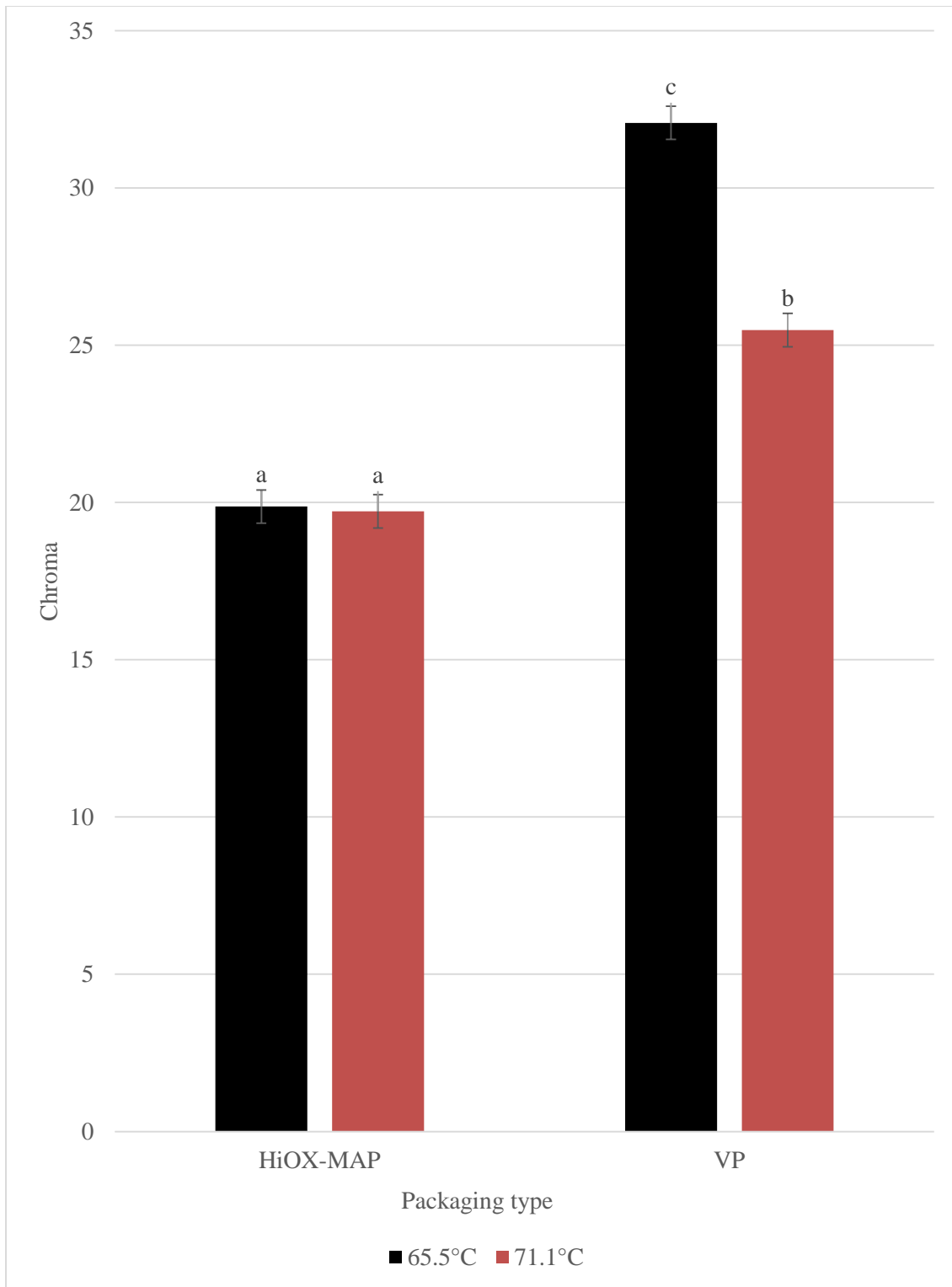
^{a-b} Least squares means with different superscripts are significantly different ($P < 0.05$)

Figure 2: Effects of packaging and temperature on internal redness (a*) of ground beef patties (n = 6) in high-oxygen modified atmosphere packaging (HiOX-MAP) and vacuum packaging (VP)



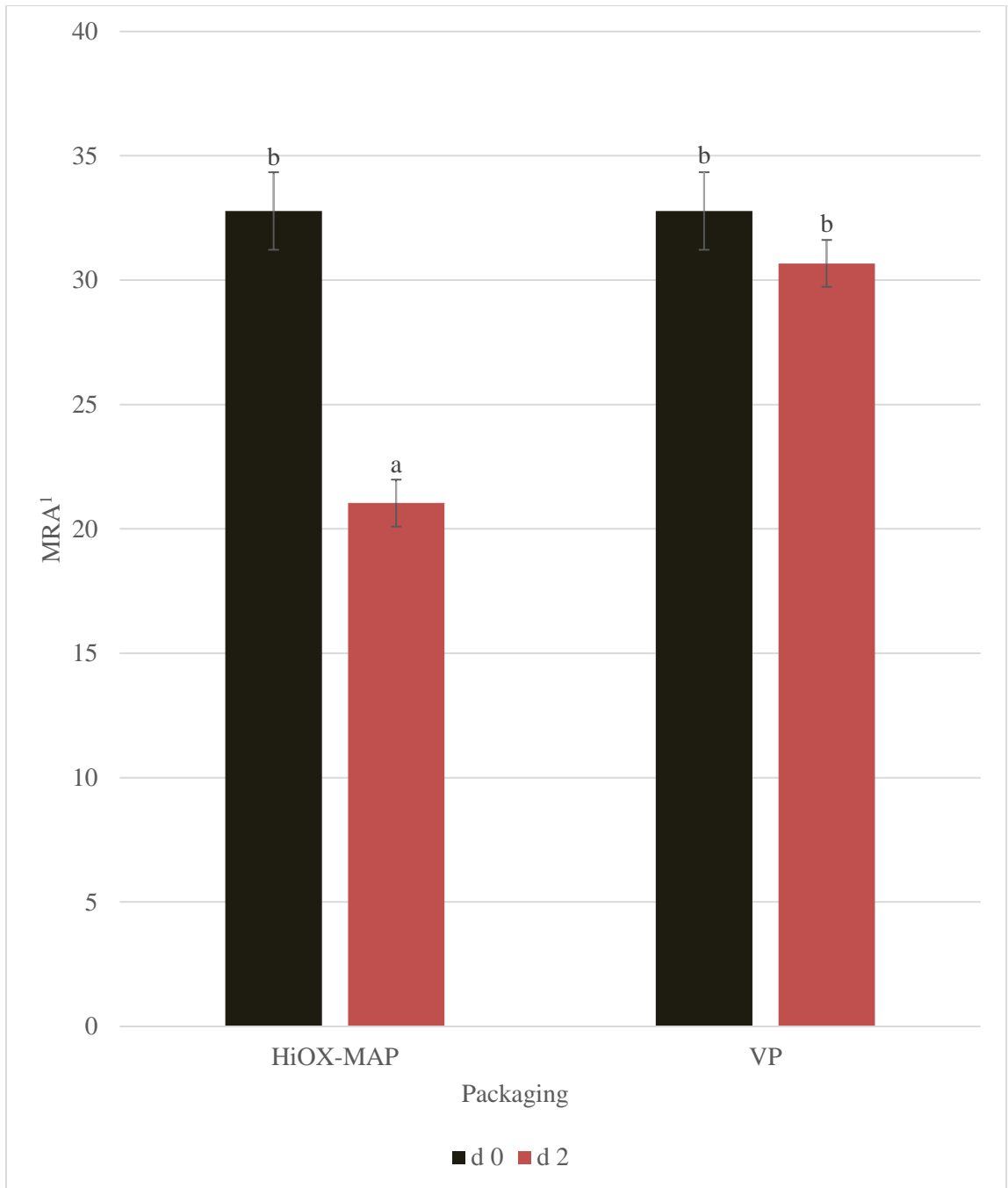
^{a-c} Least squares means with different superscripts are significantly different ($P < 0.05$)

Figure 3: Effects of packaging and temperature on intensity of the internal color (chroma) of ground beef patties (n = 6) in high-oxygen modified atmosphere packaging (HiOX-MAP) and vacuum packaging (VP)



^{a-c} Least squares means with different superscripts are significantly different ($P < 0.05$)

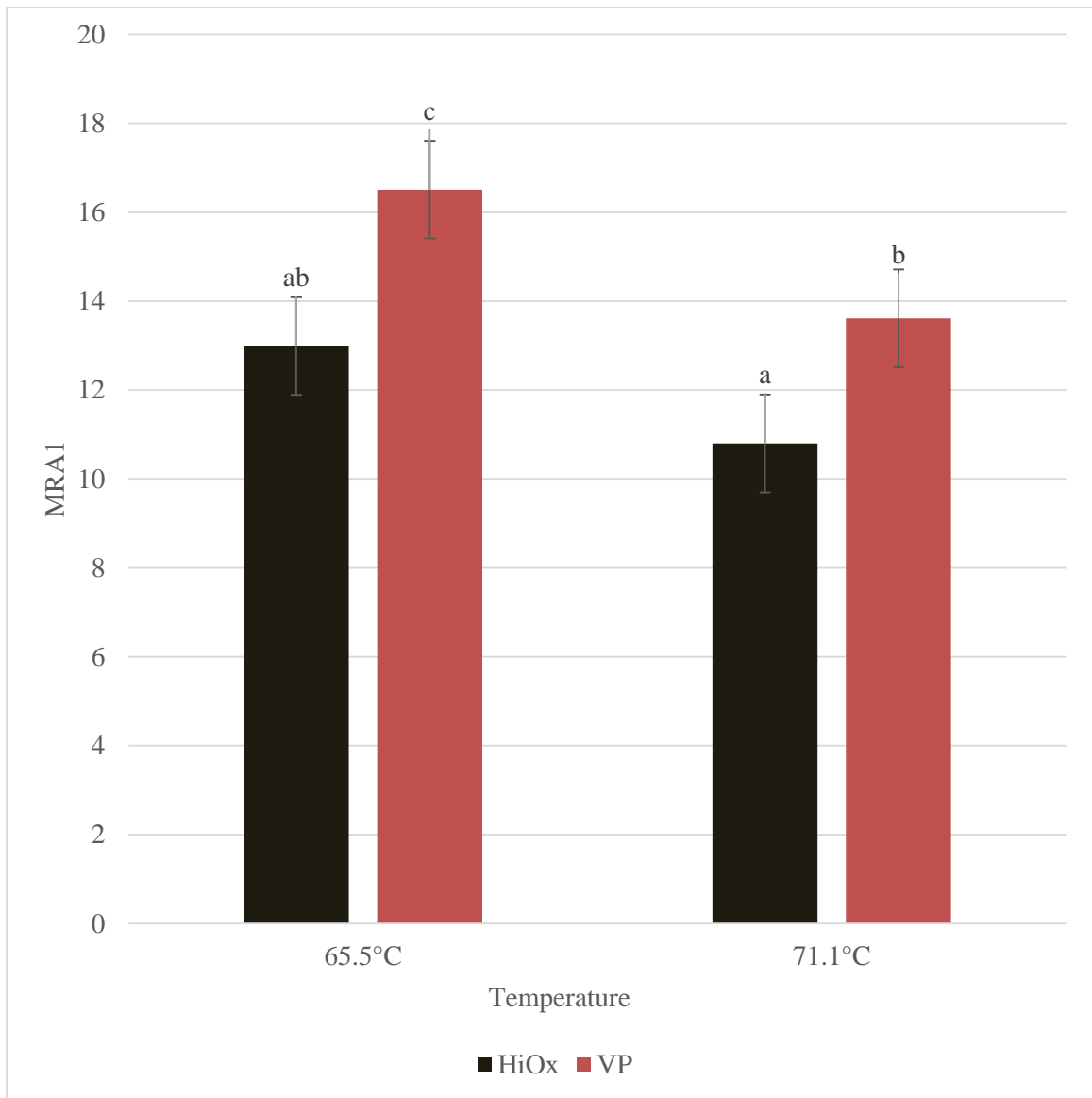
Figure 4: Effects of packaging and time on internal MRA of raw ground beef patties (n = 6) in high-oxygen modified atmosphere packaging (HiOX-MAP) and vacuum packaging (VP)



¹MRA: Change in MRA expressed as difference in absorbance at 630 and 580 nm according to the 2012 AMSA guidelines.

^{a-b} Least squares means with different superscripts are significantly different ($P < 0.05$)

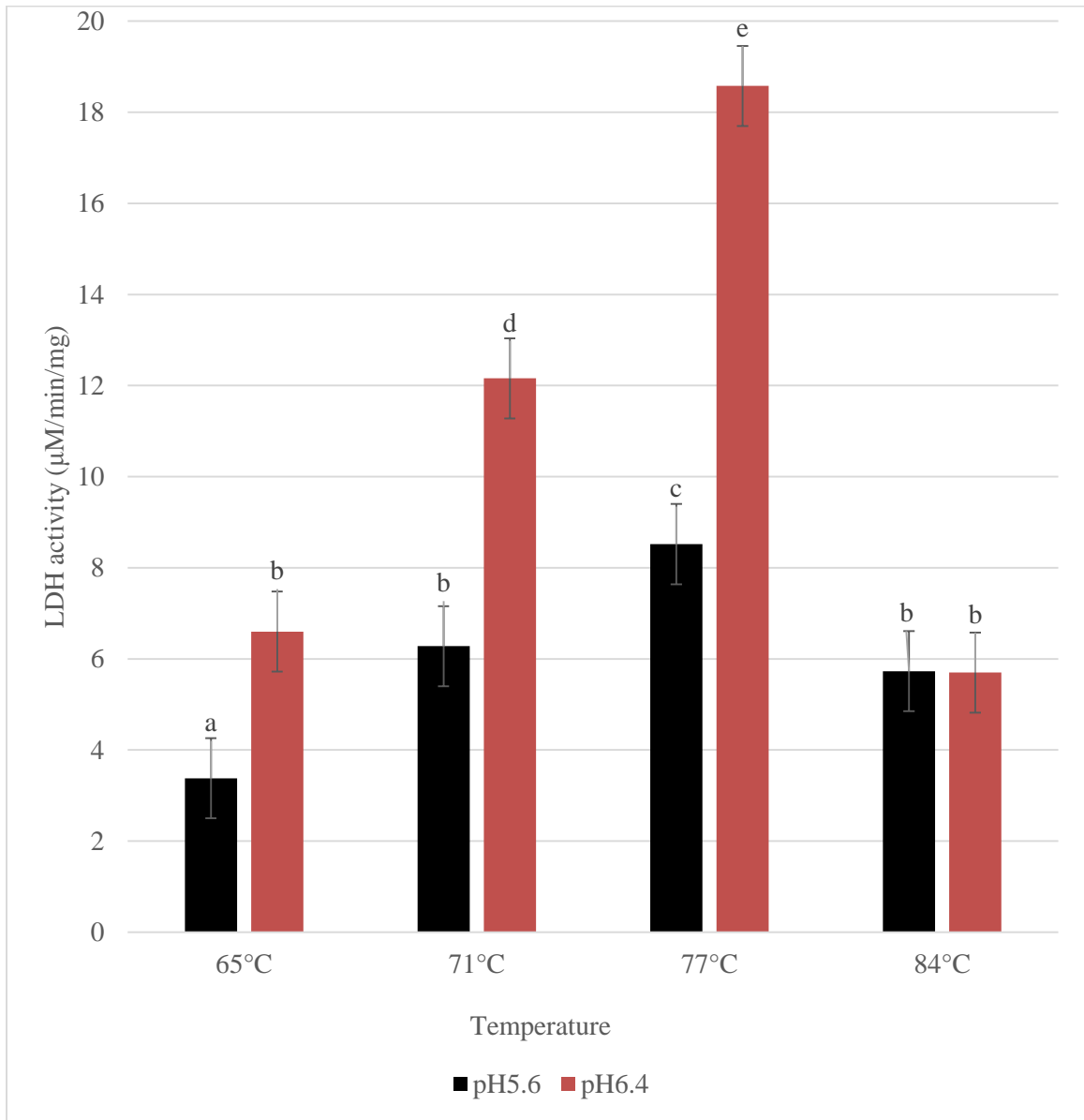
Figure 5: Effects of packaging and temperature on internal MRA of cooked ground beef patties (n = 6) in high-oxygen modified atmosphere packaging (HiOX-MAP) and vacuum packaging (VP)



¹MRA: Change in MRA expressed as difference in absorbance at 630 and 580 nm according to AMSA (2012) guidelines.

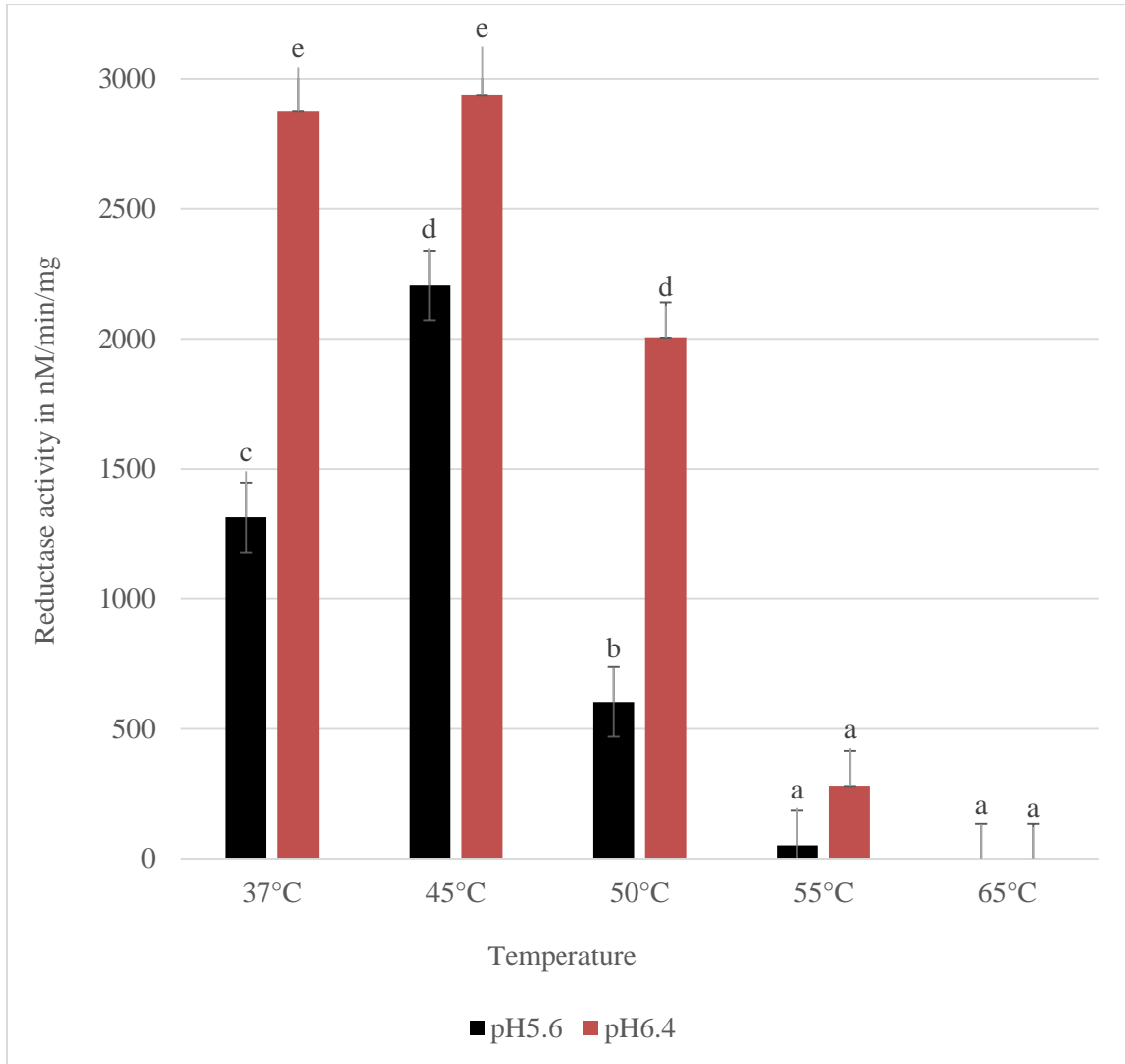
^{a-c} Least squares means with different superscripts are significantly different ($P < 0.05$)

Figure 6: Effects of pH and temperature on lactate dehydrogenase activity (LDH, n = 6)



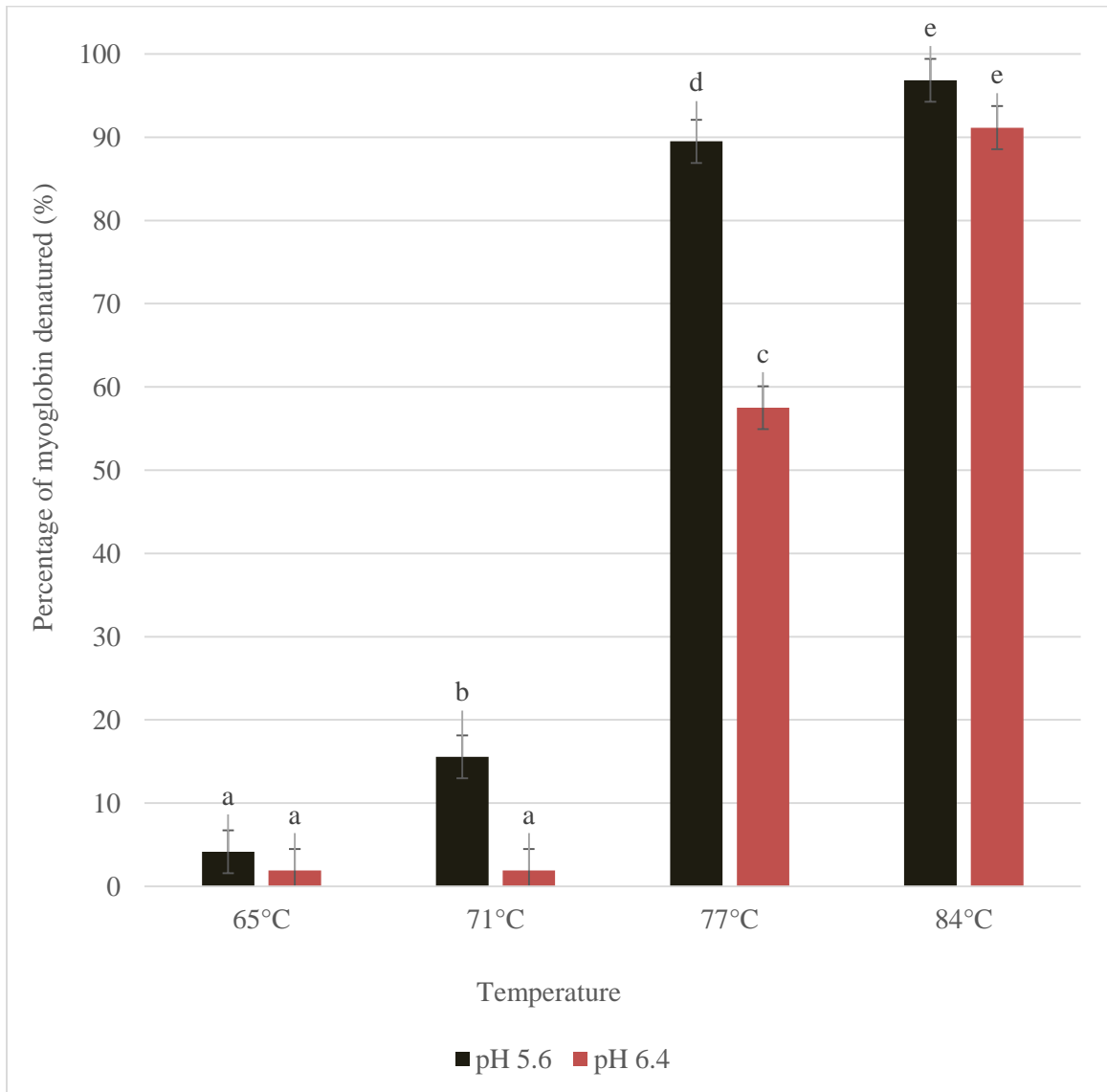
^{a-e} Least squares means with different superscripts are significantly different ($P < 0.05$)

Figure 7: Effects of pH and temperature on nicotinamide adenine dinucleotide hydrogen-dependent reductase activity (n = 6)



^{a-e} Least squares means with different superscripts are significantly different ($P < 0.05$)

Figure 8: Effects of pH and temperature on myoglobin denaturation (n = 6)



^{a-e} Least squares means with different superscripts are significantly different ($P < 0.05$)

CHAPTER V

CONCLUSIONS

Raw ground beef patties with greater reducing activity have been shown to retain more predictable red cooked color (a^* values) in the interior. Further, these patties had cooked reducing activity suggesting that enzymes involved in MRA may be functional at cooking temperatures. The current study suggested that a lactate-LDH reducing system is most likely responsible for cooked reducing activity in cooked ground beef patties due to its greater thermal stability compared with NADH-dependent reductase. Developing strategies to improve reducing activity may practically result in more predictable internal cooked color development.

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