Increased protein digestibility of beef with aging in an infant *in vitro* digestion model

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ABSTRACT

This study investigated the protein digestibility of aged beef using an infant *in vitro* digestion model. Semitendinosus muscles were vacuum-packed and aged for 0, 7, and 14 days at 4°C. Malondialdehyde content of raw and cooked beef increased with aging ($P < .05$). However, no changes in carbonyl content were observed for aged raw and cooked beef samples. The α-amino group content and myofibrillar fragmentation index of beef increased with aging ($P < .05$). The α-amino group content of cooked beef digesta increased upon aging ($P < .05$). Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed the decrease in band intensities of actin in the digesta of aged beef. These results highlight the improvement in the protein digestibility of beef aged for 7 and 14 days. Therefore, aged beef is more desirable as complementary food owing to increased protein digestibility.

1. Introduction

Six months postpartum, breast milk is not enriched with all the essential nutrients, necessitating supplementation with complementary food to infants (Nguyen, Bhandari, Cichero, & Prakash, 2015a). As this period is characterized with emotional and physical development, sufficient supply of proteins, vitamins, and minerals is imperative to meet the needs of a growing infant.

Digestibility is an important characteristic that dictates protein quality. The digestibility of proteins in the gastrointestinal tract is significantly lower for infants than that for adults (Gan, Bornhorst, Henrick, & German, 2018; Nguyen, Bhandari, Cichero, & Prakash, 2015b). Proteolysis in infant stomach is significantly lower than that in adult stomach owing to reduced pepsin secretion and increased gastric pH, which is higher than the optimum pH for pepsin activity (Bourlieu et al., 2014; Gan et al., 2018). In contrast, the trypsin content and activity in the duodenum are similar between adults and 1-month-old infants (Dallas, Underwood, Zivkovic, & German, 2012). However, total protein digestibility in infants is lower than that in adults, given the much reduced digestion rate in the infant stomach.

Undigested proteins in infants can induce undesirable effects following their fermentation and conversion to phenols, p-cresol, ammonia, and sulfide in the colon, resulting in diarrhea, pain, and allergy (Gan et al., 2018; Santé-Lhoutellier, Astruc, Marinova, Greve, & Gatellier, 2008; Windey, De Preter, & Verbeke, 2012). As growth and development largely occur during infancy, protein digestion and absorption in large quantities is imperative. Poor protein digestibility may hinder growth and development and cause diseases.

Beef represents a great protein source rich in essential amino acids and iron, which is generally deficient in infants (Williams, 2007). Lee et al. (2019) reported the reduced digestibility of beef proteins in infants as compared with that in adults.

Aging improves meat quality in terms of tenderness and flavor (Kim et al., 2019). Various changes in the meat proteins occur during aging, including the degradation of myofibrillar proteins due to proteolysis by endogenous proteases such as calpain, cathepsin, and caspase (Ha et al., 2019). Protein degradation is related to the loss of structural integrity and molecular size reduction, and significantly affects protein digestibility. The accessibility of digestive proteases is increased through skeletal protein disruption and protein unfolding upon structural modifications (Schøyen, Frøyland, Sahlström, Knutsen, & Skrede, 2005). Therefore, aged beef is thought to exhibit improved protein digestibility.

In the present study, the changes in the physicochemical properties

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and protein digestibility of aged beef were investigated in a simulated infant *in vitro* digestion model.

2. Materials and methods

2.1. Beef sample preparation

Three semitendinosus muscles from three steer carcasses at day 7 postmortem were obtained from a local market. Each muscle was divided into nine pieces and randomly allocated to three treatment groups as follows: aging for 0 days (D0), 7 days (D7), and 14 days (D14). The muscle samples were vacuum-packed and stored at 4°C for aging. At the end of treatment, one half of each sample was removed and stored at −50°C for raw meat analysis, while the other half was vacuum-packed and cooked in a water bath until its core temperature reached 80°C. The cooked muscles were stored at −50°C before analysis.

2.2. Lipid and protein oxidation

Lipid oxidation was determined using the 2-thiobarbituric acid reactive substance (TBARS) value measured as per the method developed by Jung, Nam, and Jo (2016).

Protein oxidation in beef was monitored from the total carbonyl content measured according to the method developed by Armenteros, Morcuende, Ventanas, and Estévez (2016).

2.3. α-Amino group content

In brief, 2 g beef sample was mixed with 10 mL of a trichloroacetic acid solution (12% *w/v*) and homogenized (T25 basic, IKA GmbH & Co. KG, Staufen, Germany) at 13,000 rpm for 1 min. The mixture was subsequently centrifuged (1580 R, LABOGENE Co., Ltd., Lynge, Denmark) at 2063 ×*g* for 20 min, and the supernatant was collected after filtration through No. 4 filter paper (Whatman, Maidstone, England).

Quantification of α-amino groups in beef filtrate and digesta was performed using o-phthaldialdehyde (OPA) as per the method of Church, Swaisgood, Porter, and Catignani (1983).

2.4. Myofibrillar fragmentation index (MFI)

MFI was measured according to the method developed by Culler, Smith, and Cross (1978).

2.5. In vitro digestion

To prepare beef puree as complementary food for infants between 6 and 24 months, a beef homogenate was used as the sample for *in vitro* digestion (Lee et al., 2019). Beef sample (3 g) was mixed with distilled water (9 mL) and heated in a water bath at 80°C for 30 min. The mixture was homogenized (T25 basic, IKA GmbH & Co. KG, Staufen, Germany) at 13,000 rpm for 1 min, and the protein digestibility of the beef homogenate was investigated in the gastrointestinal digesta. Digestive fluid contents such as pepsin from porcine mucosa, gastric lipase from *Rhizopus oryzae*, bile extract, trypsin from bovine pancreas, chymotrypsin from bovine pancreas, and pancreatic lipase from porcine pancreas were purchased from Sigma-Aldrich (St. Louis, MO, USA). The simulated digestive fluids were prepared as described by Dupont et al. (2010), Nguyen et al. (2015b), and Nguyen, Bhandari, Cichero, and Prakash (2018) with some modifications as follows: gastric juice (pepsin 22.75 unit/mg and gastric lipase 21 unit/mg in 0.15 M sodium chloride [NaCl], pH 3.8 adjusted using 0.1 M hydrochloric acid [HCl]), duodenal juice (trypsin 34.5 unit/mg, chymotrypsin 0.04 unit/mg, and pancreatic lipase 200 unit/mg in 0.1 M NaCl, pH 7.5 adjusted using 1.0 M NaOH), and bile juice (4 mM bile extract in distilled water,

pH 7.5 adjusted using 1.0 M NaOH). Enzyme unit refers to the target unit of the digestive enzyme per milligram of total protein. The pH of the puree and digestive fluid mixture were not additionally controlled during digestion.

In vitro digestion of beef homogenate was conducted according to the procedure described by Kim and Hur (2018). A total of 4 mL of beef homogenate was mixed with 10 mL of gastric juice and digested for 2 h in a shaking water bath at 37°C. The gastric digesta sample was immediately collected after digestion and stored at −70°C until analysis. To simulate gastrointestinal digestion of beef homogenate, the gastric digesta was mixed with 10 mL of simulated duodenal juice and 5 mL of bile juice. The mixture was digested for 2 h in a shaking water bath at 37°C, and then immediately cooled on ice and stored at −70°C until analysis. Digestion was performed thrice on different days for statistical analysis.

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using a 12.5% polyacrylamide gel comprising 30% acrylamide solution, 1.5 M Tris-HCl (pH 8.8), 0.5 M Tris-HCl (pH 6.8), 10% ammonium persulfate, and *N,N,N′,N′*-tetramethyl-ethylenediamine. The analysis of digesta sample by SDS-PAGE was conducted as per the methods described in the study of Lee et al. (2019). The proteins in the gel were stained in a staining solution containing Coomassie Brilliant Blue and then destained with 10% acetic acid. The stained gel was scanned using a GS-710 densitometer (Bio-Rad Laboratories Inc., Hercules, Ca, USA), and analyzed by Image Master 2D Platinum v5.0 (GE Healthcare, formerly Amersham Biosciences, Seoul, Korea). The relative protein composition was estimated from the observed band intensity (pixel intensity × band area).

2.7. Statistical analysis

The study was conducted in three iterations (three batches), and the results were statistically analyzed using a general linear model with a randomized complete block design (batch as a block). The results were expressed as least-square mean with standard error, and significance of the main effect was tested by Tukey's multiple comparison test ($P < .05$). Statistical analysis was performed using the SAS program (version 9.3, SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1. Lipid oxidation

Malondialdehyde (MDA), a secondary product of lipid oxidation, exhibits potential mutagenic and genotoxic properties (Marnett, 1999). Oxidized lipids in infants may cause health problems such as growth inhibition, intestinal irritation, and pathological changes in the gastrointestinal mucous, enzyme activity, and lipid metabolism (Martysiak-Żurowska & Stołyhwo, 2006). Therefore, consumption of food containing lipid oxidation products may be detrimental for the health of infants and toddlers.

MDA content was not significantly different between D0 to D7 raw beef samples but significantly increased in D14 raw beef samples (Fig. 1). Continuous lipid oxidation has been reported in meat during storage (Filgueras et al., 2010; Park, Kim, & Choe, 2019). The extent of lipid oxidation in meat is dictated by the antagonistic effects of antioxidants and prooxidants. Meat is rich in endogenous antioxidants such as glutathione peroxidase, superoxide dismutase, anserine, and carnosine (Xiong, 2010), but their activities gradually decrease during storage as the levels of prooxidants such as free radicals and metal ions increase (Baron & Andersen, 2002; Descalzo et al., 2008). However, new endogenous antioxidants such as free amino acids and peptides are generated by the proteolysis of myofibrillar proteins during meat aging
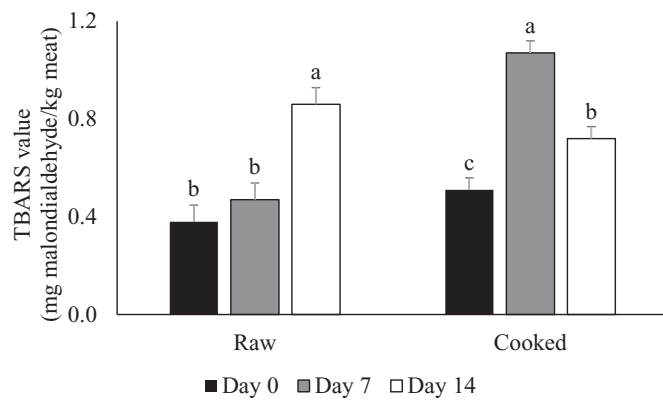
Fig. 1. TBARS values (mg malondialdehyde/kg meat) of the beef during aging for 14 days.

Standard error of the means for raw beef = 0.068 (n = 9) and cooked beef = 0.049 (n = 9).

[a-c] Different letters indicate significant differences between the means (P < .05).

(Escudero, Mora, Fraser, Aristoy, & Toldrá, 2013; Fu, Young, & Therkildsen, 2017; Saiga, Tanabe, & Nishimura, 2003).

The MDA content of cooked beef was significantly higher on D7 than on D0 (Fig. 1). However, the MDA content of D14 samples was lower than that of D7 samples. This observation may be attributed to the increase in the proteolysis of myofibrillar proteins during beef aging. The 10% TCA-soluble α-amino group content of beef significantly increased during aging. This fraction may contain small peptides comprising 3–4 amino acid residues (Yvon, Chabanet, & PÉLISSIER, 1989). Meat peptides exert antioxidant activities *via* their radical-scavenging and metal-chelating properties (Domínguez et al., 2019). The free iron ions derived from myoglobin denaturation are major prooxidants in beef, and their prooxidant effects are higher in cooked beef because free irons are abundantly released during heating (Jung, Nam, Ahn, Kim, & Jo, 2013; Min, Nam, Cordray, & Ahn, 2008). A previous study found that metal chelators exert significant antioxidant activities in cooked ground beef, but no antioxidant activity was observed in raw ground beef (Jung et al., 2013). Therefore, antioxidant peptides generated from the degradation of myofibrillar proteins may inhibit lipid oxidation in cooked beef at D14 *via* their metal-chelating and free radical-scavenging activities.

3.2. Protein oxidation

The side chains of basic amino acids may be attacked by active oxygen species and converted into carbonyl derivatives (Santé-Lhoutellier et al., 2008). These carbonyl groups may subsequently react with free amino acid groups and form amide bonds, initiate polymerization, and induce aggregation (Sante-Lhoutellier, Aubry, & Gatellier, 2007). Structural changes in that influence the active sites of proteases may affect protein susceptibility and digestibility (Bhat, Morton, Mason, & Bekhit, 2018b; Schøyen et al., 2005). Considerable oxidation can reduce protein digestibility owing to induced protein aggregation and precipitation (Sante-Lhoutellier et al., 2007).

No significant difference was observed in total carbonyl content during the entire aging period in both raw and cooked beef (Fig. 2). Herein, beef was aged and cooked after vacuum packaging. Similar results were observed by Filgueras et al. (2010) who reported no significant increase in carbonyl content over entire storage period.

3.3. α-Amino group content and MFI of beef

The protease-mediated cleavage of the peptide bond may result in an increase in the α-amino group content. The α-amino group content
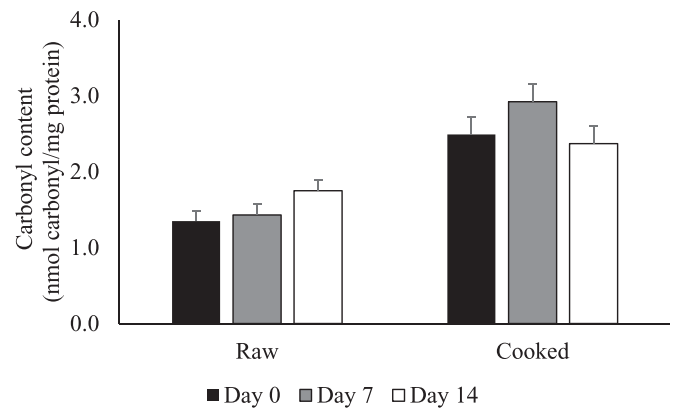

Fig. 2. Carbonyl content (nmol carbonyl/mg protein) of the beef during aging for 14 days.

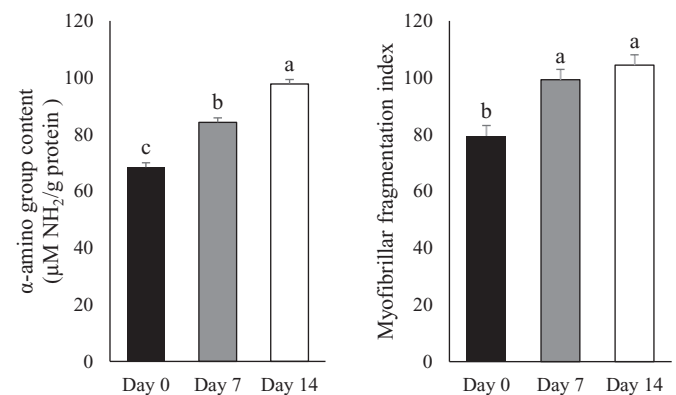Standard error of the means for raw beef = 0.139 (n = 9) and cooked beef = 0.229 (n = 9).

Fig. 3. α-Amino group content (μM NH$_2$/g protein) and myofibrillar fragmentation index of the beef during aging for 14 days.

Standard error of the means for α-amino group content = 1.559 (n = 9) and myofibrillar fragmentation index = 3.637 (n = 9).

[a-c] Different letters indicate significant differences between the means (P < .05).

of beef samples significantly increased with aging. MFI value significantly increased at D7 and was maintained thereafter (Fig. 3). As MFI value is positively correlated with protein hydrolysis, the increased MFI value is indicative of the increased proteolysis in the sarcomere I-band and weakening of inter-linkages in myofibrillar proteins, possibly owing to the action of muscle proteases (Bhat, Morton, Mason, & Bekhit, 2018a; Wang, Han, Ma, Yu, & Zhao, 2017).

Calpains and cathepsins are considered as the major enzymes participating in postmortem aging (Lomiwes, Farouk, Wu, & Young, 2014). As calpains are autolyzed upon exposure to sufficient Ca^{2+} required for their activation, autolysis is accompanied with calpain proteolysis. The autolyzed forms of calpains determine their activity (Bhat et al., 2018a). Although extended autolysis inactivates calpains, the reduced Ca^{2+} requirement of their autolyzed forms maintains their enzymatic activities (Edmunds, Nagainis, Sathe, Thompson, & Goll, 1991). Previous studies have reported autolysis and loss of calpain-1 activity at day 7 postmortem; however, potent calpain-2 activity was observed until day 56 after slaughter (Camou, Marchello, Thompson, Mares, & Goll, 2007; Geesink & Koohmaraie, 1999). In the present study, the beef used for aging was treated 7 days after slaughter, during which calpain may have undergone autolysis and the produced calpain-2 would have significantly contributed to proteolysis during aging. Moreover, the pH of beef samples was 5.50, 5.47, and 5.52 at D0, D7, and D14, respectively (data not shown). Previous studies have reported high cathepsin
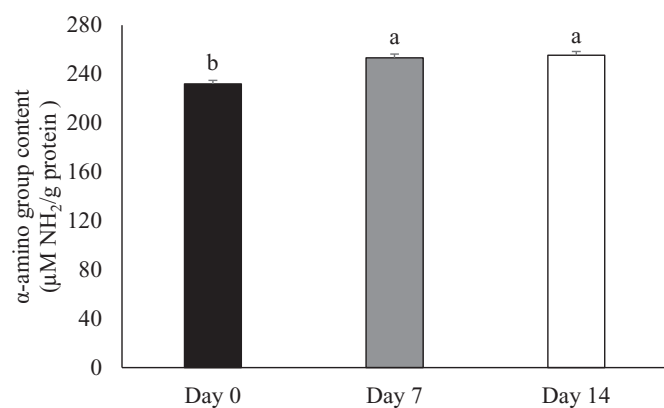
Fig. 4. α-Amino group content (μM NH$_2$/g protein) in the beef digesta after *in vitro* gastrointestinal digestion during aging for 14 days.
Standard error of the means = 3.051 (n = 9).
[a-b] Different letters indicate significant differences between the means (*P* < .05).

B activity for beef with low ultimate pH (Lomiwes et al., 2014) because cathepsins must be released from the lysosome and their optimum pH is pH 5.0–6.0 (Ouali, 1992; Sentandreu, Coulis, & Ouali, 2002). Therefore, cathepsins may have contributed to proteolysis during storage. The activity of endogenous proteases may have increased the fragmentation of myofibrillar proteins, while protein hydrolysis may have likely contributed to the increase in α-amino group content in beef samples.

3.4. α-Amino group content and SDS-PAGE of beef digesta after in vitro gastrointestinal digestion

The content of α-amino groups significantly increased in the digesta subjected to gastrointestinal digestion from D0 to D7, while no difference was observed between D7 and D14 samples (Fig. 4). This phenomenon may be attributed to the differences in the degree of protein digestion depending on the aging period. Thus, the digestibility of proteins in beef samples increased with aging.

On SDS-PAGE gels, the bands for actin (approximately 43–48 kDa) and troponin T (approximately 37 kDa) from the digesta subjected to gastrointestinal digestion faded with an increase in the aging period (Fig. 5). The relative band intensities of actin at D7 and D14 were 32% and 27%, respectively, which were significantly lower than 41% reported on D0. Further, the relative intensity of troponin T band gradually decreased from 35% at D0 to 33% on D7 and 32% on D14, although no significant difference was found (data not shown).

Actin is an abundant protein in the thin filament, accounting for 15–30% of total muscular proteins (Ahmed, Donkor, Street, & Vasiljevic, 2015). A previous study showed that the low protein digestibility of beef puree in an infant digestion model was associated with the low digestion of actin (Lee et al., 2019). Therefore, the improved digestibility of aged beef demonstrated herein may be related to the increase in actin digestion. However, actin degradation during postmortem aging remains controversial. Sawdy, Kaiser, St-Pierre, and Wick (2004) found that actin was not degraded under normal postmortem aging conditions. However, Lametsch, Roepstorff, Møller, and Bendixen (2004) reported slight degradation of actin during postmortem meat aging. The degradation of nebulin that spans the distal regions of thin filament and *Z*-disk is suggested to reduce the structural integrity of thin filament (Watanabe & Devine, 1996). Wu, Farouk, Clerens, and Rosenvold (2014) reported that nebulin was degraded almost completely after 7 days of aging and Ilian, Bekhit, and Bickerstaffe (2004) observed complete fragmentation of nebulin in 3 days postmortem. Since this large structural protein plays an important role in muscle myofibrillar proteins, the disruption of nebulin may have occurred the loss of structural integrity of thin filament (Wu
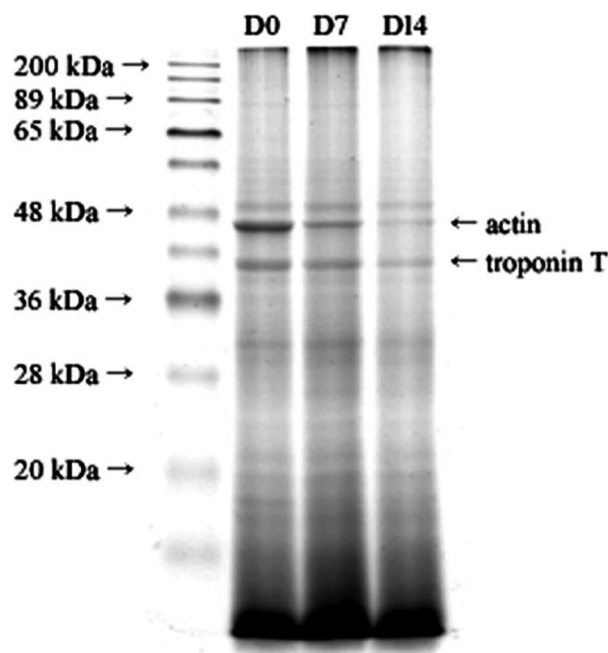

Fig. 5. SDS-PAGE electrophoretogram of beef digesta proteins after *in vitro* gastrointestinal digestion during aging for 14 days.
D0, beef aged for 0 days; D7, beef aged for 7 days; D14, beef aged for 14 days.

et al., 2014). Besides, previous study reported that because nebulin plays a direct role of promoting actin and myosin interaction, the absence of nebulin decreased the attachment probability between actin and myosin (Bang et al., 2009). Also, Takahashi (1996) reported the weakened rigor linkage formed between actin and myosin by fragmentation of nebulin filament. Therefore, the breakdown of nebulin may have also increased the substrate availability for proteases to actin.

As troponin T facilitates the interaction between actin and myosin, troponin T degradation may increase protease accessibility by reducing the structural integrity of myofibrillar filaments (Kitamura et al., 2005). Li, Xu, and Zhou (2012) showed that troponin T degradation weakens the actin-myosin cross-linkage and improves the substrate availability for endogenous proteases. Therefore, protein digestibility increases following the degradation and loss of the structural integrity of myofibrillar proteins during aging.

4. Conclusion

Lipid oxidation of raw and cooked beef increased after aging but was lower in D14 cooked beef samples than in D7 cooked beef samples. Therefore, changes in the antioxidative potential of aged beef warrant further studies. The myofibrillar proteins in beef were degraded during aging, while the α-amino group content of the cooked beef digesta from the infant *in vitro* digestion model increased with aging. The increased α-amino group content of the digesta after *in vitro* gastrointestinal digestion indicates the improved digestibility of beef proteins following aging. The improved protein digestibility of aged beef was related to the increased digestion of actin. Therefore, we conclude that aged beef is more desirable as complementary food owing to its increased protein digestibility.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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