Study on the Bioactivity of Chicken Tenderloin Protein Bars and Soy Beverages Enhanced with Myofibrillar Protein Hydrolysates from Korean Native Cattle

Running Title: Bioactivity of Products with Protein Hydrolysates from Korean Native Cattle

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ABSTRACT

This study comparatively analyzed protein digestibility, molecular weight, free amino acid composition, and changes in bioactivity during *in vitro* digestion of chicken tenderloin protein bars and soy beverages fortified with myofibrillar protein hydrolysates from various Korean native cattle breeds— Chikso, Jeju Black Cattle, Jeju Black Hanwoo, and Hanwoo—and a foreign breed, Black Angus. The results indicated that chicken tenderloin protein bars and soy beverages containing 0.5% and 1.0% protein hydrolysate exhibited enhanced bioactivity. Comparative analysis of protein digestibility before and after *in vitro* digestion revealed that myofibrillar protein hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus significantly improved protein digestibility. Molecular weight analysis demonstrated a breakdown into smaller molecular forms, suggesting potential improvements in digestibility and possible associations with bioactivity. On comparing the antioxidant and antihypertensive activities of protein hydrolysates from loin and shank, the loin protein hydrolysate from Jeju Black Cattle exhibited the highest bioactivity prior to *in vitro* digestion. Antioxidant and antihypertensive activities were considerably higher post-digestion compared with pre-digestion. This study concludes that myofibrillar protein hydrolysates derived from Jeju Black Cattle loin—at concentrations of 0.5% and 1% in chicken tenderloin protein bars and soy beverages—exhibit relatively high antioxidant and antihypertensive activities. These findings may have potential as functional food ingredients associated with antioxidant and antihypertensive activities.

Keywords: Korean native cattle, Protein hydrolysate, Bioactivity, Functional food

1. Introduction

Korean native cattle breeds possess distinct genetic and physicochemical characteristics that may influence the composition and bioactivity of muscle-derived protein hydrolysates. Previous studies have reported that protein hydrolysates derived from Hanwoo and other Korean native cattle breeds exhibit antioxidant and antihypertensive activities; however, the extent to which these functional properties differ among breeds remains insufficiently understood (Lee et al., 2017; Kim et al., 2024).

Myofibrillar proteins, which constitute a major portion of muscle proteins, can generate bioactive peptides through enzymatic hydrolysis. Protein hydrolysates generated from myofibrillar proteins have been reported to exhibit diverse biological activities, including antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities (Kim et al., 2024). In addition, low-molecular-weight peptides derived from animal proteins generally exhibit high digestibility and bioactivity, generally exhibit high digestibility and bioactivity (Hu et al., 2023). Previous studies have also reported that protein hydrolysates from Korean native cattle breeds, such as Chikso, Jeju Black Cattle, and Hanwoo, may exhibit different antioxidant and antihypertensive activities depending on breed characteristics (Lee et al., 2017; Kim et al., 2024). These findings suggest that breed origin may influence the functional properties of cattle-derived protein hydrolysates.

In addition to protein source, the bioactivity and digestibility of protein hydrolysates can also be affected by the physicochemical properties of food matrices. Factors such as protein composition, fat content, texture, and structural characteristics may influence the incorporation, stability and digestion behavior of bioactive peptides in food systems. Therefore, evaluating protein hydrolysates in different food matrices is important for understanding matrix-dependent differences in their functional behavior and bioactivity. Chicken tenderloin protein bars and soy beverages were selected in this study as representative solid and liquid food matrices with distinct protein compositions and structural

characteristics. Chicken tenderloin protein bars provide an animal protein-based solid matrix with relatively high protein content and complex texture characteristics, whereas soy beverages represent a plant-based liquid matrix with different physicochemical properties. These contrasting food systems enabled the evaluation of matrix-dependent differences in digestibility, antioxidant activity, and antihypertensive activity following the incorporation of cattle-derived protein hydrolysates.

Therefore, this study primarily aimed to determine whether breed origin and food matrix differentially affect the bioactivity of cattle-derived myofibrillar protein hydrolysates during *in vitro* digestion.

2. Materials and Methods

2.1 Materials

The loin and shank of Chikso, Jeju Black Cattle, and Jeju Black Hanwoo utilized in the experiments were supplied by the National Institute of Animal Science (Jeonbuk, Korea). The Chikso, Jeju Black Cattle, and Jeju Black Hanwoo were 34, 31, and 31 months old, respectively, and all were castrated steers. The loin and shank of Hanwoo were purchased from Achim Farm (Chungnam, Korea), originating from a 34-month-old castrated steer. The loin of Black Angus was procured from Haengbok Mart (Busan, Korea), while the shank was acquired from SNT Solution (Seongnam, Korea). Chicken tenderloin was sourced from Hau CM (Daegu, Korea). Additional ingredients—including canola oil, pancake mix, eggs, onion powder, refined salt, milk, soy sauce, sugar, nucleotide seasoning, beef bone powder, pepper, and lemon juice—were secured from a local mart in Anseong, Korea. Roasted peanuts, roasted almonds, and soybeans were also obtained from a local mart in Anseong.

For the *in vitro* digestion experiments, α-amylase from hog pancreas (CAS No. 9000-90-2), bile extract porcine (CAS No. 8008-63-7), lipase from porcine pancreas (CAS No. 9001-62-

1), mucin from porcine stomach Type II (CAS No. 84082-64-4), pepsin from porcine gastric mucosa (CAS No. 9001-75-6), and uric acid (≥ 99%, crystalline, CAS No. 69-93-2) were purchased from Sigma-Aldrich (St. Louis, USA). Pancreatin from porcine pancreas (CAS No. 8049-47-6) was procured from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), while bovine serum albumin (CAS No. 9048-46-8) was acquired from Biosesang (Seongnam, Korea).

For the protein hydrolysate extraction experiments, sodium phosphate monobasic anhydrous (CAS No. 7558-80-7) and sodium phosphate dibasic anhydrous (CAS No. 7558-79-4) were secured from Daejung (Siheung, Korea) to prepare a 0.04 M phosphate buffer (pH 7.4). The Amicon® Ultra-15 Centrifugal Filter Unit with an Ultracel-10 regenerated cellulose membrane (15-mL sample volume, CAT No. UFC901024) was sourced from Merck Millipore (Massachusetts, USA).

For the bicinchoninic acid (BCA) assay, trichloroacetic acid (TCA, CAS No. 76-03-9) was obtained from Daejung (Siheung, Korea), while the Pierce™ BCA protein assay kit (CAT No. 23225) was acquired from Thermo Fisher Scientific (Massachusetts, USA).For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the following reagents were secured: Tris (Catalog No. 1610719), 30% acrylamide/bis solution (37.5:1, Catalog No. 1610158), 10% (w/v) SDS solution (Catalog No. 1610416), ammonium persulfate (Catalog No. 1610700), tetramethylethylenediamine (Catalog No. 1610800), 10× Tris/Glycine/SDS buffer (Catalog No. 1610772), 4× Laemmli sample buffer (Catalog No. 1610747), Precision Plus Protein™ Dual Xtra Standards (Catalog No. 1610374), and Bio-Safe Coomassie G-250 stain (Catalog No. 1610786), all sourced from BIO-RAD (California, USA). A microcentrifuge (Catalog No. DH.WCF00010) and a heating block (Catalog No. DH.WHB00348) from Daihan Scientific (Seoul, Korea) were employed during the experiments.

To analyze amino acid composition, buffers PF-1, 2, 3, and 4, alongside RG, for the Hitachi high-speed amino acid analyzer were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The ninhydrin coloring solution kit for the Hitachi analyzer was obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). Amino acid analysis was conducted utilizing the Hitachi L-8900 amino acid analyzer from Hitachi High-Technologies (Tokyo, Japan).

To measure antioxidant activity, methyl alcohol (MeOH, 99.5%, CAS No. 67-56-1) was procured from Samchun Pure Chemical Co., Ltd. (Seoul, Korea). Additionally, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (CAS No. 30931-67-0) was acquired from Sigma-Aldrich (St. Louis, USA), L(+)-Ascorbic acid (99.5%, CAS No. 50-81-7) from Samchun Pure Chemical Co., Ltd. (Seoul, Korea), and potassium persulfate (CAS No. 7727-21-1) from Daejung Chemicals (Siheung, Korea). Furthermore, 2,2-Diphenyl-1-picrylhydrazyl (CAS No. 1898-66-4) was secured from Sigma-Aldrich (St. Louis, USA), and 2,6-Di-tert-butyl-4-methylphenol (99.0%) was obtained from Samchun Pure Chemical Co., Ltd. (Seoul, Korea).

For antihypertensive activity measurements, ethyl acetate (Catalog No. E0688), sodium chloride (Catalog No. S0476), and sodium tetraborate anhydrous (Catalog No. S3412) were acquired from Samchun Pure Chemical Co., Ltd. (Seoul, Korea). Boric acid (Catalog No. 04232-00) was sourced from Kanto Chemical Co., Inc. (Tokyo, Japan), while lung acetone powder from rabbit (L0756-5G) and N-Hippuryl-His-Leu hydrate (H1635-100MG) were purchased from Sigma-Aldrich (St. Louis, USA). The Elabscience® ACE activity assay kit (Catalog No. E-BC-K003-S) used for analyzing antihypertensive enzyme activity was obtained from Elabscience (Texas, USA).

To measure absorbance, a SpectraMax 190 microplate reader from Molecular Devices (California, USA) and a Cary 300 ultraviolet-visible (UV/VIS) spectrophotometer from Agilent Technologies (California, USA) were utilized.

2.2 Extraction of protein hydrolysates from myofibrillar protein

To extract protein hydrolysates from Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, the methodologies outlined by Kim et al. (2021) and Lee & Hur (2019) were employed (Kim et al., 2021; Lee & Hur, 2019). Figure 1 illustrates the overall experimental procedure. Initially, the loin and shank from the aforementioned cattle breeds were prepared at 1,000 × g, with connective tissue and fat meticulously removed. Distilled water (DW), in a volume 10 times the weight of the raw material, was added, and the samples were thoroughly ground using a mixer. The fat and connective tissue that surfaced were discarded along with the DW. This process was repeated approximately 10 times to completely remove blood and fat. Thereafter, the precipitate collected at the bottom was used to extract myofibrillar proteins using a 0.04 M phosphate buffer.

Protein hydrolysates were subsequently extracted from the myofibrillar proteins of the loin and shank derived from Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, using the proteolytic enzymes Alcalase 2.4L, ProteAX, and trypsin from porcine pancreas (Kim et al., 2024). DW and the extracted myofibrillar proteins were mixed in a 1:4 ratio, homogenized, and adjusted to pH 8.0. Alcalase 2.4L was added at a concentration of 0.67% relative to the weight of the myofibrillar proteins and reacted at 50 °C with agitation at 60 rpm. ProteAX was treated under identical conditions, while trypsin was reacted at 37 °C and 60 rpm.

To inactivate the enzymes, the samples were heated to 95°C for 10 min, and impurities were removed using filter paper No. 4. The resulting filtrates were centrifuged at 1,977 × g and 4 °C for 20 min with a centrifugal ultrafiltration device to obtain protein hydrolysates with

163 molecular weights < 10 kDa. The isolated protein hydrolysates were dried at 54 °C for 24 h
164 and subsequently freeze-dried for 48 h to eliminate moisture. The final samples were stored
165 at –70 °C until further analysis.

166 **2.3 Preparation of chicken tenderloin protein bars and soy beverage**

167 To prepare chicken tenderloin protein bars, myofibrillar proteins were extracted according to
168 the same methodology utilized for chicken tenderloin (Kim et al., 2021; Lee & Hur, 2019).
169 The extracted myofibrillar proteins were combined with canola oil, pancake mix, egg white,
170 onion powder, refined salt, milk, soy sauce, sugar, nucleotide seasoning, beef bone powder,
171 pepper, egg yolk, and lemon juice, as detailed in Table 1. Protein hydrolysates were
172 subsequently incorporated at varying concentrations to prepare the experimental treatments
173 for the study.

174 To prepare the soy beverage, 100 g of soybeans were soaked in 300 mL of water for 8 h.
175 After soaking, the seed coats were entirely removed, and the water was drained. The soaked
176 soybeans were subsequently boiled for 4 min with three times their weight in water. Rolled
177 oats were roasted in a frying pan for 2 min, and roasted almonds, roasted peanuts, and roasted
178 rolled oats were added to the boiled soybeans according to the composition detailed in Table
179 2 to formulate the soy beverage.

180 Thereafter, 100 and 200 g of water were added sequentially and thoroughly blended using a
181 mixer. An additional 400 g of water was added while monitoring the consistency, and the
182 mixture was blended again until smooth. The blended mixture was then filtered twice through
183 muslin cloth to eliminate impurities. The resulting soy beverage was supplemented with
184 sugar and salt in the quantities specified in Table 2, and protein hydrolysates were
185 incorporated at varying concentrations to establish the experimental treatment groups for the
186 study.

The overall procedure of producing chicken tenderloin protein bar and soy beverage is illustrated in Figure 2.

2.4 *In vitro* digestion experiment

The *in vitro* digestion process employed in this study to assess the digestibility and bioactivity changes of chicken tenderloin protein bars and soy beverages supplemented with protein hydrolysates from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus was adapted from methods outlined by Lee et al. (2016). Table 3 presents the preparation conditions for the digestive enzymes, inorganic solvents, and organic solvents utilized during the oral, gastric, and intestinal phases of the *in vitro* digestion experiment (Hur et al., 2011; Lee et al., 2016). For the colon phase, intestinal microbiota were introduced to the samples.

The liquid medium for *Escherichia coli* (American Type Culture Collection [ATCC] 25922, Manassas, VA, USA) was prepared by dissolving 2.5 g of Luria Bertani Broth (Sparks, MD, USA) in 100 mL of deionized water (DIW), while the liquid medium for *Lacticaseibacillus casei* (ATCC 393) was prepared by dissolving 5.5 g of Lactobacilli MRS Broth (Sparks, MD, USA) in 100 mL of DIW. Both media were sterilized at 121 °C for 15 min and subsequently cooled. Stocks of *Escherichia coli* and *Lacticaseibacillus casei*, stored at –80 °C, were thawed at room temperature, and 1% of each microbial stock was inoculated into 100 mL of the respective liquid medium. *Escherichia coli* was cultured at 37 °C for 12 h, while *Lacticaseibacillus casei* was cultured at 37 °C for 24 h. This procedure was repeated for up to three passages to achieve a total microbial count of 10^8–10^9 CFU/mL.

The complete *in vitro* digestion process simulating typical adult digestive conditions is illustrated in Figure 3.

To simulate the conditions of adult oral digestion, 5 g of each sample was transferred into a 50-mL conical tube, to which 5 mL of saliva was added. The mixture was subsequently incubated in a water bath at 37 °C while being agitated at 150 rpm for 5 min.

For the *in vitro* digestion process, gastric digestion was simulated by adding 10 mL of gastric juice and maintaining incubation at 37 °C and 150 rpm for 2 h. Subsequently, small intestine digestion was replicated by adding 10 mL of duodenal juice and 5 mL of bile juice under the same conditions. Large intestine digestion was modeled by incorporating 10 mL of *Lacticaseibacillus casei* and 5 mL of *Escherichia coli*, with the incubation period extended to 4 h, thereby completing the digestion process.

Control groups were established for each *in vitro* digestion experiment. One control group exclusively contained digestive enzymes, with samples replaced by DW, while the second group consisted of only the samples, with digestive enzymes replaced by DW. Upon completion of the digestion process, all samples were centrifuged at 13,000 × g and 4 °C for 20 min. The supernatants were subsequently collected and utilized as final samples.

2.5 Protein quantitative analysis via BCA assay

The protein digestibility of hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, as well as chicken tenderloin protein bars and soy beverages prepared with these protein hydrolysates, was assessed using the BCA assay. This analysis followed the protocols established by Smith et al. (1985) and Wiechelman et al. (1988).

A 25-µL aliquot of each sample was dispensed into a microplate, followed by the addition of 200 µL of BCA solution. The microplate was covered with foil and incubated at 37 °C for 30 min. After incubation, absorbance was measured at 562 nm using an enzyme-linked immunosorbent assay reader within 5 min. The obtained values were utilized to calculate the remaining protein content following digestion.

2.6 SDS-PAGE

The SDS-PAGE method was employed to analyze the electrophoretic patterns of proteins both before and after *in vitro* digestion in chicken tenderloin protein bars and soy beverages supplemented with protein hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus. Sample preparation was conducted in accordance with the protocol used in the protein digestibility analysis via the BCA assay.

A 10-μL aliquot of each sample, along with 4 μL of protein marker, was loaded into the 12% running gel. Electrophoresis was executed at 80 V for 10 min, followed by an increase to 100 V for 90 min. Upon completion of the run, the gel was removed and rinsed multiple times with DIW. Thereafter, it was stained with a staining solution at room temperature for over 3 h. Finally, destaining was performed using a destaining solution, followed by additional rinses with DIW.

2.7 Analysis of free amino acids

The free amino acid composition of protein hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus was analyzed using a Hitachi L-8900 amino acid analyzer. Each sample was diluted to the appropriate concentration, and 1 mL was combined with an equal volume of 5% TCA solution. The mixture was stirred and centrifuged at 13,500 × g and 20 °C for 20 min. Subsequently, 4 mL of n-hexane was added to 2 mL of the supernatant, followed by shaking for 15 min and centrifugation at 1,977 × g at 20 °C for 20 min. The lower layer was collected; to ensure complete removal of n-hexane, the centrifugation process was repeated twice at 13,500 × g and 20 °C for 20 min. The final sample was filtered through a 0.2-μm syringe filter prior to analysis.

2.8 Analysis of antioxidant activity

To evaluate the antioxidant activity of the protein hydrolysates sourced from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, as well as chicken tenderloin protein bars and soy beverages supplemented with these protein hydrolysates, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays were conducted before and after *in vitro* digestion.

The ABTS assay was performed following a modified protocol based on Re et al. (1999). An ABTS stock solution was prepared by combining a 7 mM ABTS solution with 2.45 mM potassium persulfate. This mixture was shielded with aluminum foil and incubated at 25 °C for 12–16 h.

The ABTS working solution was obtained by diluting the stock solution with MeOH until the absorbance at 734 nm reached 0.7 ± 0.02. Ascorbic acid served as the standard solution, prepared with DW. MeOH was utilized as the control. In the assay, 20 μL of each sample and 180 μL of the ABTS working solution were added to a 96-well plate and allowed to react in the dark for 10 min. Absorbance was subsequently measured at 734 nm using a microplate reader.

The ABTS scavenging activity was calculated using the following formula:

$$ABTS\ scavenging\ activity\ (\%) = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100$$

The DPPH assay was conducted by modifying the method outlined by Tepe et al. (2005). A DPPH solution was prepared by dissolving 2 mg of 2,2-diphenyl-1-picrylhydrazyl in 25 mL of MeOH in a 50-mL conical tube.

Ascorbic acid, prepared with DW as the solvent, functioned as the standard solution. MeOH also served as the control. For the assay, 100 μL of each sample and 100 μL of the DPPH

283 solution were added to a 96-well plate and allowed to react in the dark for 30 min.
284 Absorbance was subsequently measured at 517 nm using a microplate reader.
285 The DPPH scavenging activity was calculated using the following formula:

286 $$DPPH\ scavenging\ activity\ (\%) = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100$$

287

288 **2.9 Analysis of antihypertensive activity**

289 The angiotensin-converting enzyme (ACE) inhibitory activity assay was conducted to
290 evaluate the antihypertensive effects of protein hydrolysates derived from the loin and shank
291 of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, as well as from
292 chicken tenderloin protein bars and soy beverages supplemented with these hydrolysates,
293 both before and after *in vitro* digestion.
294 This assay was adapted from the methodology described by Cushman and Cheung (1971). To
295 prepare the ACE solution, a 0.1 M sodium borate buffer (pH 8.3) was created by combining
296 sodium tetraborate and boric acid, followed by the addition of 0.5 M sodium chloride. Lung
297 acetone powder from rabbit was extracted at a concentration of 50 mg/mL (w/v) by stirring at
298 4 °C for 24 h. The mixture was subsequently centrifuged at 9,400 × g at 4 °C for 30 min, and
299 the supernatant was collected and stored. The ACE substrate was prepared by dissolving N-
300 Hippuryl-His-Leu hydrate in 0.1 M sodium borate buffer (pH 8.3) to achieve a concentration
301 of 8.3 mM.
302 For the assay, 50 µL of each sample was added to 2 mL tubes, with DW serving as the
303 control. Additional samples and controls were prepared by adding 1 M HCl at the outset to
304 terminate the reaction. To each sample, 50 µL of the ACE substrate was added and allowed
305 to react at 37 °C for 10 min. Thereafter, 50 µL of ACE solution (25 mM/mL) was added to
306 the reaction mixtures, which were incubated at 37 °C for 30 min. For samples without initial

HCl addition, 250 µL of 1 M HCl was added to terminate the reaction, followed by the addition of 500 µL of ethyl acetate. The mixtures were vortexed for 1 min and centrifuged at 1,977 × g for 10 min. From the supernatants, 200 µL was collected, dried at 60 °C for 30 min, and reconstituted with 1 mL of distilled water to prepare the final samples.

The absorbance of the final samples was measured at 228 nm using a UV/VIS spectrophotometer.

$$ACE\ inhibitory\ activity\ (\%) = \left(\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control - Absorbance\ of\ control_{HCl}}\right) \times 100$$

2.10 Statistical analysis

The experimental results were analyzed using SPSS Statistics 26 (IBM, Armonk, NY, USA). Mean values and standard deviations were calculated, followed by statistical analyses. Differences between two variables were assessed using Student's t-test, while *post hoc* comparisons among mean values were conducted using the Student–Newman–Keuls test at a significance level of $p < 0.05$.

3. Results and Discussion

3.1 Antioxidant and antihypertensive analysis of products supplemented with different concentrations of protein hydrolysates

3.1.1 Chicken tenderloin protein bar

ABTS and DPPH radical scavenging assays showed no significant differences in antioxidant activity among hydrolysate concentrations ranging from 0.5% to 2.0%, although the highest activity was consistently observed at 0.5%. Similarly, ACE inhibitory activity did not differ significantly among concentrations, with 0.5% exhibiting the highest activity in Figure 8.

The physiological activity of protein hydrolysates is influenced by factors such as hydrolysis conditions, molecular weight distribution of peptides, and amino acid composition. Previous studies have suggested that antioxidant and ACE inhibitory activities tend to increase with higher concentrations of protein hydrolysates; however, these activities may plateau or decline beyond a certain concentration (Chen et al., 2012; Senadheera et al., 2023). The absence of further increases in antioxidant and ACE inhibitory activities at higher concentrations may be associated with peptide aggregation or reduced peptide solubility, which can limit interactions between bioactive peptides and target radicals or enzymes. Because no significant improvement in antioxidant or ACE inhibitory activities was observed at concentrations above 0.5%, this concentration was selected for subsequent analyses because no further significant improvement was observed at higher concentrations.

3.1.2 Soy beverage

Consistent with the chicken tenderloin protein bar results, ABTS and DPPH radical scavenging assays showed no significant differences in antioxidant activity among hydrolysate concentrations ranging from 0.5% to 2.0%, although antioxidant activity was highest at 0.5%. In contrast, ACE inhibitory activity was significantly greater at concentrations of 1.0% or higher than at 0.5%, with the highest activity observed at 1.0% in Figure 9. These findings suggest that incorporating protein hydrolysates at a concentration of 1.0% into soy beverages optimally enhances both antioxidant and antihypertensive activities. These findings suggest that relatively low supplementation levels were sufficient to maintain antioxidant activity, whereas slightly higher concentrations were required to enhance ACE inhibitory activity in the soy beverage matrix.

The different concentration-dependent trends observed between antioxidant and ACE inhibitory activities may reflect differences in peptide interactions within the soy beverage matrix and differences in the mechanisms underlying radical scavenging and ACE inhibition.

3.2 Analysis of protein digestibility and composition changes in products supplemented with protein hydrolysates before and after *in vitro* digestion

3.2.1 Analysis of protein digestibility

Protein digestibility of chicken tenderloin protein bars and soy beverages supplemented with cattle-derived protein hydrolysates was evaluated during *in vitro* digestion using the BCA assay in Figure 4. Treatment groups receiving protein hydrolysate supplementation exhibited significantly greater protein digestibility than the non-supplemented controls. This increase in digestibility may be associated with the presence of low-molecular-weight peptides generated during enzymatic hydrolysis, which can improve enzyme accessibility and facilitate protein breakdown during digestion. Differences in digestibility among treatment groups may also reflect interactions between protein hydrolysates and food matrix components, including variations in protein composition and structural properties between the chicken tenderloin protein bar and soy beverage systems. These findings indicate that incorporation of cattle-derived protein hydrolysates may improve protein digestibility during *in vitro* digestion, although the extent of improvement may vary depending on breed origin and food matrix characteristics.

3.2.2 Analysis of changes in protein molecular weight

SDS-PAGE analysis revealed substantial changes in protein molecular weight distributions before and after *in vitro* digestion in both chicken tenderloin protein bars and soy beverages in Figures 5–6. Before digestion, protein bands were broadly distributed across higher molecular weight regions, whereas post-digestion samples predominantly exhibited bands below 5 kDa. Soy beverages exhibited a broader initial molecular weight distribution than chicken tenderloin protein bars, possibly reflecting differences in matrix composition and protein structure. Conversely, following *in vitro* digestion, proteins with low molecular weights of 5 kDa were predominantly observed.

A comparison of protein bands before and after *in vitro* digestion in soy beverages, contrasting the control group without protein hydrolysate supplementation with the treatment group that received supplementation, revealed that proteins with molecular weights ranging from 5 to 75 kDa were evenly distributed, as illustrated in Figures 5–6. Nonetheless, following *in vitro* digestion, a predominance of low-molecular-weight proteins, specifically those below 5 kDa, was observed, mirroring trends noted in chicken tenderloin protein bars. Low-molecular-weight peptides generated via protein hydrolysis can exhibit various physiological activities, including antioxidant and antihypertensive effects. Previous studies have reported that low-molecular-weight peptide fractions exhibit enhanced antioxidant and ACE inhibitory activities (Chen et al., 2023; Zou et al., 2020). The predominance of low-molecular-weight peptides after digestion suggests that gastrointestinal digestion further hydrolyzed proteins and incorporated hydrolysates into smaller peptide fractions, which may contribute to the increased antioxidant and ACE inhibitory activities observed after digestion. These findings support the possibility that digestion-induced generation of low-molecular-weight peptides contributed to the enhanced bioactivity observed after *in vitro* digestion. However, the specific peptide sequences responsible for these functional changes were not identified in the present study.

3.3 Free amino acid content and bioactivity of protein hydrolysates

3.3.1 Composition analysis of free amino acids

Differences in free amino acid composition were observed among protein hydrolysates derived from different cattle breeds and muscle.

Previous studies have reported that amino acids such as lysine and phenylalanine contribute to antioxidant and ACE inhibitory activities (Cheng et al., 2020; Huang et al., 2021). In the current study, protein hydrolysates derived from Jeju Black Cattle loin exhibited the highest

concentrations of lysine and phenylalanine among all treatment groups, measuring 14.0 ± 0.1 and 7.2 ± 0.4, respectively.

In the present study, protein hydrolysates derived from Jeju Black Cattle loin exhibited the highest lysine and phenylalanine contents, which may partially explain the relatively high antioxidant and antihypertensive activities observed.

These findings suggest that breed-dependent differences in amino acid composition may partially contribute to variations in the bioactivity of cattle-derived protein hydrolysates. However, further studies are needed to identify the specific peptides and amino acid interactions responsible for these functional differences.

3.3.2 Analysis of antioxidant and antihypertensive activity

To assess the antioxidant and antihypertensive activities of protein hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, we employed the ABTS radical scavenging assay, DPPH radical scavenging assay, and ACE inhibitory assay. The results of these analyses are presented in Figure 7.

The ABTS radical scavenging assay results demonstrated that the antioxidant activity of the protein hydrolysates was generally lower than that of ascorbic acid ($p < 0.05$). Among the treatment groups, the protein hydrolysates derived from the loin of Jeju Black Cattle exhibited the highest antioxidant activity. A similar trend was observed in the DPPH radical scavenging assay results.

While the ABTS radical scavenging assay evaluates antioxidant activity using cation radicals, the DPPH radical scavenging assay employs free radicals, reflecting distinct mechanisms (Song et al., 2018). Although ABTS and DPPH assays are based on different radical scavenging mechanisms, both assays showed similar trends among treatment groups.

The ACE inhibitory activity assay results indicated that protein hydrolysates from the loin of Jeju Black Cattle demonstrated the highest ACE inhibitory activity, followed by those

derived from the shank of Hanwoo and the loin of Black Angus. These findings suggest that protein hydrolysates from the loin of Jeju Black Cattle exhibited relatively high ACE inhibitory activity

Additionally, research on the antihypertensive activity of free amino acids has demonstrated that phenylalanine is particularly effective in inhibiting ACE activity and regulating blood pressure (Yu et al., 2018; Olalere et al., 2023). These findings align with the antihypertensive activity results presented in Figure 7, suggesting that amino acid composition may partially contribute to the observed ACE inhibitory activity.

However, the specific bioactive peptides responsible for these activities were not identified in the present study.

3.4 Analysis of bioactivity in products supplemented with protein hydrolysates before and after *in vitro* digestion

3.4.1 Chicken tenderloin protein bar

The antioxidant activity of chicken tenderloin protein bars before and after *in vitro* digestion was assessed using the ABTS and DPPH radical scavenging assays, with results depicted in Figure 10. The assays revealed a significant increase in antioxidant activity following digestion compared with pre-digestion levels. This increase in antioxidant activity after digestion may be associated with previous studies reporting that gastrointestinal digestion can further hydrolyze proteins and generate bioactive low-molecular-weight peptides (Wijesekara et al., 2024; Xi et al., 2024).

Among the pre-digestion results from the ABTS and DPPH radical scavenging assays, the chicken tenderloin protein bar supplemented with protein hydrolysates from the loin of Jeju Black Cattle exhibited the highest antioxidant activity. Post-digestion, the ABTS radical scavenging assay results indicated significantly higher antioxidant activity in the treatment groups supplemented with protein hydrolysates from the loin and shank of Jeju Black Cattle

and Jeju Black Hanwoo. In the DPPH radical scavenging assay, the highest antioxidant activity was observed in the treatment group supplemented with protein hydrolysates from the loin of Hanwoo. These shifts in antioxidant activity patterns after digestion suggest that breed-specific peptide compositions responded differently to gastrointestinal digestion. These results indicate that chicken tenderloin protein bars supplemented with protein hydrolysates exhibit distinct trends in antioxidant activity before and after digestion. Notably, as protein hydrolysates undergo further breakdown during the colonic phase owing to microbial activity, their bioactivity may be enhanced. Differences in peptide release and transformation during digestion may have contributed to the altered post-digestion antioxidant activity rankings observed among breeds.

An ACE inhibitory assay was performed to assess antihypertensive activity before and after *in vitro* digestion (**Figure 11**). Consistent with the antioxidant activity results, antihypertensive activity post-digestion was significantly greater than that observed pre-digestion. Moreover, both pre- and post-digestion, chicken tenderloin protein bars supplemented with protein hydrolysates demonstrated higher antihypertensive activity than the non-supplemented CTL group, aligning with the findings from the analysis of the protein hydrolysates. Among the pre-digestion results, treatment groups supplemented with protein hydrolysates derived from the loin of Chikso, the loin of Jeju Black Cattle, the shank of Hanwoo, and the loin of Black Angus exhibited the highest antihypertensive activity. However, post-digestion ACE inhibitory activity exhibited a different pattern from that observed before digestion, suggesting that gastrointestinal digestion differentially altered antihypertensive peptide profiles among breeds. The ACE inhibitory assay results post-digestion revealed that the treatment group supplemented with protein hydrolysates from the loin of Black Angus exhibited the highest activity. This transition in activity suggests that digestion may have facilitated the breakdown of proteins and protein hydrolysates into lower-

480 molecular-weight fractions, potentially contributing to enhanced ACE inhibitory activity, as
481 smaller peptides are often more effective inhibitors (Erdmann et al., 2008; Udenigwe & Aluko,
482 2012). These findings are consistent with the predominance of low-molecular-weight protein
483 fractions observed after digestion in the SDS-PAGE analysis. Previous studies have
484 demonstrated that digestion can modify peptide structures, influencing their bioactivity and
485 absorption (Amigo & Hernández-Ledesma, 2020). These findings underscore the necessity of
486 considering digestion-induced changes when evaluating the functional potential of protein
487 hydrolysates in antihypertensive applications.

488 **3.4.2 Soy beverage**

489 Before digestion, soy beverages supplemented with protein hydrolysates derived from Jeju
490 Black Cattle loin exhibited the highest antioxidant activity in both ABTS and DPPH assays.
491 However, post-digestion antioxidant activity patterns differed, with Black Angus-derived
492 protein hydrolysates exhibiting the greatest activity. Similarly, in the DPPH radical
493 scavenging assay, treatment groups supplemented with protein hydrolysates from both the
494 loin and shank of Black Angus demonstrated significantly higher antioxidant activity in
495 Figure 12.

496 In the DPPH radical scavenging assay, the treatment group supplemented with protein
497 hydrolysates from the shank of Chikso exhibited no significant difference in antioxidant
498 activity before and after digestion. The limited change in antioxidant activity observed in the
499 Chikso shank treatment group may reflect the relatively high baseline antioxidant activity
500 observed before digestion.

501 These results indicate that soy beverages supplemented with protein hydrolysates exhibit
502 distinct trends in antioxidant activity both before and after digestion. This finding is
503 consistent with previous research, which demonstrated that protein hydrolysates enhance
504 antioxidant activity post-digestion owing to the release of bioactive peptides with improved

505 radical scavenging abilities (Zhao et al., 2020). These digestion-dependent shifts in
506 antioxidant activity suggest that peptide release and transformation during digestion differed
507 according to cattle breed and food matrix interactions.
508 The ACE inhibitory assay was performed to assess the antihypertensive activity of soy
509 beverages before and after *in vitro* digestion, with results illustrated in Figure 13. Similar to
510 the antioxidant activity findings, antihypertensive activity post-digestion was significantly
511 elevated compared with pre-digestion levels. This increase in ACE inhibitory activity after
512 digestion may be associated with the formation of bioactive low-molecular-weight peptides
513 during digestion (Suo et al., 2022). Furthermore, in alignment with the chicken tenderloin
514 protein bar experiment, soy beverages supplemented with protein hydrolysates demonstrated
515 greater antihypertensive activity than the non-supplemented CTL group, reinforcing trends
516 observed in the antihypertensive activity analysis of protein hydrolysates.
517 On examining antihypertensive activity measurements for soy beverages prior to *in vitro*
518 digestion, the treatment group supplemented with protein hydrolysates from Jeju Black Cattle
519 loin exhibited the highest activity. Nevertheless, post-digestion, the group supplemented with
520 protein hydrolysates from Black Angus loin demonstrated the highest antihypertensive
521 activity. This variation may stem from differences in peptide profiles generated during
522 digestion, influenced by the specific protein sources employed. Previous studies have
523 demonstrated that the enzymatic hydrolysis of different protein sources potentially yields
524 peptides with varying bioactivity, thus affecting their potential antihypertensive properties
525 (Peighambardoust et al., 2021). These findings suggest that gastrointestinal digestion
526 differentially modified antihypertensive peptide profiles among cattle breeds, resulting in
527 altered post-digestion activity rankings.
528 These findings suggest that although the soy beverage supplemented with Jeju Black Cattle
529 loin protein hydrolysates exhibited the highest activity prior to *in vitro* digestion, variations in

antihypertensive activity trends before and after digestion imply that proteins and their hydrolysates are further degraded into lower-molecular-weight components during the digestive process. These findings are consistent with the SDS-PAGE results showing increased low-molecular-weight protein fractions after digestion, which may have contributed to the enhanced post-digestion bioactivity.

These digestion-dependent shifts may reflect differences in peptide release, peptide stability, or peptide transformation among breeds and muscle. However, because peptide profiles were not directly characterized in the present study, the possibility that assay variability or unmeasured peptide composition differences contributed to these changes cannot be excluded.

4. Conclusion

In this study, myofibrillar protein hydrolysates derived from Korean native cattle breeds and Black Angus were incorporated into chicken tenderloin protein bars and soy beverages to evaluate and bioactivity before and after *in vitro* digestion. Supplementation with protein hydrolysates improved protein digestibility, antioxidant activity, and ACE inhibitory activity compared with non-supplemented controls. Among the tested protein hydrolysates, Jeju Black Cattle loin generally exhibited relatively high bioactivity before digestion, whereas post-digestion activity patterns differed among treatment groups, particularly in foods supplemented with Black Angus protein hydrolysates. These findings suggest that breed origin and food matrix may influence bioactivity of cattle-derived protein hydrolysates. However, because specific peptide profiles were not identified, further peptidomic analyses are needed to clarify the mechanisms underlying breed- and matrix-dependent differences in bioactivity.

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Conflicts of Interest

The authors declare no potential conflicts of interest.

Data Availability

Data available upon reasonable request to the authors

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650 # Tables

651 **Table 1. Composition of chicken tenderloin protein bar supplemented with protein**
652 **hydrolysates**

Materials	Chicken tenderloin protein bar				
	Proportion (%)				
	0.0	0.5	1.0	1.5	2.0
Chicken tenderloin myofibrillar protein	43.6	43.4	43.2	42.9	42.7
Canola oil	7.9	7.9	7.8	7.8	7.8
Korean pancake mix	11.1	11.0	11.0	10.9	10.9
Egg white	4.8	4.7	4.7	4.7	4.7
Onion powder	0.5	0.5	0.5	0.5	0.5
Salt	0.8	0.8	0.8	0.8	0.8
Sugar	28.0	27.9	27.8	27.6	27.5
Milk	0.6	0.6	0.6	0.6	0.6
Soy sauce	0.6	0.6	0.6	0.6	0.6
Nucleic acid seasoning	0.3	0.3	0.3	0.3	0.3
Beef bone stew powder	0.3	0.3	0.3	0.3	0.3
Pepper	0.5	0.5	0.5	0.5	0.5
Egg york	0.8	0.8	0.8	0.8	0.8
Lemon juice	0.2	0.2	0.2	0.2	0.2
Protein hydrolysates	0.0	0.5	1.0	1.5	2.0

Total	100.0	100.0	100.0	100.0	100.0

[1)] The numbers indicate the addition of protein hydrolysates to the chicken tenderloin protein bar.

655 **Table 2. Composition of soy beverage supplemented with protein hydrolysates**

Materials	Soy beverage				
	Proportion (%)				
	0.0	0.5	1.0	1.5	2.0
Soybean	20.34	20.24	20.14	20.03	19.93
Roasted almond	4.58	4.56	4.54	4.51	4.49
Roasted oatmeal	4.58	4.56	4.54	4.51	4.49
Roasted peanut	2.29	2.28	2.27	2.26	2.25
Water	64.16	63.84	63.52	63.20	62.88
Sugar	4.03	4.01	3.99	3.97	3.95
Salt	0.01	0.01	0.01	0.01	0.01
Protein hydrolysates	0.00	0.50	1.00	1.50	2.00
Total	100.00	100.00	100.00	100.00	100.00

656

Table 3. Constituents and concentrations of synthetic digestive juices used on the *in vitro* digestion model representing post-feeding conditions

	Saliva (mouth step)	Gastric juice (stomach step)	Duodenal juice (small intestine step)	Bile juice (small intestine step)
Inorganic and organic components	1.7 mL NaCl[1)] (175.3 g/L)[2)] 8 mL urea (25 g/L) 15 mg uric acid	6.5 mL HCl (37 g/L) 18 mL $CaCl_2 \cdot 2H_2O$ (22.2 g/L) 1 g bovine serum albumin	6.3 mL KCl (89.6 g/L) 9 mL $CaCl_2 \cdot 2H_2O$ (22.2 g/L) 1 g bovine serum albumin	69.3 mL $NaHCO_3$ (84.7 g/L) 10 mL $CaCl_2 \cdot 2H_2O$ (22.2 g/L) 1.8 g bovine serum albumin 30 g bile
Enzyme	290 mg α-amylase 25 mg mucin	2.5 g pepsin 3 g mucin	9 g pancreatin 1.5 g lipase	
pH	6.8 ± 0.2	1.50 ± 0.02	8.0 ± 0.2	7.0 ± 0.2

[1)] The numbers indicate the concentration of chemicals used to prepare digestive juices.

[2)] The numbers in parentheses indicate the concentrations of inorganic or organic components per 1 L of distilled water.

After mixing all components (inorganic components, organic components, and enzymes), the volume was increased to 500 mL with distilled water. If necessary, the pH of the digestive juices was adjusted to the appropriate value.

664 **Table 4. Percentage (%) of free amino acids in protein hydrolysates from Korean native cattle and Black Angus**

Content (%)	Chikso		Jeju Black Cattle		Jeju black Hanwoo		Hanwoo		Black Angus	
	Loin	Shank	Loin	Shank	Loin	Shank	Loin	Shank	Loin	Shank
Histidine	2.7±0.1^{a}	2.4±0.2^{a}	2.8±0.0^{a}	2.3±0.0^{a}	1.5±0.5^{b}	1.4±0.3^{b}	1.2±0.2^{b}	1.3±0.1^{b}	1.4±0.3^{b}	1.4±0.3^{b}
Isoleucine	8.1±0.1^{a}	8.7±0.5^{a}	8.8±0.3^{a}	8.5±0.1^{a}	4.3±4.3^{a}	4.2±4.2^{a}	4.2±4.2^{a}	3.7±3.7^{a}	4.5±4.5^{a}	4.2±4.2^{a}
Leucine	13.0±0.3 ^{a}	14.5±1.3 ^{a}	14.2±0.5 ^{a}	14.4±0.1 ^{a}	10.9±2.5 ^{a}	10.9±2.7 ^{a}	11.2±2.4 ^{a}	10.3±1.8 ^{a}	11.1±2.7 ^{a}	10.7±2.0 ^{a}
Lysine	11.8±0.3 ^{b}	11.1±0.9 ^{b}	14.0±0.1 ^{a}	11.7±0.0 ^{b}	10.1±3.4 ^{b}	10±2.7^{b}	7.0±3.6^{c}	6.8±5.0^{c}	6.7±5.7^{c}	6.9±5.9^{c}
Methionine	4.5±0.2^{a}	4.8±0.2^{a}	4.9±0.3^{a}	4.9±0.1^{a}	2.5±2.0^{a}	2.6±2.0^{a}	2.3±1.9^{a}	2.5±1.8^{a}	2.9±2.1^{a}	2.7±1.8^{a}
Phenylalanine	6.6±0.2^{a} ^{b}	6.1±0.6^{a} ^{b}	7.2±0.4^{a}	7.0±0.1^{a} ^{b}	1.5±0.8^{c}	3.9±2.9^{b}	6.0±1.0^{a} ^{b}	5.0±1.1^{a} ^{b}	6.1±1.0^{b}	5.9±0.8^{a} ^{b}
Threonine	6.0±0.0^{a}	5.9±0.1^{a}	5.3±0.1^{a} ^{b}	5.5±0.1^{a} ^{b}	3.5±2.6^{a} ^{bc}	5.1±0.5^{a} ^{b}	4.2±1.9^{a} ^{bc}	4.9±1.0^{a} ^{b}	2.0±0.5^{c}	2.7±0.1^{b} ^{c}
Tryptophan	1.4±0.1^{a}	1.3±0.1^{a}	1.4±0.0^{a}	1.4±0.0^{a}	0.8±0.8^{a}	0.8±0.8^{a}	0.7±0.7^{a}	0.7±0.7^{a}	0.8±0.8^{a}	0.7±0.7^{a}
Valine	6.6±0.0^{a}	6.7±0.2^{a}	6.1±0.1^{a}	6.6±0.0^{a}	3.6±3.2^{a}	3.5±3.0^{a}	3.1±3.1^{a}	3.0±3.0^{a}	3.7±3.7^{a}	3.5±3.5^{a}
Arginine	3.6±3.4^{a}	3.3±3.1^{a}	4.3±0.8^{a}	2.4±1.2^{a}	0.2±0.2^{a}	0.3±0.3^{a}	5.0±5.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}

Cystine	0.6±0.0[a]	0.3±0.2[a]	0.9±0.1[a]	0.2±0.0[a]	3.6±3.0[a]	3.5±3.0[a]	3.8±3.1[a]	3.5±2.9[a]	3.1±3.1[a]	3.2±2.9[a]
Glutamine	1.6±0.2[a]	0.7±0.5[a]	0.0±0.0[a]	0.6±0.0[a]	5.2±4.2[a]	4.6±4.4[a]	4.9±2.2[a]	3.9±2.6[a]	2.6±2.6[a]	2.6±2.4[a]
Glycine	1.7±0.0[a]	2.0±0.2[a]	1.0±0.0[a]	1.3±0.0[a]	0.7±0.7[a]	0.7±0.7[a]	0.7±0.7[a]	0.8±0.8[a]	1.3±1.3[a]	1.2±1.2[a]
Proline	0.3±0.2[a]	0.4±0.3[a]	0.4±0.2[a]	0.9±0.5[a]	0.3±0.3[a]	0.3±0.3[a]	0.3±0.3[a]	0.2±0.2[a]	0.3±0.3[a]	0.4±0.4[a]
Tyrosine	3.9±1.0[a]	2.6±2.0[a]	4.9±0.2[a]	3.4±0.0[a]	7.5±6.3[a]	7.6±5.2[a]	9.0±5.1[a]	9.2±4.2[a]	7.9±5.1[a]	8.1±5.3[a]
Alanine	6.7±0.4[a]	6.6±0.0[a]	5.4±0.1[a]	7.8±0.1[a]	4.2±2.7[a]	5.0±3.7[a]	3.0±1.1[a]	3.6±2.2[a]	4.6±3.1[a]	5.0±3.6[a]
Aspartic acid	2.3±0.4[a]	3.3±1.3[a]	1.8±0.2[a]	1.3±0.1[a]	2.5±2.5[a]	0.5±0.5[a]	1.3±1.3[a]	2.6±2.6[a]	0.8±0.8[a]	1.2±1.2[a]
Asparagine	1.8±0.1[a] [b]	2.3±1.0[a] [b]	0.0±0.0[b]	1.4±0.0[a] [b]	1.3±0.8[a] [b]	1.2±1.2[a] [b]	3.2±0.5[a]	1.0±0.8[a] [b]	1.8±1.8[a] [b]	1.7±1.7[a] [b]
Glutamic acid	8.1±1.0[a]	8.7±1.1[a]	7.3±0.5[a]	8.2±0.1[a]	4.9±4.8[a]	4.8±4.4[a]	3.2±1.9[a]	5.6±4.0[a]	7.3±3.8[a]	7.4±4.2[a]
Serine	2.0±1.2[a]	2.6±0.6[a]	1.9±0.3[a]	2.4±0.0[a]	4.2±1.5[a]	4.0±1.8[a]	4.9±1.2[a]	3.9±2.0[a]	3.6±2.3[a]	3.4±2.3[a]
1-Methylhistidine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	6.4±6.4[a]	6.4±6.4[a]	5.9±5.9[a]	6.1±6.1[a]	5.2±5.2[a]	5.2±5.2[a]
…										

Content (%)	Chikso		Jeju Black Cattle		Jeju Black Hanwoo		Hanwoo		Black Angus	
	Loin	Shank	Loin	Shank	Loin		Loin	Shank	Loin	Shank
3-Methylhistidine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	1.4±1.4[a]	1.4±1.4[a]	1.3±1.3[a]	1.4±1.4[a]	1.3±1.3[a]	1.3±1.3[a]

Citrulline	1.2±1.0[a]	0.4±0.2[a]	0.8±0.2[a]	1.1±0.5[a]	4.8±3.8[a]	4.0±2.6[a]	2.9±2.8[a]	3.2±1.6[a]	3.1±1.2[a]	2.7±1.4[a]
Cystathionine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	2.3±2.3[a]	2.1±2.1[a]	2.4±2.4[a]	2.4±2.4[a]	2.2±2.2[a]	2.3±2.3[a]
Ethanol amine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.7±0.7[a]	0.7±0.7[a]	0.6±0.6[a]	0.7±0.7[a]	0.8±0.8[a]	0.6±0.6[a]
Hydroxy proline	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.3±0.3[a]	0.2±0.2[a]	2.4±2.4[a]	3.8±3.8[a]	5.0±5.0[a]	5.3±5.3[a]
Hydroxylysine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	1.3±1.3[a]	1.3±1.3[a]	0.6±0.6[a]	0.7±0.7[a]	0.3±0.3[a]	0.3±0.3[a]
Ornithine	3.6±1.6[a]	3.6±1.5[a]	4.6±1.4[a]	4.6±0.1[a]	3.8±3.8[a]	3.3±3.3[a]	0.4±0.4[a]	2.8±2.8[a]	3.3±3.3[a]	2.8±2.8[a]
Sarcosine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.1±0.1[a]	0.5±0.5[a]	0.4±0.4[a]	0.8±0.8[a]	1.2±1.2[a]	1.1±1.1[a]
α-amino-n-butyric acid	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.6±0.6[a]	0.4±0.4[a]	0.1±0.1[a]	0.2±0.2[a]	0.0±0.0[a]	0.0±0.0[a]
β-Alanine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	3.5±3.5[a]	3.3±3.3[a]	3.5±3.5[a]	3.5±3.5[a]	3.7±3.7[a]	3.7±3.7[a]
Citrulline	1.2±1.0[a]	0.4±0.2[a]	0.8±0.2[a]	1.1±0.5[a]	4.8±3.8[a]	4.0±2.6[a]	2.9±2.8[a]	3.2±1.6[a]	3.1±1.2[a]	2.7±1.4[a]
SUM	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

665 [a-c]Lowercase letters indicate significant differences after *in vitro* digestion ($p < 0.05$).

Figures

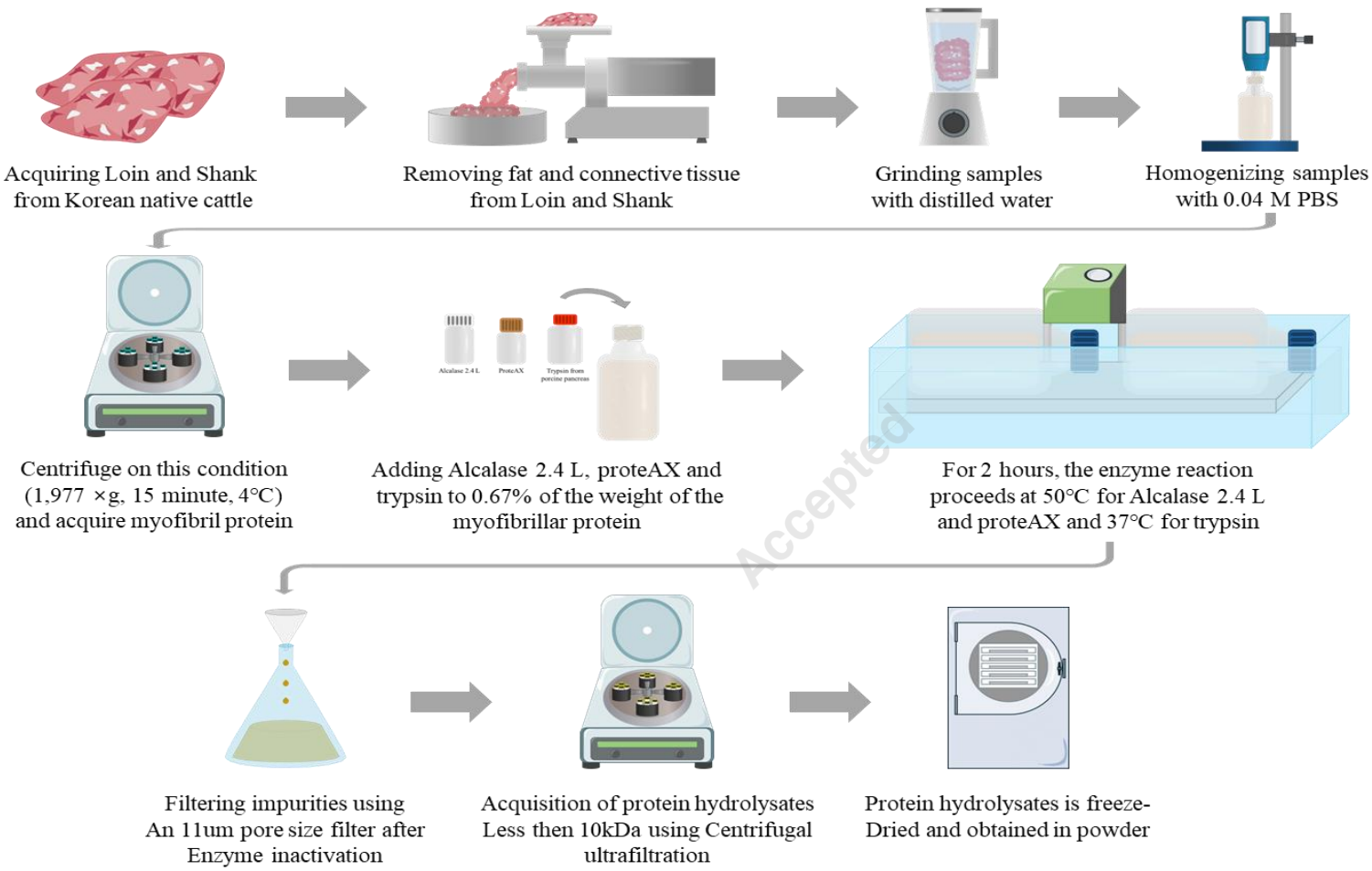


Figure 1. Process for the preparation of protein hydrolysates (< 10 kDa) derived from the myofibrillar proteins of Korean native cattle and Black Angus

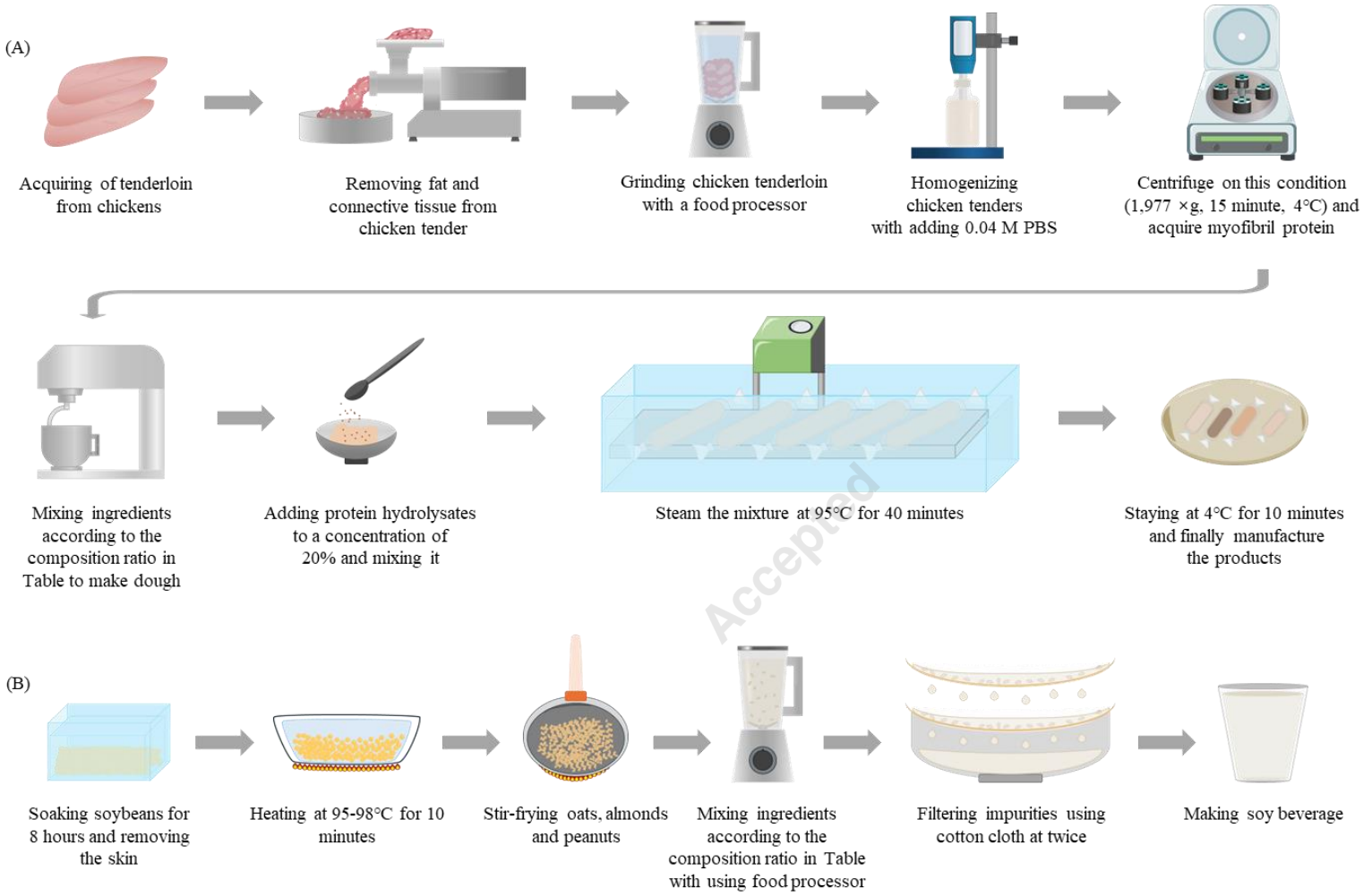
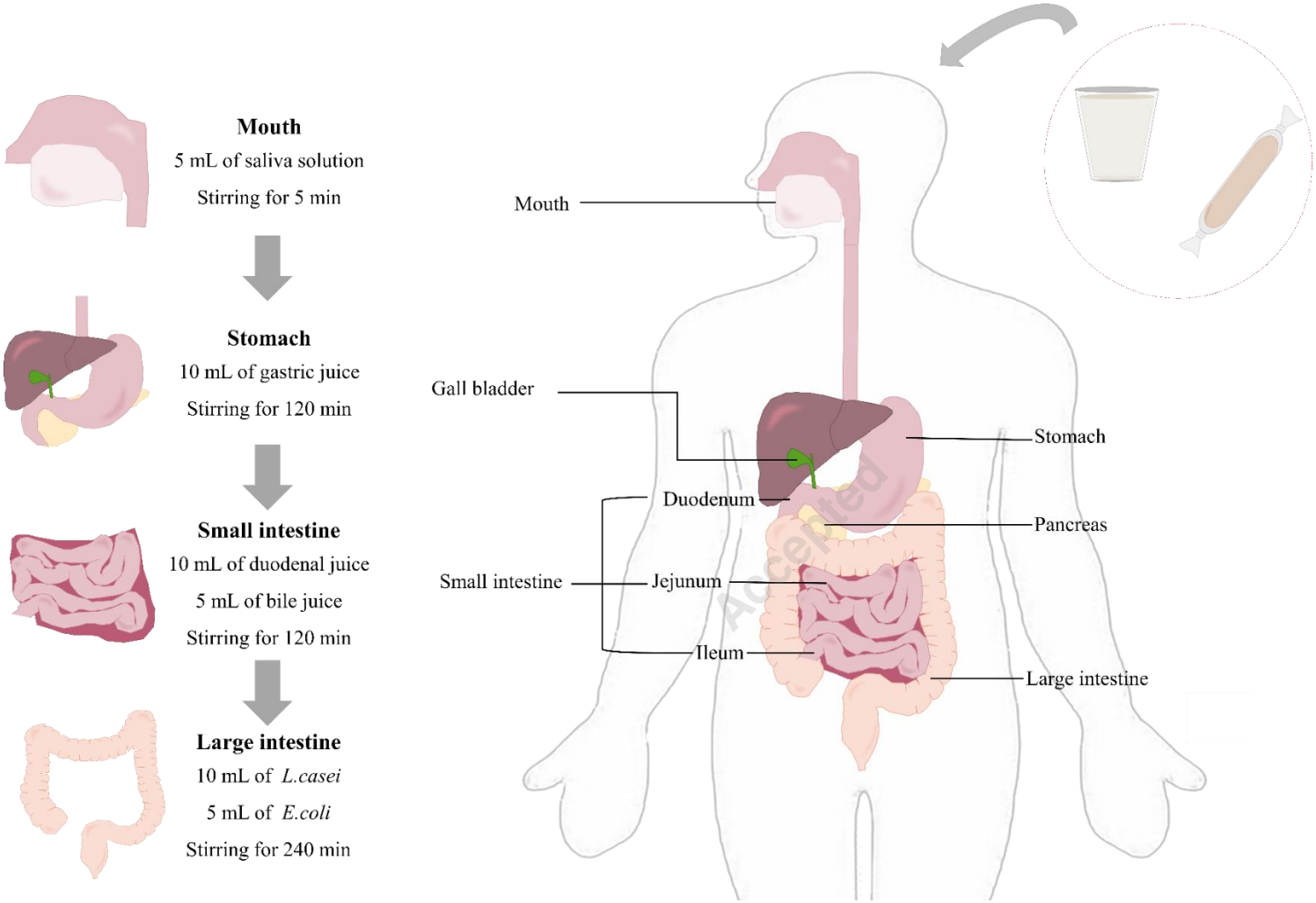


Figure 2. Manufacturing procedure for chicken tenderloin protein bars and soy beverages. (A) Chicken tenderloin protein bar (B) Soy beverage



673

674 **Figure 3. *In vitro* digestion process for soy beverages and chicken tenderloin protein bars**

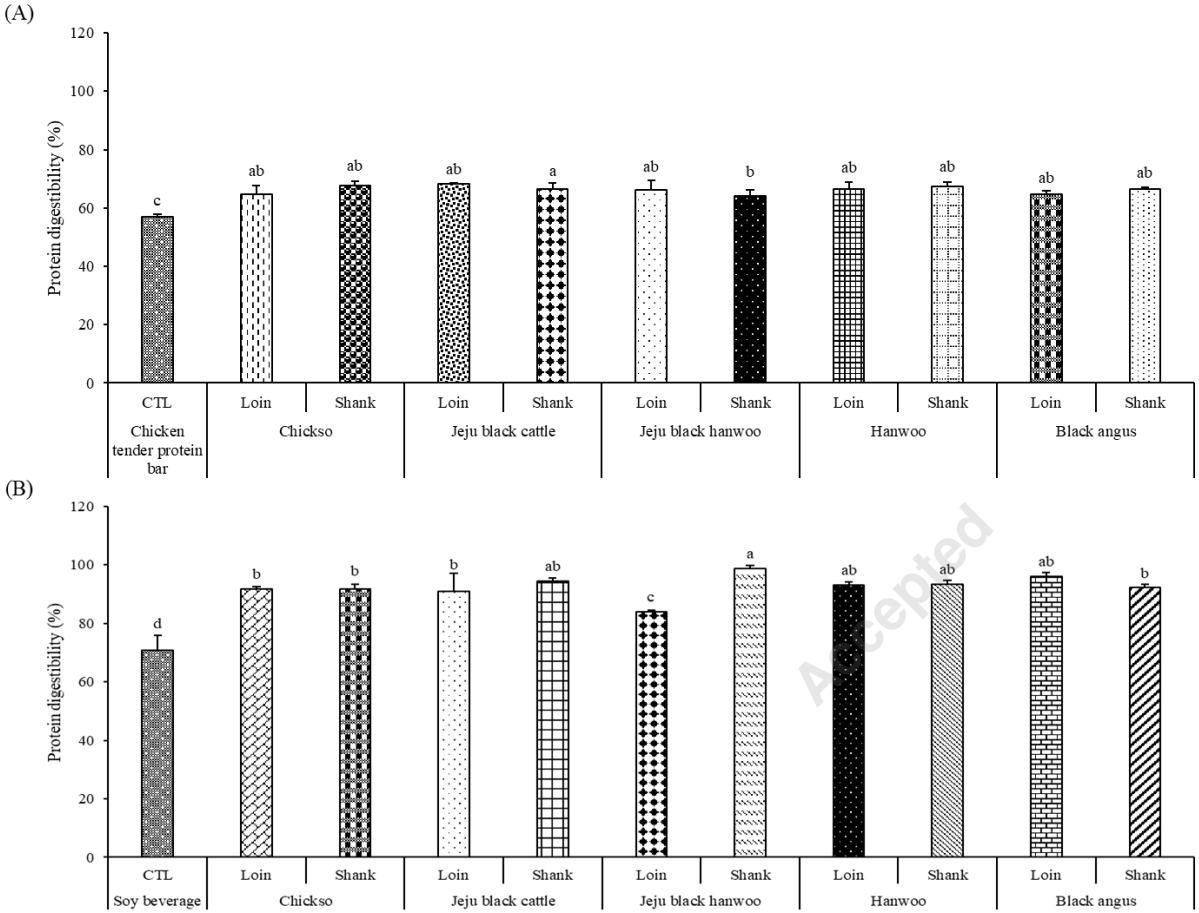
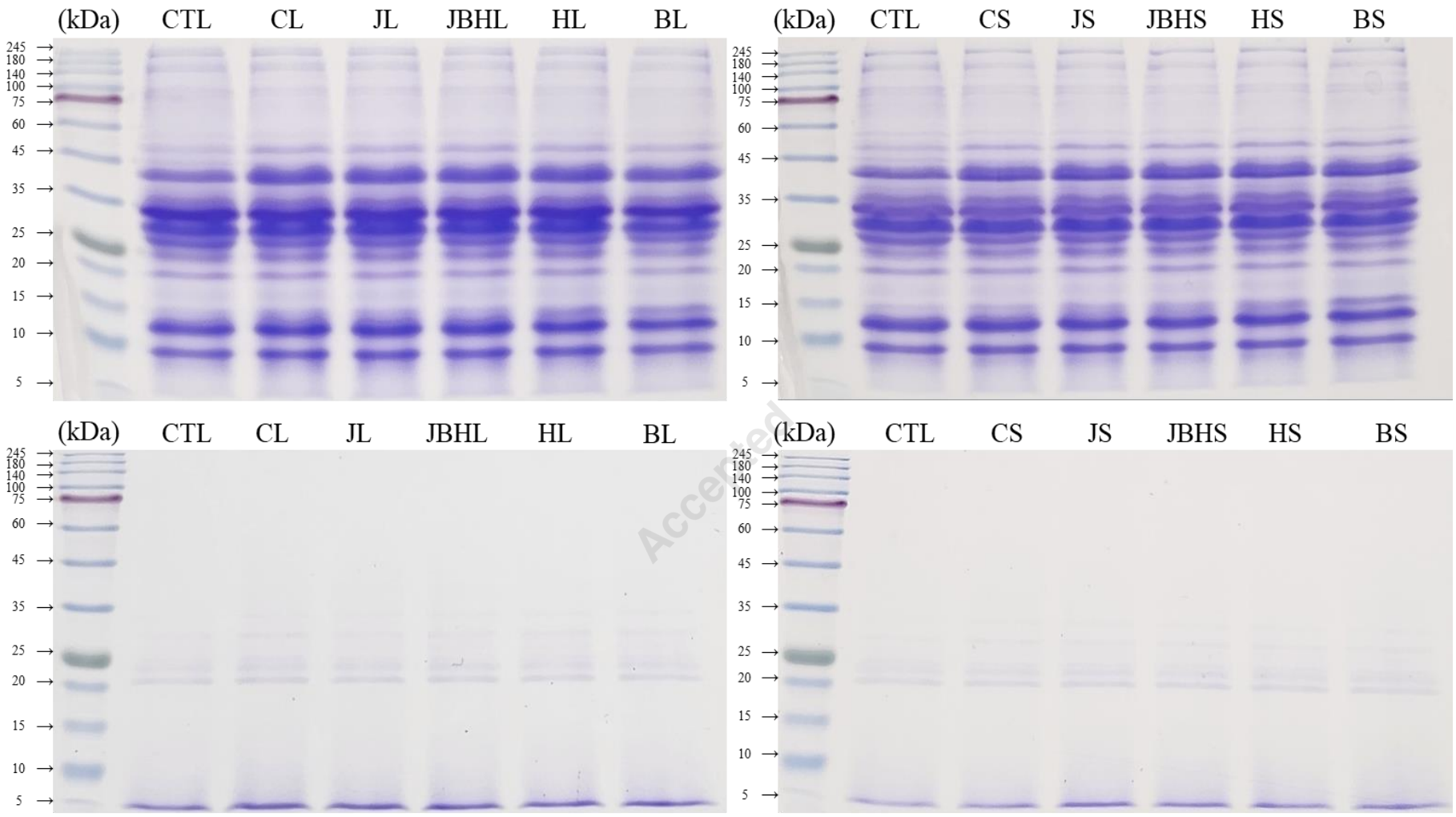
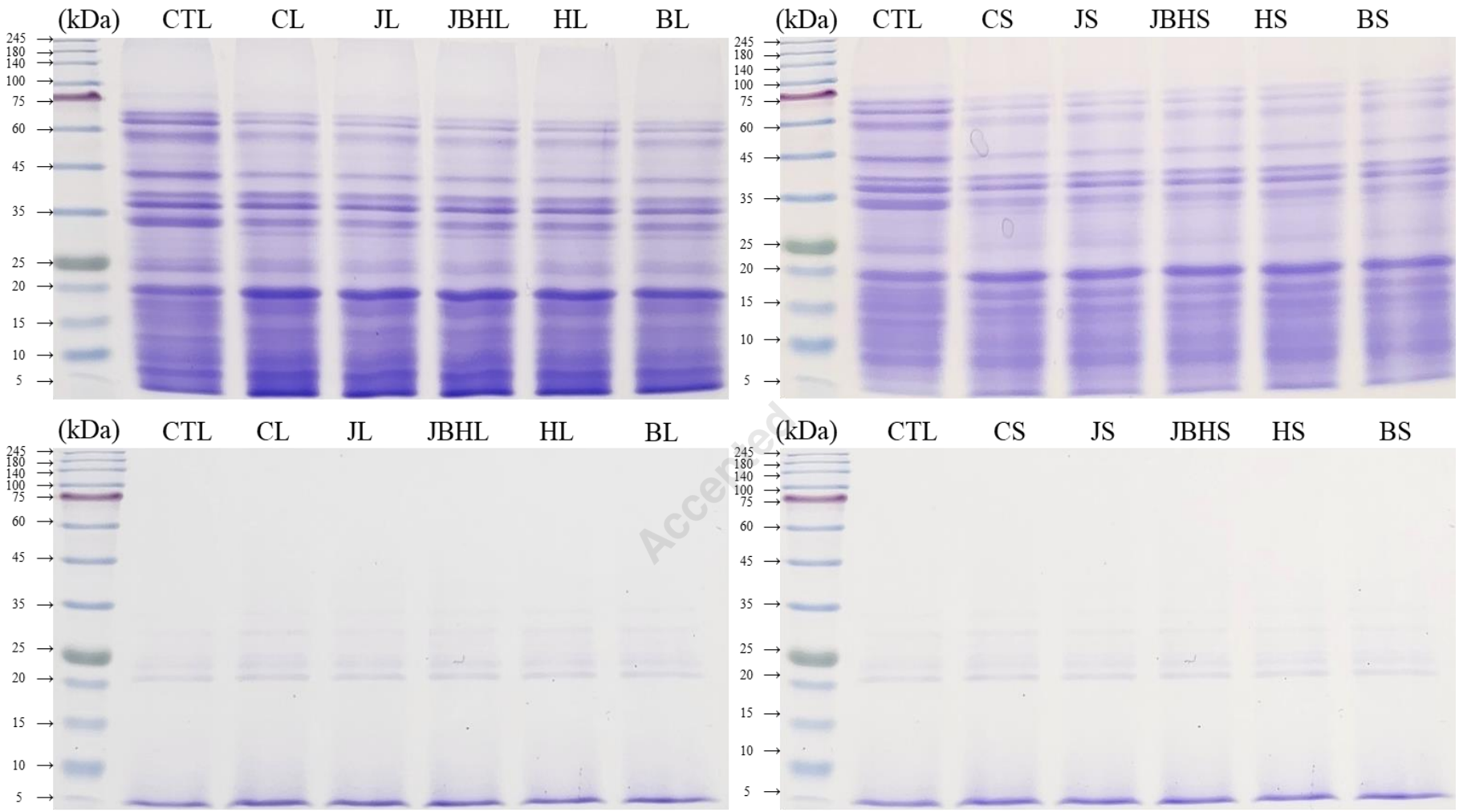


Figure 4. Protein digestibility of chicken tenderloin protein bars and soy beverages with varying percentages of protein hydrolysates following *in vitro* digestion (A) Protein digestibility of chicken tenderloin protein bars; (B) Protein digestibility of soy beverages. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$).



679

680 **Figure 5. Changes in the protein molecular weights of chicken tenderloin protein bars before and after *in vitro* digestion.** (A) Protein
681 molecular weights before *in vitro* digestion; (B) Protein molecular weights after *in vitro* digestion. Protein amount per well: 20 μg/well.



682

683 **Figure 6. Changes in protein molecular weights of soy beverages before and after *in vitro* digestion.** A) Protein molecular weights before *in*
684 *vitro* digestion; (B) Protein molecular weights after *in vitro* digestion. Protein amount per well: 20 µg/well.

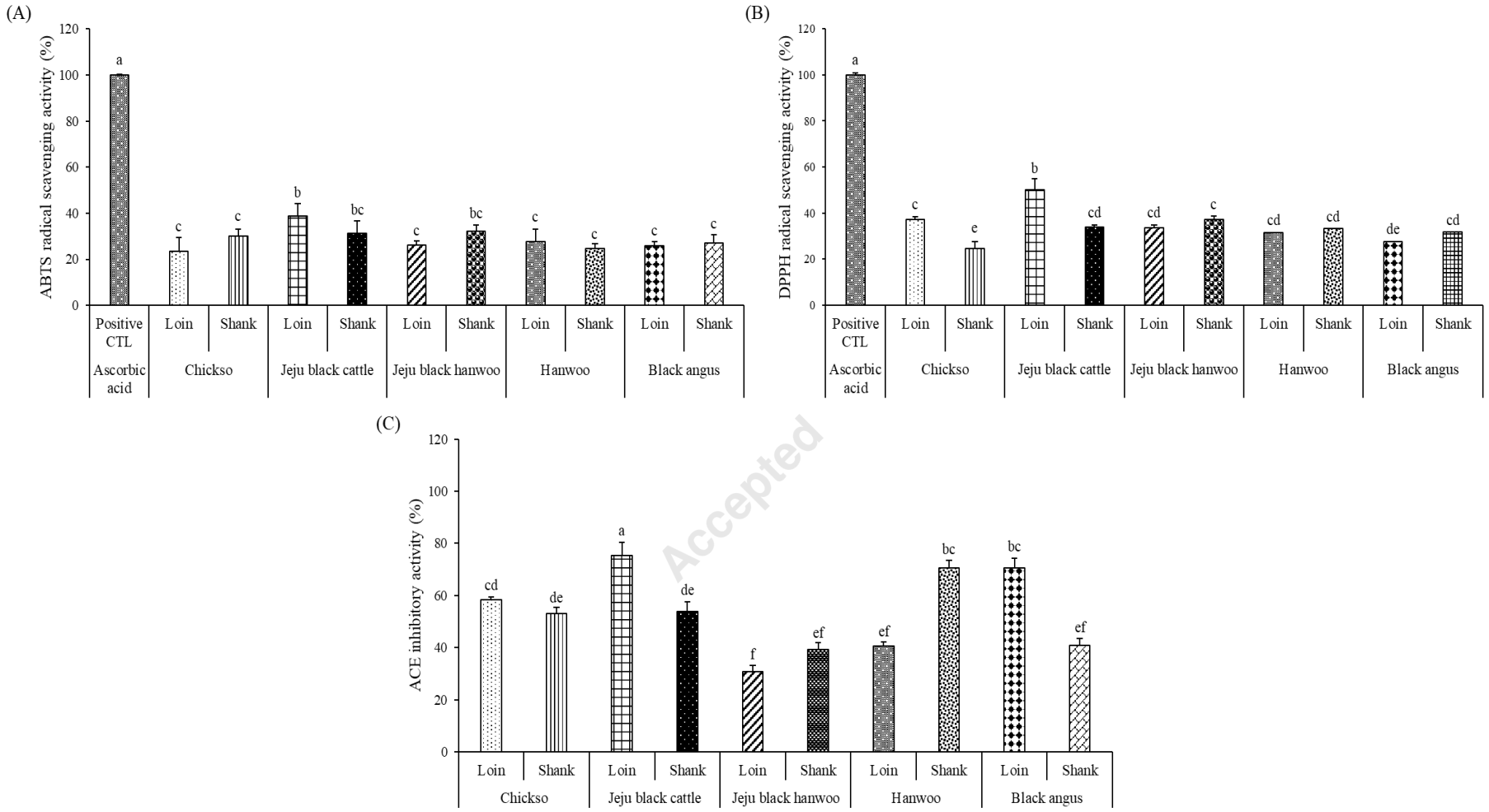


Figure 7. Comparison of DPPH and ABTS radical scavenging activities, as well as the ACE inhibitory activity of protein hydrolysates from the loin and shank of Korean native cattle. (A) ABTS radical scavenging activity; (B) DPPH radical scavenging activity; (C) ACE inhibitory assay. Lowercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$).

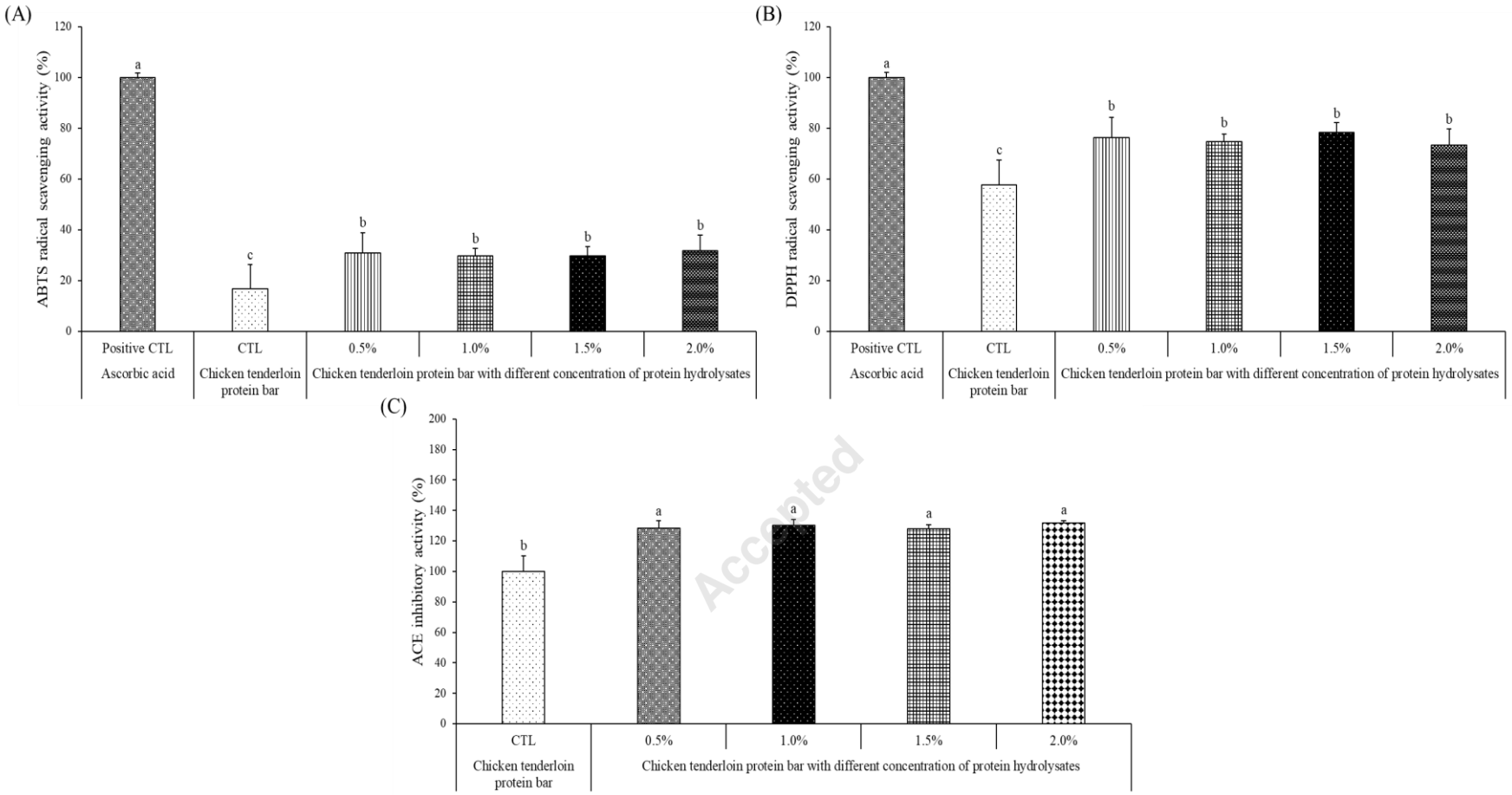
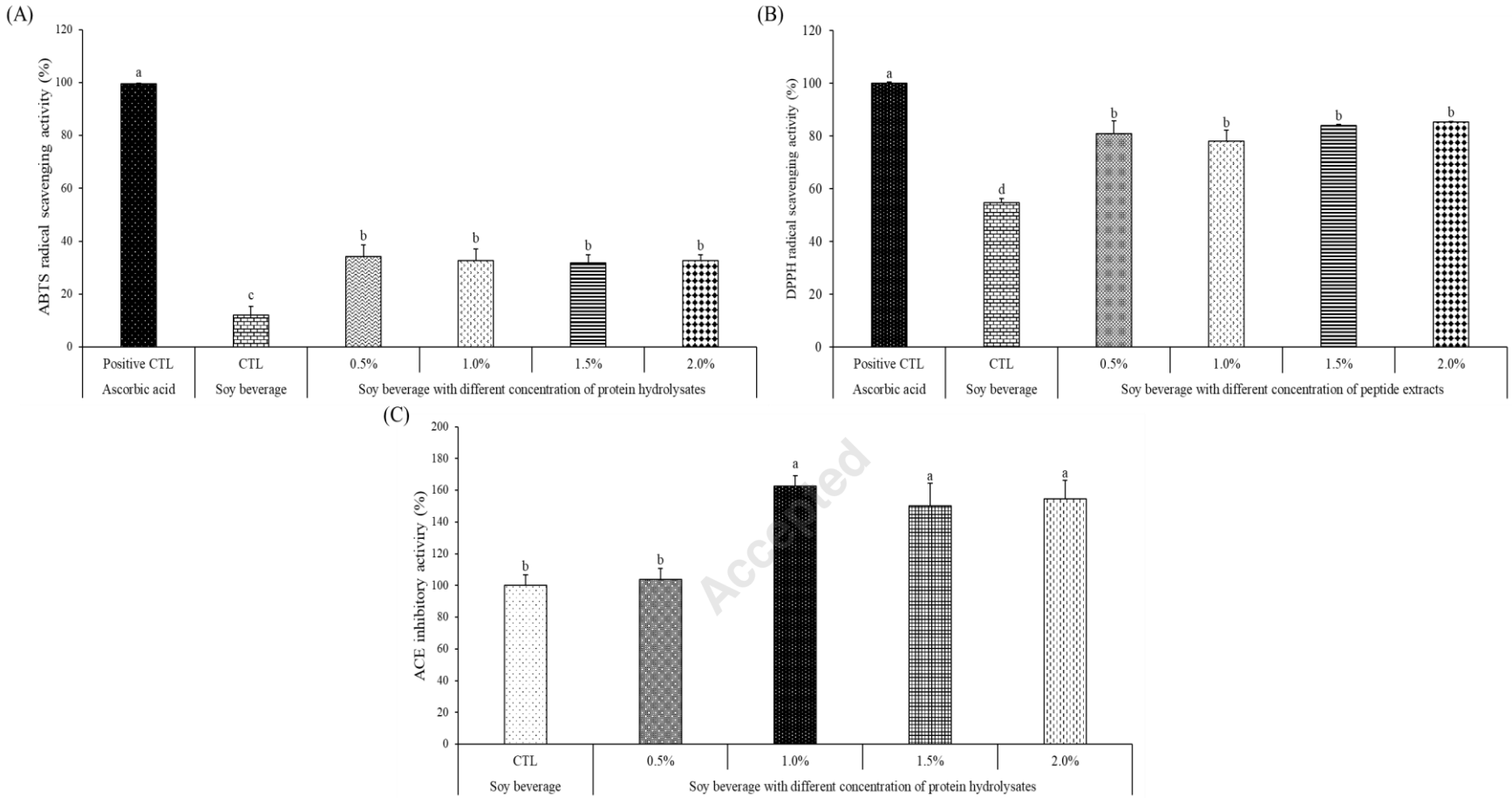


Figure 8. Comparison of antioxidant and antihypertensive activities in chicken tenderloin protein bars with varying protein hydrolysate concentrations. (A) ABTS radical scavenging assay; (B) DPPH radical scavenging assay; (C) ACE inhibitory assay. Uppercase letters indicate significant differences prior to *in vitro* digestion.



693

694 **Figure 9. Comparison of antioxidant and antihypertensive activities in soy beverages with varying protein hydrolysate concentrations.** (A)
695 ABTS radical scavenging assay; (B) DPPH radical scavenging assay; (C) ACE inhibitory assay. Uppercase letters indicate significant differences
696 prior to *in vitro* digestion, while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$).

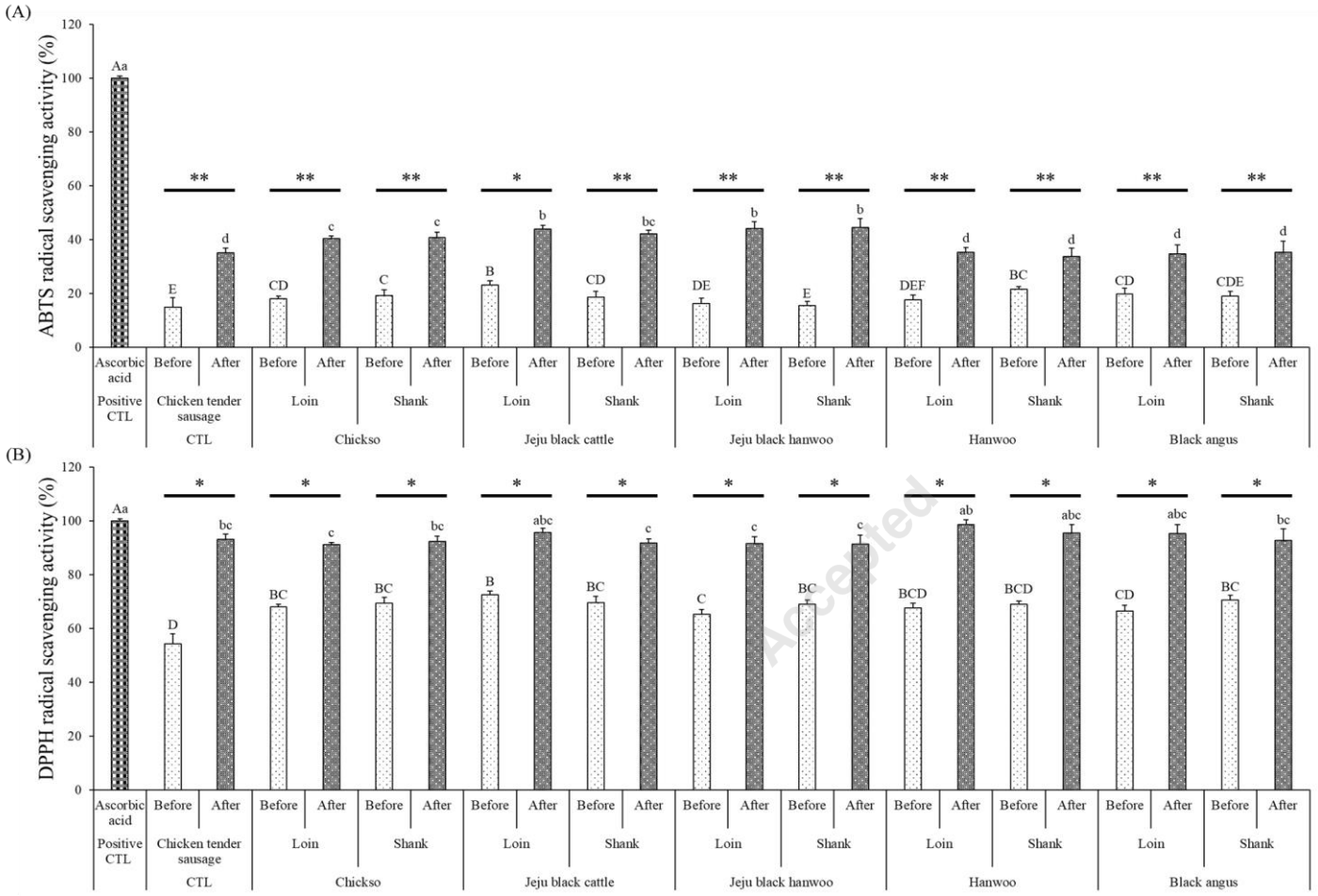


Figure 10. Comparison of antioxidant activity in chicken tenderloin protein bars before and after *in vitro* digestion. (A) ABTS radical scavenging assay; (B) DPPH radical scavenging assay. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$), while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$). A single asterisk ($p < 0.05$) and double asterisks ($p < 0.001$) denote significant differences before and after *in vitro* digestion.

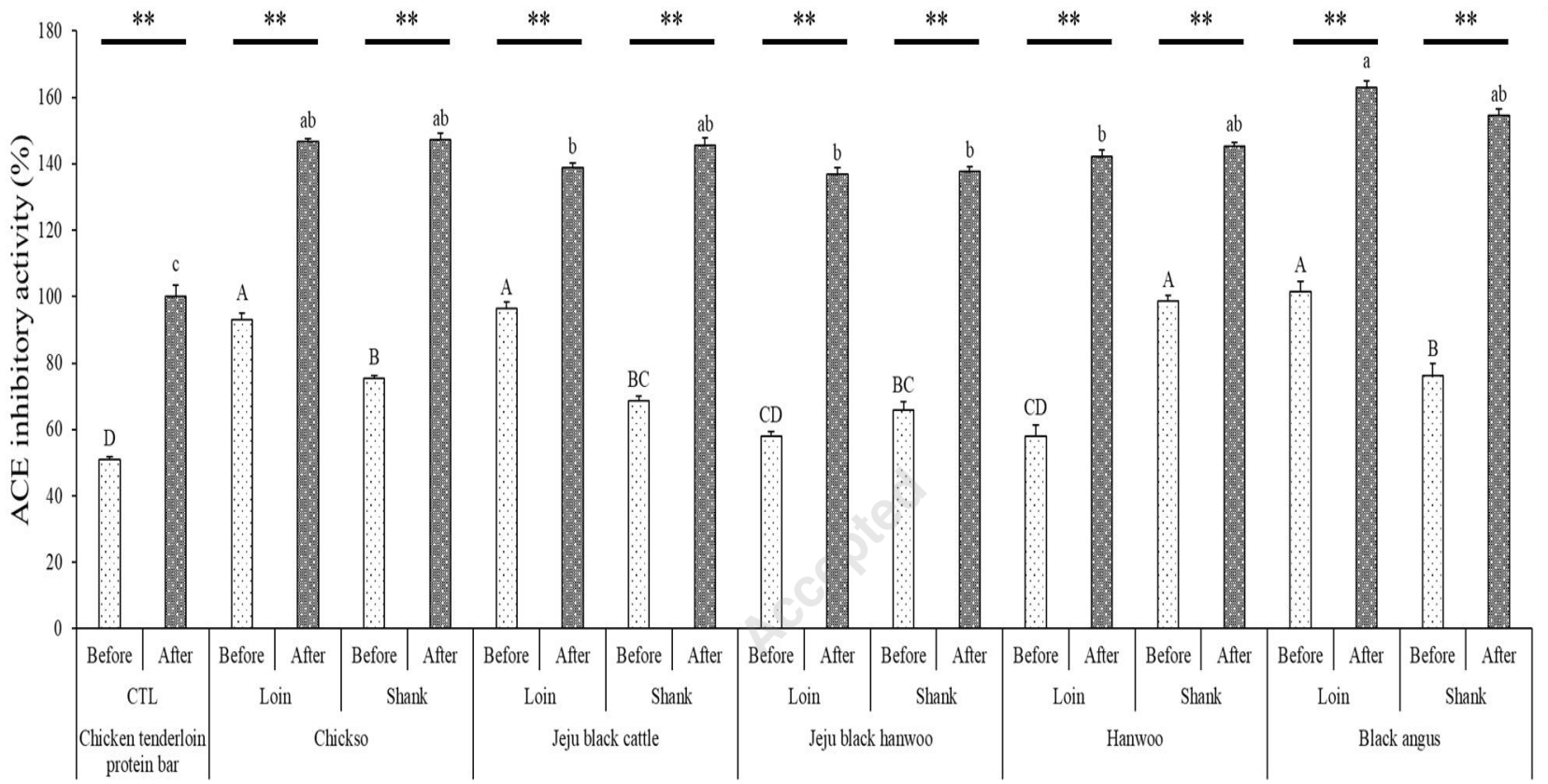


Figure 11. Comparison of antihypertensive activity in chicken tenderloin protein bars before and after *in vitro* digestion. (A) Chicken tenderloin protein bar; (B) Soy beverage. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$), while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$). A single asterisk ($p < 0.05$) and double asterisks ($p < 0.001$) denote significant differences before and after *in vitro* digestion.

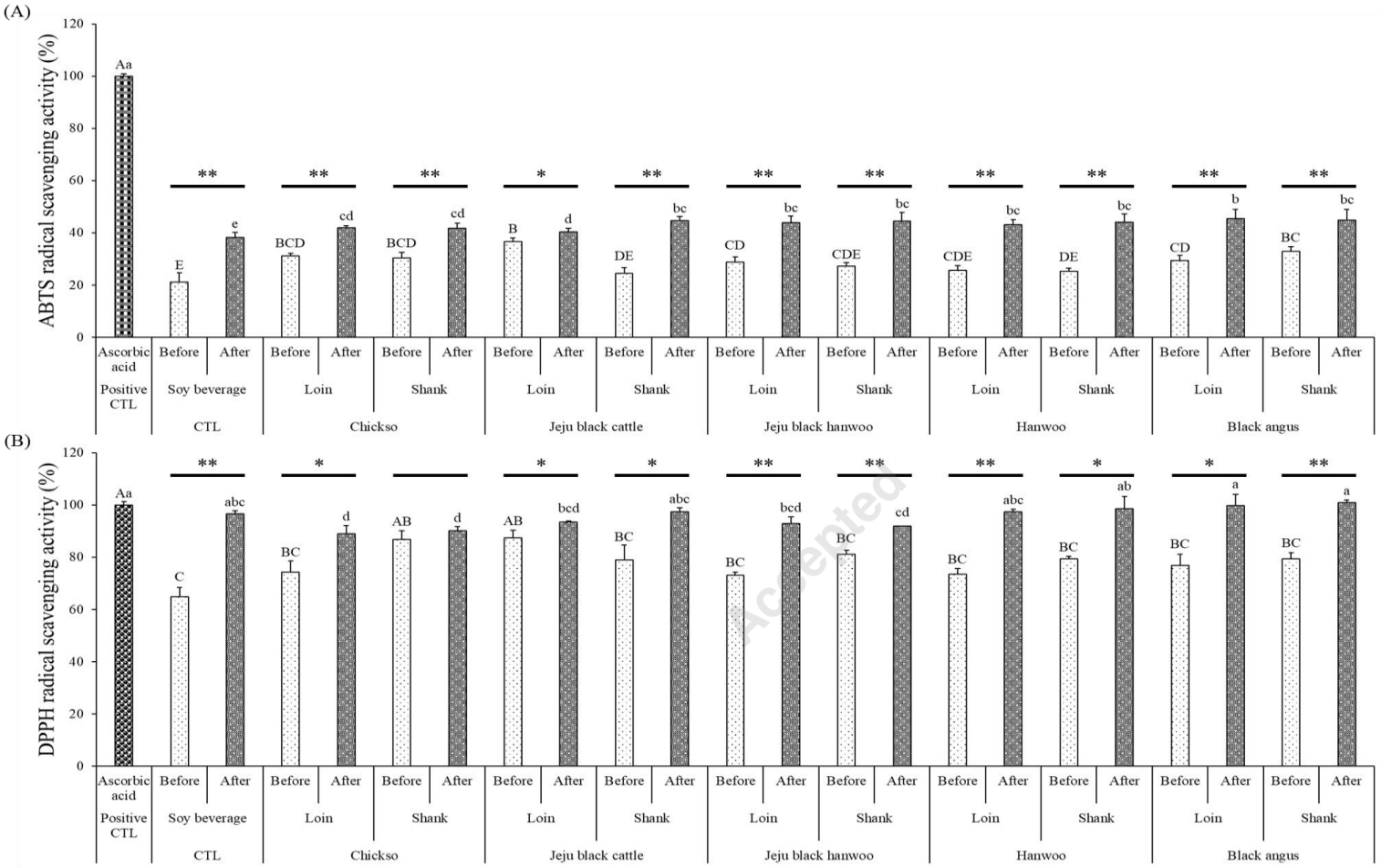


Figure 12. Comparison of angiotensin-converting enzyme inhibitory activity in soy beverages before and after *in vitro* digestion. (A) Chicken tenderloin protein bar; (B) Soy beverage. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$), while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$). A single asterisk ($p < 0.05$) and double asterisks ($p < 0.001$) denote significant differences before and after *in vitro* digestion.

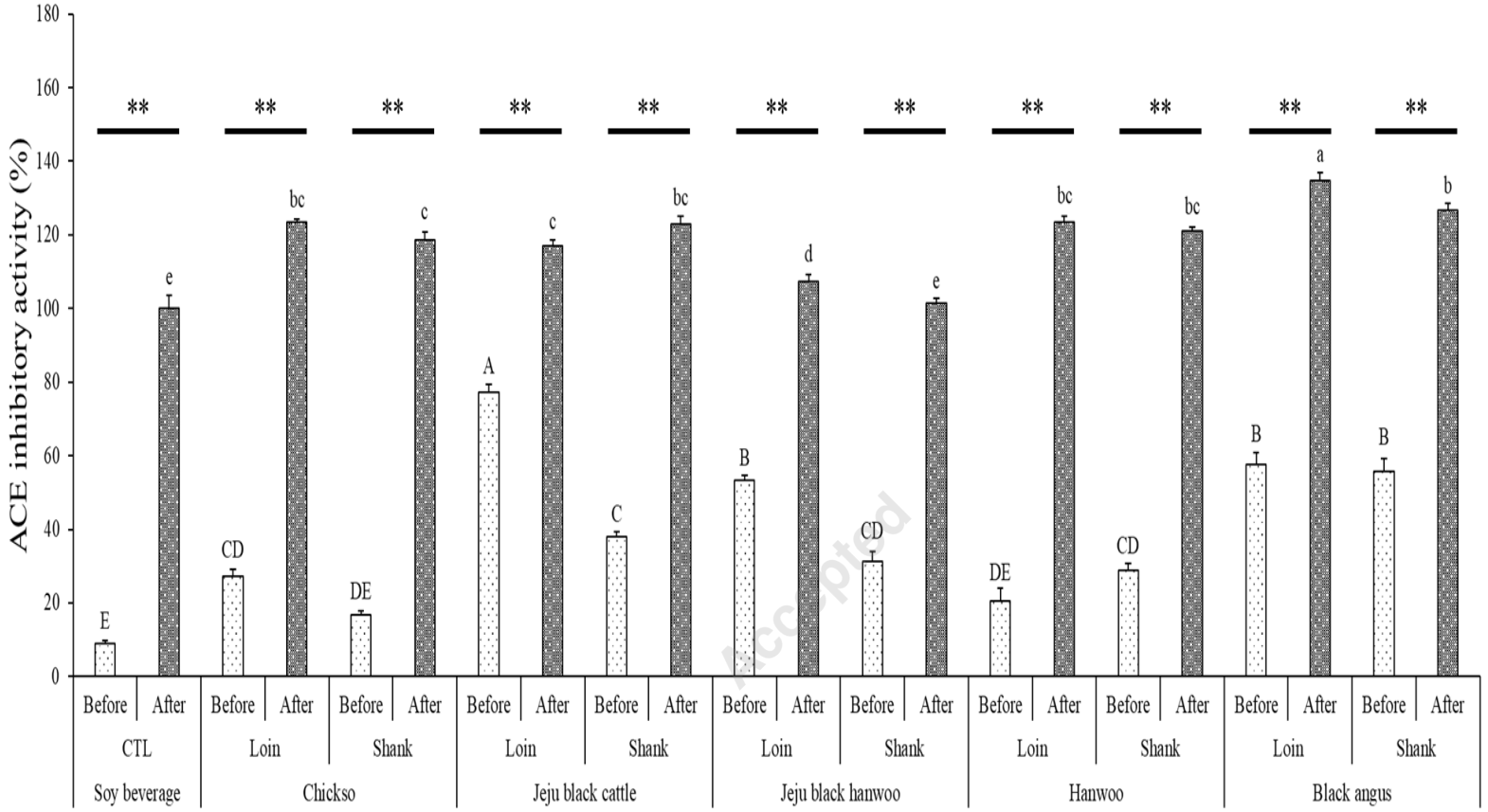


Figure 13. Comparison of antihypertensive activity in soy beverages before and after *in vitro* digestion. (A) Chicken tenderloin protein bar; (B) Soy beverage. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$), while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$). A single asterisk ($p < 0.05$) and double asterisks ($p < 0.001$) denote significant differences before and after *in vitro* digestion.

ABSTRACT

This study comparatively analyzed protein digestibility, molecular weight, free amino acid composition, and changes in bioactivity during *in vitro* digestion of chicken tenderloin protein bars and soy beverages fortified with myofibrillar protein hydrolysates from various Korean native cattle breeds— Chikso, Jeju Black Cattle, Jeju Black Hanwoo, and Hanwoo—and a foreign breed, Black Angus. The results indicated that chicken tenderloin protein bars and soy beverages containing 0.5% and 1.0% protein hydrolysate exhibited enhanced bioactivity. Comparative analysis of protein digestibility before and after *in vitro* digestion revealed that myofibrillar protein hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus significantly improved protein digestibility. Molecular weight analysis demonstrated a breakdown into smaller molecular forms, suggesting potential improvements in digestibility and possible associations with bioactivity. On comparing the antioxidant and antihypertensive activities of protein hydrolysates from loin and shank, the loin protein hydrolysate from Jeju Black Cattle exhibited the highest bioactivity prior to *in vitro* digestion. Antioxidant and antihypertensive activities were considerably higher post-digestion compared with pre-digestion. This study concludes that myofibrillar protein hydrolysates derived from Jeju Black Cattle loin—at concentrations of 0.5% and 1% in chicken tenderloin protein bars and soy beverages—exhibit relatively high antioxidant and antihypertensive activities. These findings may have potential as functional food ingredients associated with antioxidant and antihypertensive activities.

Keywords: Korean native cattle, Protein hydrolysate, Bioactivity, Functional food

1. Introduction

Korean native cattle breeds possess distinct genetic and physicochemical characteristics that may influence the composition and bioactivity of muscle-derived protein hydrolysates. Previous studies have reported that protein hydrolysates derived from Hanwoo and other Korean native cattle breeds exhibit antioxidant and antihypertensive activities; however, the extent to which these functional properties differ among breeds remains insufficiently understood (Lee et al., 2017; Kim et al., 2024).

Myofibrillar proteins, which constitute a major portion of muscle proteins, can generate bioactive peptides through enzymatic hydrolysis. Protein hydrolysates generated from myofibrillar proteins have been reported to exhibit diverse biological activities, including antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities (Kim et al., 2024). In addition, low-molecular-weight peptides derived from animal proteins generally exhibit high digestibility and bioactivity, generally exhibit high digestibility and bioactivity (Hu et al., 2023). Previous studies have also reported that protein hydrolysates from Korean native cattle breeds, such as Chikso, Jeju Black Cattle, and Hanwoo, may exhibit different antioxidant and antihypertensive activities depending on breed characteristics (Lee et al., 2017; Kim et al., 2024). These findings suggest that breed origin may influence the functional properties of cattle-derived protein hydrolysates.

In addition to protein source, the bioactivity and digestibility of protein hydrolysates can also be affected by the physicochemical properties of food matrices. Factors such as protein composition, fat content, texture, and structural characteristics may influence the incorporation, stability and digestion behavior of bioactive peptides in food systems. Therefore, evaluating protein hydrolysates in different food matrices is important for understanding matrix-dependent differences in their functional behavior and bioactivity.

Chicken tenderloin protein bars and soy beverages were selected in this study as representative solid and liquid food matrices with distinct protein compositions and structural characteristics. Chicken tenderloin protein bars provide an animal protein-based solid matrix with relatively high protein content and complex texture characteristics, whereas soy beverages represent a plant-based liquid matrix with different physicochemical properties. These contrasting food systems enabled the evaluation of matrix-dependent differences in digestibility, antioxidant activity, and antihypertensive activity following the incorporation of cattle-derived protein hydrolysates.

Therefore, this study primarily aimed to determine whether breed origin and food matrix differentially affect the bioactivity of cattle-derived myofibrillar protein hydrolysates during *in vitro* digestion.

2. Materials and Methods

2.1 Materials

The loin and shank of Chikso, Jeju Black Cattle, and Jeju Black Hanwoo utilized in the experiments were supplied by the National Institute of Animal Science (Jeonbuk, Korea). The Chikso, Jeju Black Cattle, and Jeju Black Hanwoo were 34, 31, and 31 months old, respectively, and all were castrated steers. The loin and shank of Hanwoo were purchased from Achim Farm (Chungnam, Korea), originating from a 34-month-old castrated steer. The loin of Black Angus was procured from Haengbok Mart (Busan, Korea), while the shank was acquired from SNT Solution (Seongnam, Korea). Chicken tenderloin was sourced from Hau CM (Daegu, Korea). Additional ingredients—including canola oil, pancake mix, eggs, onion powder, refined salt, milk, soy sauce, sugar, nucleotide seasoning, beef bone powder, pepper, and lemon juice—were secured from a local mart in Anseong, Korea. Roasted peanuts, roasted almonds, and soybeans were also obtained from a local mart in Anseong.

For the *in vitro* digestion experiments, α-amylase from hog pancreas (CAS No. 9000-90-2), bile extract porcine (CAS No. 8008-63-7), lipase from porcine pancreas (CAS No. 9001-62-1), mucin from porcine stomach Type II (CAS No. 84082-64-4), pepsin from porcine gastric mucosa (CAS No. 9001-75-6), and uric acid (≥ 99%, crystalline, CAS No. 69-93-2) were purchased from Sigma-Aldrich (St. Louis, USA). Pancreatin from porcine pancreas (CAS No. 8049-47-6) was procured from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), while bovine serum albumin (CAS No. 9048-46-8) was acquired from Biosesang (Seongnam, Korea).

For the protein hydrolysate extraction experiments, sodium phosphate monobasic anhydrous (CAS No. 7558-80-7) and sodium phosphate dibasic anhydrous (CAS No. 7558-79-4) were secured from Daejung (Siheung, Korea) to prepare a 0.04 M phosphate buffer (pH 7.4). The Amicon® Ultra-15 Centrifugal Filter Unit with an Ultracel-10 regenerated cellulose membrane (15-mL sample volume, CAT No. UFC901024) was sourced from Merck Millipore (Massachusetts, USA).

For the bicinchoninic acid (BCA) assay, trichloroacetic acid (TCA, CAS No. 76-03-9) was obtained from Daejung (Siheung, Korea), while the Pierce™ BCA protein assay kit (CAT No. 23225) was acquired from Thermo Fisher Scientific (Massachusetts, USA).For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the following reagents were secured: Tris (Catalog No. 1610719), 30% acrylamide/bis solution (37.5:1, Catalog No. 1610158), 10% (w/v) SDS solution (Catalog No. 1610416), ammonium persulfate (Catalog No. 1610700), tetramethylethylenediamine (Catalog No. 1610800), 10× Tris/Glycine/SDS buffer (Catalog No. 1610772), 4× Laemmli sample buffer (Catalog No. 1610747), Precision Plus Protein™ Dual Xtra Standards (Catalog No. 1610374), and Bio-Safe Coomassie G-250 stain (Catalog No. 1610786), all sourced from BIO-RAD (California, USA). A microcentrifuge (Catalog No. DH.WCF00010) and a heating block (Catalog No.

DH.WHB00348) from Daihan Scientific (Seoul, Korea) were employed during the experiments.

To analyze amino acid composition, buffers PF-1, 2, 3, and 4, alongside RG, for the Hitachi high-speed amino acid analyzer were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). The ninhydrin coloring solution kit for the Hitachi analyzer was obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). Amino acid analysis was conducted utilizing the Hitachi L-8900 amino acid analyzer from Hitachi High-Technologies (Tokyo, Japan).

To measure antioxidant activity, methyl alcohol (MeOH, 99.5%, CAS No. 67-56-1) was procured from Samchun Pure Chemical Co., Ltd. (Seoul, Korea). Additionally, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (CAS No. 30931-67-0) was acquired from Sigma-Aldrich (St. Louis, USA), L(+)-Ascorbic acid (99.5%, CAS No. 50-81-7) from Samchun Pure Chemical Co., Ltd. (Seoul, Korea), and potassium persulfate (CAS No. 7727-21-1) from Daejung Chemicals (Siheung, Korea). Furthermore, 2,2-Diphenyl-1-picrylhydrazyl (CAS No. 1898-66-4) was secured from Sigma-Aldrich (St. Louis, USA), and 2,6-Di-tert-butyl-4-methylphenol (99.0%) was obtained from Samchun Pure Chemical Co., Ltd. (Seoul, Korea).

For antihypertensive activity measurements, ethyl acetate (Catalog No. E0688), sodium chloride (Catalog No. S0476), and sodium tetraborate anhydrous (Catalog No. S3412) were acquired from Samchun Pure Chemical Co., Ltd. (Seoul, Korea). Boric acid (Catalog No. 04232-00) was sourced from Kanto Chemical Co., Inc. (Tokyo, Japan), while lung acetone powder from rabbit (L0756-5G) and N-Hippuryl-His-Leu hydrate (H1635-100MG) were purchased from Sigma-Aldrich (St. Louis, USA). The Elabscience® ACE activity assay kit (Catalog No. E-BC-K003-S) used for analyzing antihypertensive enzyme activity was obtained from Elabscience (Texas, USA).

To measure absorbance, a SpectraMax 190 microplate reader from Molecular Devices (California, USA) and a Cary 300 ultraviolet-visible (UV/VIS) spectrophotometer from Agilent Technologies (California, USA) were utilized.

2.2 Extraction of protein hydrolysates from myofibrillar protein

To extract protein hydrolysates from Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, the methodologies outlined by Kim et al. (2021) and Lee & Hur (2019) were employed (Kim et al., 2021; Lee & Hur, 2019). Figure 1 illustrates the overall experimental procedure. Initially, the loin and shank from the aforementioned cattle breeds were prepared at 1,000 × g, with connective tissue and fat meticulously removed. Distilled water (DW), in a volume 10 times the weight of the raw material, was added, and the samples were thoroughly ground using a mixer. The fat and connective tissue that surfaced were discarded along with the DW. This process was repeated approximately 10 times to completely remove blood and fat. Thereafter, the precipitate collected at the bottom was used to extract myofibrillar proteins using a 0.04 M phosphate buffer.

Protein hydrolysates were subsequently extracted from the myofibrillar proteins of the loin and shank derived from Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, using the proteolytic enzymes Alcalase 2.4L, ProteAX, and trypsin from porcine pancreas (Kim et al., 2024). DW and the extracted myofibrillar proteins were mixed in a 1:4 ratio, homogenized, and adjusted to pH 8.0. Alcalase 2.4L was added at a concentration of 0.67% relative to the weight of the myofibrillar proteins and reacted at 50 °C with agitation at 60 rpm. ProteAX was treated under identical conditions, while trypsin was reacted at 37 °C and 60 rpm.

To inactivate the enzymes, the samples were heated to 95°C for 10 min, and impurities were removed using filter paper No. 4. The resulting filtrates were centrifuged at 1,977 × g and 4 °C for 20 min with a centrifugal ultrafiltration device to obtain protein hydrolysates with

146 molecular weights < 10 kDa. The isolated protein hydrolysates were dried at 54 °C for 24 h
147 and subsequently freeze-dried for 48 h to eliminate moisture. The final samples were stored
148 at –70 °C until further analysis.

149 **2.3 Preparation of chicken tenderloin protein bars and soy beverage**

150 To prepare chicken tenderloin protein bars, myofibrillar proteins were extracted according to
151 the same methodology utilized for chicken tenderloin (Kim et al., 2021; Lee & Hur, 2019).
152 The extracted myofibrillar proteins were combined with canola oil, pancake mix, egg white,
153 onion powder, refined salt, milk, soy sauce, sugar, nucleotide seasoning, beef bone powder,
154 pepper, egg yolk, and lemon juice, as detailed in Table 1. Protein hydrolysates were
155 subsequently incorporated at varying concentrations to prepare the experimental treatments
156 for the study.

157 To prepare the soy beverage, 100 g of soybeans were soaked in 300 mL of water for 8 h.
158 After soaking, the seed coats were entirely removed, and the water was drained. The soaked
159 soybeans were subsequently boiled for 4 min with three times their weight in water. Rolled
160 oats were roasted in a frying pan for 2 min, and roasted almonds, roasted peanuts, and roasted
161 rolled oats were added to the boiled soybeans according to the composition detailed in Table
162 2 to formulate the soy beverage.

163 Thereafter, 100 and 200 g of water were added sequentially and thoroughly blended using a
164 mixer. An additional 400 g of water was added while monitoring the consistency, and the
165 mixture was blended again until smooth. The blended mixture was then filtered twice through
166 muslin cloth to eliminate impurities. The resulting soy beverage was supplemented with
167 sugar and salt in the quantities specified in Table 2, and protein hydrolysates were
168 incorporated at varying concentrations to establish the experimental treatment groups for the
169 study.

The overall procedure of producing chicken tenderloin protein bar and soy beverage is illustrated in Figure 2.

2.4 *In vitro* digestion experiment

The *in vitro* digestion process employed in this study to assess the digestibility and bioactivity changes of chicken tenderloin protein bars and soy beverages supplemented with protein hydrolysates from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus was adapted from methods outlined by Lee et al. (2016). Table 3 presents the preparation conditions for the digestive enzymes, inorganic solvents, and organic solvents utilized during the oral, gastric, and intestinal phases of the *in vitro* digestion experiment (Hur et al., 2011; Lee et al., 2016). For the colon phase, intestinal microbiota were introduced to the samples.

The liquid medium for *Escherichia coli* (American Type Culture Collection [ATCC] 25922, Manassas, VA, USA) was prepared by dissolving 2.5 g of Luria Bertani Broth (Sparks, MD, USA) in 100 mL of deionized water (DIW), while the liquid medium for *Lacticaseibacillus casei* (ATCC 393) was prepared by dissolving 5.5 g of Lactobacilli MRS Broth (Sparks, MD, USA) in 100 mL of DIW. Both media were sterilized at 121 °C for 15 min and subsequently cooled. Stocks of *Escherichia coli* and *Lacticaseibacillus casei*, stored at –80 °C, were thawed at room temperature, and 1% of each microbial stock was inoculated into 100 mL of the respective liquid medium. *Escherichia coli* was cultured at 37 °C for 12 h, while *Lacticaseibacillus casei* was cultured at 37 °C for 24 h. This procedure was repeated for up to three passages to achieve a total microbial count of 10^8–10^9 CFU/mL.

The complete *in vitro* digestion process simulating typical adult digestive conditions is illustrated in Figure 3.

To simulate the conditions of adult oral digestion, 5 g of each sample was transferred into a 50-mL conical tube, to which 5 mL of saliva was added. The mixture was subsequently incubated in a water bath at 37 °C while being agitated at 150 rpm for 5 min.

For the *in vitro* digestion process, gastric digestion was simulated by adding 10 mL of gastric juice and maintaining incubation at 37 °C and 150 rpm for 2 h. Subsequently, small intestine digestion was replicated by adding 10 mL of duodenal juice and 5 mL of bile juice under the same conditions. Large intestine digestion was modeled by incorporating 10 mL of *Lacticaseibacillus casei* and 5 mL of *Escherichia coli*, with the incubation period extended to 4 h, thereby completing the digestion process.

Control groups were established for each *in vitro* digestion experiment. One control group exclusively contained digestive enzymes, with samples replaced by DW, while the second group consisted of only the samples, with digestive enzymes replaced by DW. Upon completion of the digestion process, all samples were centrifuged at 13,000 × g and 4 °C for 20 min. The supernatants were subsequently collected and utilized as final samples.

2.5 Protein quantitative analysis via BCA assay

The protein digestibility of hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, as well as chicken tenderloin protein bars and soy beverages prepared with these protein hydrolysates, was assessed using the BCA assay. This analysis followed the protocols established by Smith et al. (1985) and Wiechelman et al. (1988).

A 25-µL aliquot of each sample was dispensed into a microplate, followed by the addition of 200 µL of BCA solution. The microplate was covered with foil and incubated at 37 °C for 30 min. After incubation, absorbance was measured at 562 nm using an enzyme-linked immunosorbent assay reader within 5 min. The obtained values were utilized to calculate the remaining protein content following digestion.

2.6 SDS-PAGE

The SDS-PAGE method was employed to analyze the electrophoretic patterns of proteins both before and after *in vitro* digestion in chicken tenderloin protein bars and soy beverages supplemented with protein hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus. Sample preparation was conducted in accordance with the protocol used in the protein digestibility analysis via the BCA assay.

A 10-μL aliquot of each sample, along with 4 μL of protein marker, was loaded into the 12% running gel. Electrophoresis was executed at 80 V for 10 min, followed by an increase to 100 V for 90 min. Upon completion of the run, the gel was removed and rinsed multiple times with DIW. Thereafter, it was stained with a staining solution at room temperature for over 3 h. Finally, destaining was performed using a destaining solution, followed by additional rinses with DIW.

2.7 Analysis of free amino acids

The free amino acid composition of protein hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus was analyzed using a Hitachi L-8900 amino acid analyzer. Each sample was diluted to the appropriate concentration, and 1 mL was combined with an equal volume of 5% TCA solution. The mixture was stirred and centrifuged at 13,500 × g and 20 °C for 20 min. Subsequently, 4 mL of n-hexane was added to 2 mL of the supernatant, followed by shaking for 15 min and centrifugation at 1,977 × g at 20 °C for 20 min. The lower layer was collected; to ensure complete removal of n-hexane, the centrifugation process was repeated twice at 13,500 × g and 20 °C for 20 min. The final sample was filtered through a 0.2-μm syringe filter prior to analysis.

2.8 Analysis of antioxidant activity

To evaluate the antioxidant activity of the protein hydrolysates sourced from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, as well as chicken tenderloin protein bars and soy beverages supplemented with these protein hydrolysates, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays were conducted before and after *in vitro* digestion.

The ABTS assay was performed following a modified protocol based on Re et al. (1999). An ABTS stock solution was prepared by combining a 7 mM ABTS solution with 2.45 mM potassium persulfate. This mixture was shielded with aluminum foil and incubated at 25 °C for 12–16 h.

The ABTS working solution was obtained by diluting the stock solution with MeOH until the absorbance at 734 nm reached 0.7 ± 0.02. Ascorbic acid served as the standard solution, prepared with DW. MeOH was utilized as the control. In the assay, 20 μL of each sample and 180 μL of the ABTS working solution were added to a 96-well plate and allowed to react in the dark for 10 min. Absorbance was subsequently measured at 734 nm using a microplate reader.

The ABTS scavenging activity was calculated using the following formula:

$$ABTS\ scavenging\ activity\ (\%) = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100$$

The DPPH assay was conducted by modifying the method outlined by Tepe et al. (2005). A DPPH solution was prepared by dissolving 2 mg of 2,2-diphenyl-1-picrylhydrazyl in 25 mL of MeOH in a 50-mL conical tube.

Ascorbic acid, prepared with DW as the solvent, functioned as the standard solution. MeOH also served as the control. For the assay, 100 μL of each sample and 100 μL of the DPPH

266 solution were added to a 96-well plate and allowed to react in the dark for 30 min.
267 Absorbance was subsequently measured at 517 nm using a microplate reader.
268 The DPPH scavenging activity was calculated using the following formula:

269 $$DPPH\ scavenging\ activity\ (\%) = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100$$

270

271 **2.9 Analysis of antihypertensive activity**

272 The angiotensin-converting enzyme (ACE) inhibitory activity assay was conducted to
273 evaluate the antihypertensive effects of protein hydrolysates derived from the loin and shank
274 of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, as well as from
275 chicken tenderloin protein bars and soy beverages supplemented with these hydrolysates,
276 both before and after *in vitro* digestion.
277 This assay was adapted from the methodology described by Cushman and Cheung (1971). To
278 prepare the ACE solution, a 0.1 M sodium borate buffer (pH 8.3) was created by combining
279 sodium tetraborate and boric acid, followed by the addition of 0.5 M sodium chloride. Lung
280 acetone powder from rabbit was extracted at a concentration of 50 mg/mL (w/v) by stirring at
281 4 °C for 24 h. The mixture was subsequently centrifuged at 9,400 × g at 4 °C for 30 min, and
282 the supernatant was collected and stored. The ACE substrate was prepared by dissolving N-
283 Hippuryl-His-Leu hydrate in 0.1 M sodium borate buffer (pH 8.3) to achieve a concentration
284 of 8.3 mM.
285 For the assay, 50 µL of each sample was added to 2 mL tubes, with DW serving as the
286 control. Additional samples and controls were prepared by adding 1 M HCl at the outset to
287 terminate the reaction. To each sample, 50 µL of the ACE substrate was added and allowed
288 to react at 37 °C for 10 min. Thereafter, 50 µL of ACE solution (25 mM/mL) was added to
289 the reaction mixtures, which were incubated at 37 °C for 30 min. For samples without initial

HCl addition, 250 µL of 1 M HCl was added to terminate the reaction, followed by the addition of 500 µL of ethyl acetate. The mixtures were vortexed for 1 min and centrifuged at 1,977 × g for 10 min. From the supernatants, 200 µL was collected, dried at 60 °C for 30 min, and reconstituted with 1 mL of distilled water to prepare the final samples.

The absorbance of the final samples was measured at 228 nm using a UV/VIS spectrophotometer.

$$ACE\ inhibitory\ activity\ (\%) = \left(\frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control - Absorbance\ of\ control_{HCl}}\right) \times 100$$

2.10 Statistical analysis

The experimental results were analyzed using SPSS Statistics 26 (IBM, Armonk, NY, USA). Mean values and standard deviations were calculated, followed by statistical analyses. Differences between two variables were assessed using Student's t-test, while *post hoc* comparisons among mean values were conducted using the Student–Newman–Keuls test at a significance level of $p < 0.05$.

3. Results and Discussion

3.1 Antioxidant and antihypertensive analysis of products supplemented with different concentrations of protein hydrolysates

3.1.1 Chicken tenderloin protein bar

ABTS and DPPH radical scavenging assays showed no significant differences in antioxidant activity among hydrolysate concentrations ranging from 0.5% to 2.0%, although the highest activity was consistently observed at 0.5%. Similarly, ACE inhibitory activity did not differ significantly among concentrations, with 0.5% exhibiting the highest activity in Figure 8.

The physiological activity of protein hydrolysates is influenced by factors such as hydrolysis conditions, molecular weight distribution of peptides, and amino acid composition. Previous studies have suggested that antioxidant and ACE inhibitory activities tend to increase with higher concentrations of protein hydrolysates; however, these activities may plateau or decline beyond a certain concentration (Chen et al., 2012; Senadheera et al., 2023). The absence of further increases in antioxidant and ACE inhibitory activities at higher concentrations may be associated with peptide aggregation or reduced peptide solubility, which can limit interactions between bioactive peptides and target radicals or enzymes. Because no significant improvement in antioxidant or ACE inhibitory activities was observed at concentrations above 0.5%, this concentration was selected for subsequent analyses because no further significant improvement was observed at higher concentrations.

3.1.2 Soy beverage

Consistent with the chicken tenderloin protein bar results, ABTS and DPPH radical scavenging assays showed no significant differences in antioxidant activity among hydrolysate concentrations ranging from 0.5% to 2.0%, although antioxidant activity was highest at 0.5%. In contrast, ACE inhibitory activity was significantly greater at concentrations of 1.0% or higher than at 0.5%, with the highest activity observed at 1.0% in Figure 9. These findings suggest that incorporating protein hydrolysates at a concentration of 1.0% into soy beverages optimally enhances both antioxidant and antihypertensive activities. These findings suggest that relatively low supplementation levels were sufficient to maintain antioxidant activity, whereas slightly higher concentrations were required to enhance ACE inhibitory activity in the soy beverage matrix.

The different concentration-dependent trends observed between antioxidant and ACE inhibitory activities may reflect differences in peptide interactions within the soy beverage matrix and differences in the mechanisms underlying radical scavenging and ACE inhibition.

3.2 Analysis of protein digestibility and composition changes in products supplemented with protein hydrolysates before and after *in vitro* digestion

3.2.1 Analysis of protein digestibility

Protein digestibility of chicken tenderloin protein bars and soy beverages supplemented with cattle-derived protein hydrolysates was evaluated during *in vitro* digestion using the BCA assay in Figure 4. Treatment groups receiving protein hydrolysate supplementation exhibited significantly greater protein digestibility than the non-supplemented controls. This increase in digestibility may be associated with the presence of low-molecular-weight peptides generated during enzymatic hydrolysis, which can improve enzyme accessibility and facilitate protein breakdown during digestion. Differences in digestibility among treatment groups may also reflect interactions between protein hydrolysates and food matrix components, including variations in protein composition and structural properties between the chicken tenderloin protein bar and soy beverage systems. These findings indicate that incorporation of cattle-derived protein hydrolysates may improve protein digestibility during *in vitro* digestion, although the extent of improvement may vary depending on breed origin and food matrix characteristics.

3.2.2 Analysis of changes in protein molecular weight

SDS-PAGE analysis revealed substantial changes in protein molecular weight distributions before and after *in vitro* digestion in both chicken tenderloin protein bars and soy beverages in Figures 5–6. Before digestion, protein bands were broadly distributed across higher molecular weight regions, whereas post-digestion samples predominantly exhibited bands below 5 kDa. Soy beverages exhibited a broader initial molecular weight distribution than chicken tenderloin protein bars, possibly reflecting differences in matrix composition and protein structure. Conversely, following *in vitro* digestion, proteins with low molecular weights of 5 kDa were predominantly observed.

A comparison of protein bands before and after *in vitro* digestion in soy beverages, contrasting the control group without protein hydrolysate supplementation with the treatment group that received supplementation, revealed that proteins with molecular weights ranging from 5 to 75 kDa were evenly distributed, as illustrated in Figures 5–6. Nonetheless, following *in vitro* digestion, a predominance of low-molecular-weight proteins, specifically those below 5 kDa, was observed, mirroring trends noted in chicken tenderloin protein bars. Low-molecular-weight peptides generated via protein hydrolysis can exhibit various physiological activities, including antioxidant and antihypertensive effects. Previous studies have reported that low-molecular-weight peptide fractions exhibit enhanced antioxidant and ACE inhibitory activities (Chen et al., 2023; Zou et al., 2020). The predominance of low-molecular-weight peptides after digestion suggests that gastrointestinal digestion further hydrolyzed proteins and incorporated hydrolysates into smaller peptide fractions, which may contribute to the increased antioxidant and ACE inhibitory activities observed after digestion. These findings support the possibility that digestion-induced generation of low-molecular-weight peptides contributed to the enhanced bioactivity observed after *in vitro* digestion. However, the specific peptide sequences responsible for these functional changes were not identified in the present study.

3.3 Free amino acid content and bioactivity of protein hydrolysates

3.3.1 Composition analysis of free amino acids

Differences in free amino acid composition were observed among protein hydrolysates derived from different cattle breeds and muscle.

Previous studies have reported that amino acids such as lysine and phenylalanine contribute to antioxidant and ACE inhibitory activities (Cheng et al., 2020; Huang et al., 2021). In the current study, protein hydrolysates derived from Jeju Black Cattle loin exhibited the highest

concentrations of lysine and phenylalanine among all treatment groups, measuring 14.0 ± 0.1 and 7.2 ± 0.4, respectively.

In the present study, protein hydrolysates derived from Jeju Black Cattle loin exhibited the highest lysine and phenylalanine contents, which may partially explain the relatively high antioxidant and antihypertensive activities observed.

These findings suggest that breed-dependent differences in amino acid composition may partially contribute to variations in the bioactivity of cattle-derived protein hydrolysates. However, further studies are needed to identify the specific peptides and amino acid interactions responsible for these functional differences.

3.3.2 Analysis of antioxidant and antihypertensive activity

To assess the antioxidant and antihypertensive activities of protein hydrolysates derived from the loin and shank of Chikso, Jeju Black Cattle, Jeju Black Hanwoo, Hanwoo, and Black Angus, we employed the ABTS radical scavenging assay, DPPH radical scavenging assay, and ACE inhibitory assay. The results of these analyses are presented in Figure 7.

The ABTS radical scavenging assay results demonstrated that the antioxidant activity of the protein hydrolysates was generally lower than that of ascorbic acid ($p < 0.05$). Among the treatment groups, the protein hydrolysates derived from the loin of Jeju Black Cattle exhibited the highest antioxidant activity. A similar trend was observed in the DPPH radical scavenging assay results.

While the ABTS radical scavenging assay evaluates antioxidant activity using cation radicals, the DPPH radical scavenging assay employs free radicals, reflecting distinct mechanisms (Song et al., 2018). Although ABTS and DPPH assays are based on different radical scavenging mechanisms, both assays showed similar trends among treatment groups.

The ACE inhibitory activity assay results indicated that protein hydrolysates from the loin of Jeju Black Cattle demonstrated the highest ACE inhibitory activity, followed by those

413 derived from the shank of Hanwoo and the loin of Black Angus. These findings suggest that
414 protein hydrolysates from the loin of Jeju Black Cattle exhibited relatively high ACE
415 inhibitory activity
416 Additionally, research on the antihypertensive activity of free amino acids has demonstrated
417 that phenylalanine is particularly effective in inhibiting ACE activity and regulating blood
418 pressure (Yu et al., 2018; Olalere et al., 2023). These findings align with the antihypertensive
419 activity results presented in Figure 7, suggesting that amino acid composition may partially
420 contribute to the observed ACE inhibitory activity.
421 However, the specific bioactive peptides responsible for these activities were not identified in
422 the present study.
423 **3.4 Analysis of bioactivity in products supplemented with protein hydrolysates before**
424 **and after *in vitro* digestion**
425 **3.4.1 Chicken tenderloin protein bar**
426 The antioxidant activity of chicken tenderloin protein bars before and after *in vitro* digestion
427 was assessed using the ABTS and DPPH radical scavenging assays, with results depicted in
428 Figure 10. The assays revealed a significant increase in antioxidant activity following
429 digestion compared with pre-digestion levels. This increase in antioxidant activity after
430 digestion may be associated with previous studies reporting that gastrointestinal digestion can
431 further hydrolyze proteins and generate bioactive low-molecular-weight peptides (Wijesekara
432 et al., 2024; Xi et al., 2024).
433 Among the pre-digestion results from the ABTS and DPPH radical scavenging assays, the
434 chicken tenderloin protein bar supplemented with protein hydrolysates from the loin of Jeju
435 Black Cattle exhibited the highest antioxidant activity. Post-digestion, the ABTS radical
436 scavenging assay results indicated significantly higher antioxidant activity in the treatment
437 groups supplemented with protein hydrolysates from the loin and shank of Jeju Black Cattle

438 and Jeju Black Hanwoo. In the DPPH radical scavenging assay, the highest antioxidant
439 activity was observed in the treatment group supplemented with protein hydrolysates from
440 the loin of Hanwoo. These shifts in antioxidant activity patterns after digestion suggest that
441 breed-specific peptide compositions responded differently to gastrointestinal digestion.
442 These results indicate that chicken tenderloin protein bars supplemented with protein
443 hydrolysates exhibit distinct trends in antioxidant activity before and after digestion. Notably,
444 as protein hydrolysates undergo further breakdown during the colonic phase owing to microbial
445 activity, their bioactivity may be enhanced. Differences in peptide release and transformation
446 during digestion may have contributed to the altered post-digestion antioxidant activity
447 rankings observed among breeds.
448 An ACE inhibitory assay was performed to assess antihypertensive activity before and after
449 *in vitro* digestion (**Figure 11**). Consistent with the antioxidant activity results,
450 antihypertensive activity post-digestion was significantly greater than that observed pre-
451 digestion. Moreover, both pre- and post-digestion, chicken tenderloin protein bars
452 supplemented with protein hydrolysates demonstrated higher antihypertensive activity than
453 the non-supplemented CTL group, aligning with the findings from the analysis of the protein
454 hydrolysates. Among the pre-digestion results, treatment groups supplemented with protein
455 hydrolysates derived from the loin of Chikso, the loin of Jeju Black Cattle, the shank of
456 Hanwoo, and the loin of Black Angus exhibited the highest antihypertensive activity.
457 However, post-digestion ACE inhibitory activity exhibited a different pattern from that
458 observed before digestion, suggesting that gastrointestinal digestion differentially altered
459 antihypertensive peptide profiles among breeds. The ACE inhibitory assay results post-
460 digestion revealed that the treatment group supplemented with protein hydrolysates from the
461 loin of Black Angus exhibited the highest activity. This transition in activity suggests that
462 digestion may have facilitated the breakdown of proteins and protein hydrolysates into lower-

463 molecular-weight fractions, potentially contributing to enhanced ACE inhibitory activity, as
464 smaller peptides are often more effective inhibitors (Erdmann et al., 2008; Udenigwe & Aluko,
465 2012). These findings are consistent with the predominance of low-molecular-weight protein
466 fractions observed after digestion in the SDS-PAGE analysis. Previous studies have
467 demonstrated that digestion can modify peptide structures, influencing their bioactivity and
468 absorption (Amigo & Hernández-Ledesma, 2020). These findings underscore the necessity of
469 considering digestion-induced changes when evaluating the functional potential of protein
470 hydrolysates in antihypertensive applications.

471 **3.4.2 Soy beverage**

472 Before digestion, soy beverages supplemented with protein hydrolysates derived from Jeju
473 Black Cattle loin exhibited the highest antioxidant activity in both ABTS and DPPH assays.
474 However, post-digestion antioxidant activity patterns differed, with Black Angus-derived
475 protein hydrolysates exhibiting the greatest activity. Similarly, in the DPPH radical
476 scavenging assay, treatment groups supplemented with protein hydrolysates from both the
477 loin and shank of Black Angus demonstrated significantly higher antioxidant activity in
478 Figure 12.

479 In the DPPH radical scavenging assay, the treatment group supplemented with protein
480 hydrolysates from the shank of Chikso exhibited no significant difference in antioxidant
481 activity before and after digestion. The limited change in antioxidant activity observed in the
482 Chikso shank treatment group may reflect the relatively high baseline antioxidant activity
483 observed before digestion.

484 These results indicate that soy beverages supplemented with protein hydrolysates exhibit
485 distinct trends in antioxidant activity both before and after digestion. This finding is
486 consistent with previous research, which demonstrated that protein hydrolysates enhance
487 antioxidant activity post-digestion owing to the release of bioactive peptides with improved

488 radical scavenging abilities (Zhao et al., 2020). These digestion-dependent shifts in
489 antioxidant activity suggest that peptide release and transformation during digestion differed
490 according to cattle breed and food matrix interactions.
491 The ACE inhibitory assay was performed to assess the antihypertensive activity of soy
492 beverages before and after *in vitro* digestion, with results illustrated in Figure 13. Similar to
493 the antioxidant activity findings, antihypertensive activity post-digestion was significantly
494 elevated compared with pre-digestion levels. This increase in ACE inhibitory activity after
495 digestion may be associated with the formation of bioactive low-molecular-weight peptides
496 during digestion (Suo et al., 2022). Furthermore, in alignment with the chicken tenderloin
497 protein bar experiment, soy beverages supplemented with protein hydrolysates demonstrated
498 greater antihypertensive activity than the non-supplemented CTL group, reinforcing trends
499 observed in the antihypertensive activity analysis of protein hydrolysates.
500 On examining antihypertensive activity measurements for soy beverages prior to *in vitro*
501 digestion, the treatment group supplemented with protein hydrolysates from Jeju Black Cattle
502 loin exhibited the highest activity. Nevertheless, post-digestion, the group supplemented with
503 protein hydrolysates from Black Angus loin demonstrated the highest antihypertensive
504 activity. This variation may stem from differences in peptide profiles generated during
505 digestion, influenced by the specific protein sources employed. Previous studies have
506 demonstrated that the enzymatic hydrolysis of different protein sources potentially yields
507 peptides with varying bioactivity, thus affecting their potential antihypertensive properties
508 (Peighambardoust et al., 2021). These findings suggest that gastrointestinal digestion
509 differentially modified antihypertensive peptide profiles among cattle breeds, resulting in
510 altered post-digestion activity rankings.
511 These findings suggest that although the soy beverage supplemented with Jeju Black Cattle
512 loin protein hydrolysates exhibited the highest activity prior to *in vitro* digestion, variations in

antihypertensive activity trends before and after digestion imply that proteins and their hydrolysates are further degraded into lower-molecular-weight components during the digestive process. These findings are consistent with the SDS-PAGE results showing increased low-molecular-weight protein fractions after digestion, which may have contributed to the enhanced post-digestion bioactivity.

These digestion-dependent shifts may reflect differences in peptide release, peptide stability, or peptide transformation among breeds and muscle. However, because peptide profiles were not directly characterized in the present study, the possibility that assay variability or unmeasured peptide composition differences contributed to these changes cannot be excluded.

4. Conclusion

In this study, myofibrillar protein hydrolysates derived from Korean native cattle breeds and Black Angus were incorporated into chicken tenderloin protein bars and soy beverages to evaluate and bioactivity before and after *in vitro* digestion. Supplementation with protein hydrolysates improved protein digestibility, antioxidant activity, and ACE inhibitory activity compared with non-supplemented controls. Among the tested protein hydrolysates, Jeju Black Cattle loin generally exhibited relatively high bioactivity before digestion, whereas post-digestion activity patterns differed among treatment groups, particularly in foods supplemented with Black Angus protein hydrolysates. These findings suggest that breed origin and food matrix may influence bioactivity of cattle-derived protein hydrolysates. However, because specific peptide profiles were not identified, further peptidomic analyses are needed to clarify the mechanisms underlying breed- and matrix-dependent differences in bioactivity.

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Conflicts of Interest

The authors declare no potential conflicts of interest.

Data Availability

Data available upon reasonable request to the authors

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633 ## Tables

634 **Table 1. Composition of chicken tenderloin protein bar supplemented with protein**
635 **hydrolysates**

Materials	Chicken tenderloin protein bar				
	Proportion (%)				
	0.0	0.5	1.0	1.5	2.0
Chicken tenderloin myofibrillar protein	43.6	43.4	43.2	42.9	42.7
Canola oil	7.9	7.9	7.8	7.8	7.8
Korean pancake mix	11.1	11.0	11.0	10.9	10.9
Egg white	4.8	4.7	4.7	4.7	4.7
Onion powder	0.5	0.5	0.5	0.5	0.5
Salt	0.8	0.8	0.8	0.8	0.8
Sugar	28.0	27.9	27.8	27.6	27.5
Milk	0.6	0.6	0.6	0.6	0.6
Soy sauce	0.6	0.6	0.6	0.6	0.6
Nucleic acid seasoning	0.3	0.3	0.3	0.3	0.3
Beef bone stew powder	0.3	0.3	0.3	0.3	0.3
Pepper	0.5	0.5	0.5	0.5	0.5
Egg york	0.8	0.8	0.8	0.8	0.8
Lemon juice	0.2	0.2	0.2	0.2	0.2
Protein hydrolysates	0.0	0.5	1.0	1.5	2.0

Total	100.0	100.0	100.0	100.0	100.0

[1)] The numbers indicate the addition of protein hydrolysates to the chicken tenderloin protein bar.

638 **Table 2. Composition of soy beverage supplemented with protein hydrolysates**

Materials	Soy beverage				
	Proportion (%)				
	0.0	0.5	1.0	1.5	2.0
Soybean	20.34	20.24	20.14	20.03	19.93
Roasted almond	4.58	4.56	4.54	4.51	4.49
Roasted oatmeal	4.58	4.56	4.54	4.51	4.49
Roasted peanut	2.29	2.28	2.27	2.26	2.25
Water	64.16	63.84	63.52	63.20	62.88
Sugar	4.03	4.01	3.99	3.97	3.95
Salt	0.01	0.01	0.01	0.01	0.01
Protein hydrolysates	0.00	0.50	1.00	1.50	2.00
Total	100.00	100.00	100.00	100.00	100.00

639

Table 3. Constituents and concentrations of synthetic digestive juices used on the *in vitro* digestion model representing post-feeding conditions

	Saliva (mouth step)	Gastric juice (stomach step)	Duodenal juice (small intestine step)	Bile juice (small intestine step)
Inorganic and organic components	1.7 mL NaCl[1)] (175.3 g/L)[2)] 8 mL urea (25 g/L) 15 mg uric acid	6.5 mL HCl (37 g/L) 18 mL $CaCl_2 \cdot 2H_2O$ (22.2 g/L) 1 g bovine serum albumin	6.3 mL KCl (89.6 g/L) 9 mL $CaCl_2 \cdot 2H_2O$ (22.2 g/L) 1 g bovine serum albumin	69.3 mL $NaHCO_3$ (84.7 g/L) 10 mL $CaCl_2 \cdot 2H_2O$ (22.2 g/L) 1.8 g bovine serum albumin 30 g bile
Enzyme	290 mg α-amylase 25 mg mucin	2.5 g pepsin 3 g mucin	9 g pancreatin 1.5 g lipase	
pH	6.8 ± 0.2	1.50 ± 0.02	8.0 ± 0.2	7.0 ± 0.2

[1)] The numbers indicate the concentration of chemicals used to prepare digestive juices.

[2)] The numbers in parentheses indicate the concentrations of inorganic or organic components per 1 L of distilled water.

After mixing all components (inorganic components, organic components, and enzymes), the volume was increased to 500 mL with distilled water. If necessary, the pH of the digestive juices was adjusted to the appropriate value.

647 **Table 4. Percentage (%) of free amino acids in protein hydrolysates from Korean native cattle and Black Angus**

Content (%)	Chikso		Jeju Black Cattle		Jeju black Hanwoo		Hanwoo		Black Angus	
	Loin	Shank	Loin	Shank	Loin	Shank	Loin	Shank	Loin	Shank
Histidine	2.7±0.1^{a}	2.4±0.2^{a}	2.8±0.0^{a}	2.3±0.0^{a}	1.5±0.5^{b}	1.4±0.3^{b}	1.2±0.2^{b}	1.3±0.1^{b}	1.4±0.3^{b}	1.4±0.3^{b}
Isoleucine	8.1±0.1^{a}	8.7±0.5^{a}	8.8±0.3^{a}	8.5±0.1^{a}	4.3±4.3^{a}	4.2±4.2^{a}	4.2±4.2^{a}	3.7±3.7^{a}	4.5±4.5^{a}	4.2±4.2^{a}
Leucine	13.0±0.3 ^{a}	14.5±1.3 ^{a}	14.2±0.5 ^{a}	14.4±0.1 ^{a}	10.9±2.5 ^{a}	10.9±2.7 ^{a}	11.2±2.4 ^{a}	10.3±1.8 ^{a}	11.1±2.7 ^{a}	10.7±2.0 ^{a}
Lysine	11.8±0.3 ^{b}	11.1±0.9 ^{b}	14.0±0.1 ^{a}	11.7±0.0 ^{b}	10.1±3.4 ^{b}	10±2.7^{b}	7.0±3.6^{c}	6.8±5.0^{c}	6.7±5.7^{c}	6.9±5.9^{c}
Methionine	4.5±0.2^{a}	4.8±0.2^{a}	4.9±0.3^{a}	4.9±0.1^{a}	2.5±2.0^{a}	2.6±2.0^{a}	2.3±1.9^{a}	2.5±1.8^{a}	2.9±2.1^{a}	2.7±1.8^{a}
Phenylalanine	6.6±0.2^{a} ^{b}	6.1±0.6^{a} ^{b}	7.2±0.4^{a}	7.0±0.1^{a} ^{b}	1.5±0.8^{c}	3.9±2.9^{b}	6.0±1.0^{a} ^{b}	5.0±1.1^{a} ^{b}	6.1±1.0^{b}	5.9±0.8^{a} ^{b}
Threonine	6.0±0.0^{a}	5.9±0.1^{a}	5.3±0.1^{a} ^{b}	5.5±0.1^{a} ^{b}	3.5±2.6^{a} ^{bc}	5.1±0.5^{a} ^{b}	4.2±1.9^{a} ^{bc}	4.9±1.0^{a} ^{b}	2.0±0.5^{c}	2.7±0.1^{b} ^{c}
Tryptophan	1.4±0.1^{a}	1.3±0.1^{a}	1.4±0.0^{a}	1.4±0.0^{a}	0.8±0.8^{a}	0.8±0.8^{a}	0.7±0.7^{a}	0.7±0.7^{a}	0.8±0.8^{a}	0.7±0.7^{a}
Valine	6.6±0.0^{a}	6.7±0.2^{a}	6.1±0.1^{a}	6.6±0.0^{a}	3.6±3.2^{a}	3.5±3.0^{a}	3.1±3.1^{a}	3.0±3.0^{a}	3.7±3.7^{a}	3.5±3.5^{a}
Arginine	3.6±3.4^{a}	3.3±3.1^{a}	4.3±0.8^{a}	2.4±1.2^{a}	0.2±0.2^{a}	0.3±0.3^{a}	5.0±5.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}	0.0±0.0^{a}

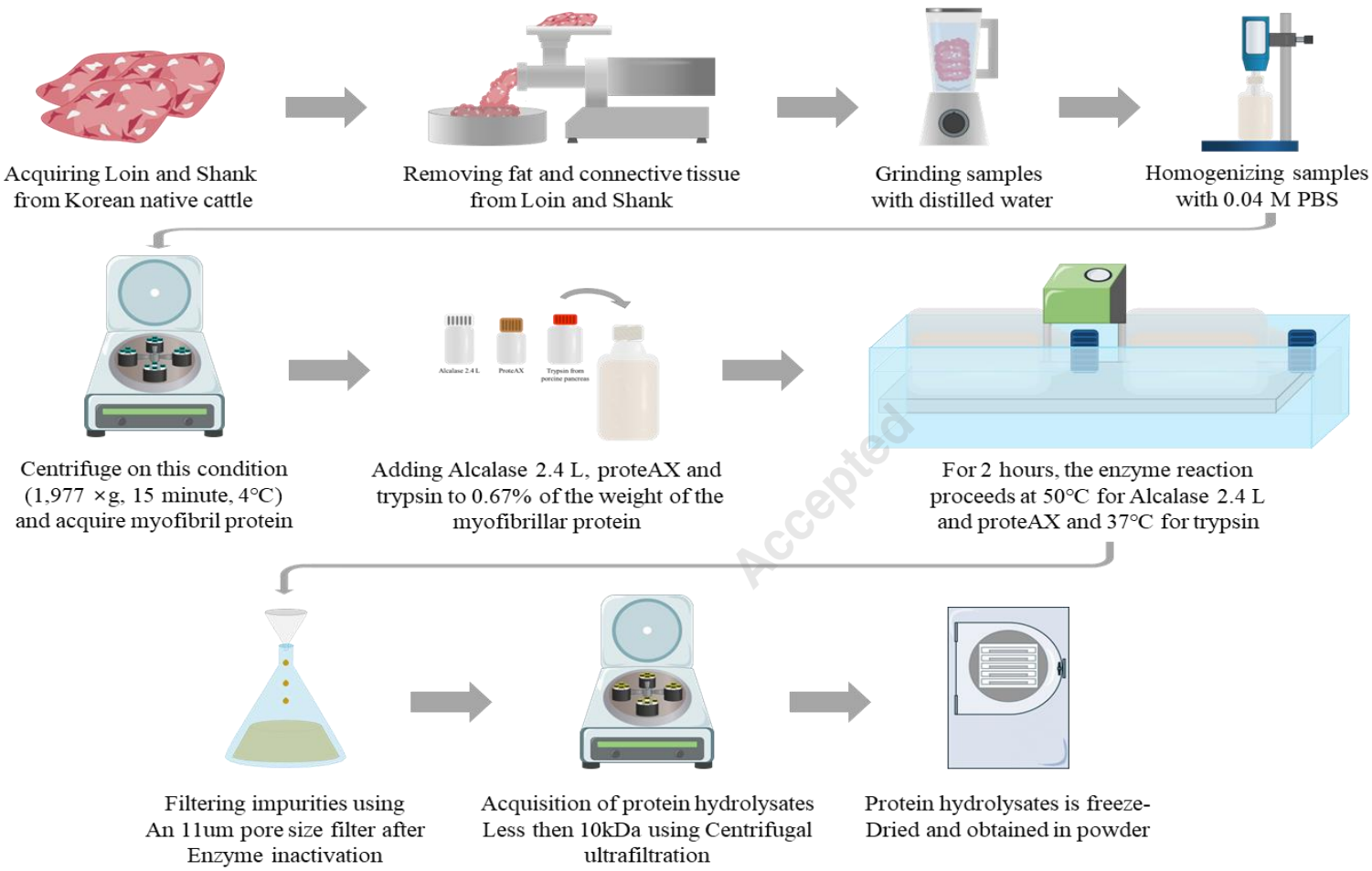
Cystine	0.6±0.0[a]	0.3±0.2[a]	0.9±0.1[a]	0.2±0.0[a]	3.6±3.0[a]	3.5±3.0[a]	3.8±3.1[a]	3.5±2.9[a]	3.1±3.1[a]	3.2±2.9[a]
Glutamine	1.6±0.2[a]	0.7±0.5[a]	0.0±0.0[a]	0.6±0.0[a]	5.2±4.2[a]	4.6±4.4[a]	4.9±2.2[a]	3.9±2.6[a]	2.6±2.6[a]	2.6±2.4[a]
Glycine	1.7±0.0[a]	2.0±0.2[a]	1.0±0.0[a]	1.3±0.0[a]	0.7±0.7[a]	0.7±0.7[a]	0.7±0.7[a]	0.8±0.8[a]	1.3±1.3[a]	1.2±1.2[a]
Proline	0.3±0.2[a]	0.4±0.3[a]	0.4±0.2[a]	0.9±0.5[a]	0.3±0.3[a]	0.3±0.3[a]	0.3±0.3[a]	0.2±0.2[a]	0.3±0.3[a]	0.4±0.4[a]
Tyrosine	3.9±1.0[a]	2.6±2.0[a]	4.9±0.2[a]	3.4±0.0[a]	7.5±6.3[a]	7.6±5.2[a]	9.0±5.1[a]	9.2±4.2[a]	7.9±5.1[a]	8.1±5.3[a]
Alanine	6.7±0.4[a]	6.6±0.0[a]	5.4±0.1[a]	7.8±0.1[a]	4.2±2.7[a]	5.0±3.7[a]	3.0±1.1[a]	3.6±2.2[a]	4.6±3.1[a]	5.0±3.6[a]
Aspartic acid	2.3±0.4[a]	3.3±1.3[a]	1.8±0.2[a]	1.3±0.1[a]	2.5±2.5[a]	0.5±0.5[a]	1.3±1.3[a]	2.6±2.6[a]	0.8±0.8[a]	1.2±1.2[a]
Asparagine	1.8±0.1[a] [b]	2.3±1.0[a] [b]	0.0±0.0[b]	1.4±0.0[a] [b]	1.3±0.8[a] [b]	1.2±1.2[a] [b]	3.2±0.5[a]	1.0±0.8[a] [b]	1.8±1.8[a] [b]	1.7±1.7[a] [b]
Glutamic acid	8.1±1.0[a]	8.7±1.1[a]	7.3±0.5[a]	8.2±0.1[a]	4.9±4.8[a]	4.8±4.4[a]	3.2±1.9[a]	5.6±4.0[a]	7.3±3.8[a]	7.4±4.2[a]
Serine	2.0±1.2[a]	2.6±0.6[a]	1.9±0.3[a]	2.4±0.0[a]	4.2±1.5[a]	4.0±1.8[a]	4.9±1.2[a]	3.9±2.0[a]	3.6±2.3[a]	3.4±2.3[a]
1-Methylhistidine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	6.4±6.4[a]	6.4±6.4[a]	5.9±5.9[a]	6.1±6.1[a]	5.2±5.2[a]	5.2±5.2[a]
…										

Content (%)	Chikso		Jeju Black Cattle		Jeju Black Hanwoo		Hanwoo		Black Angus	
	Loin	Shank	Loin	Shank	Loin		Loin	Shank	Loin	Shank
3-Methylhistidine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	1.4±1.4[a]	1.4±1.4[a]	1.3±1.3[a]	1.4±1.4[a]	1.3±1.3[a]	1.3±1.3[a]

Citrulline	1.2±1.0[a]	0.4±0.2[a]	0.8±0.2[a]	1.1±0.5[a]	4.8±3.8[a]	4.0±2.6[a]	2.9±2.8[a]	3.2±1.6[a]	3.1±1.2[a]	2.7±1.4[a]
Cystathionine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	2.3±2.3[a]	2.1±2.1[a]	2.4±2.4[a]	2.4±2.4[a]	2.2±2.2[a]	2.3±2.3[a]
Ethanol amine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.7±0.7[a]	0.7±0.7[a]	0.6±0.6[a]	0.7±0.7[a]	0.8±0.8[a]	0.6±0.6[a]
Hydroxy proline	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.3±0.3[a]	0.2±0.2[a]	2.4±2.4[a]	3.8±3.8[a]	5.0±5.0[a]	5.3±5.3[a]
Hydroxylysine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	1.3±1.3[a]	1.3±1.3[a]	0.6±0.6[a]	0.7±0.7[a]	0.3±0.3[a]	0.3±0.3[a]
Ornithine	3.6±1.6[a]	3.6±1.5[a]	4.6±1.4[a]	4.6±0.1[a]	3.8±3.8[a]	3.3±3.3[a]	0.4±0.4[a]	2.8±2.8[a]	3.3±3.3[a]	2.8±2.8[a]
Sarcosine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.1±0.1[a]	0.5±0.5[a]	0.4±0.4[a]	0.8±0.8[a]	1.2±1.2[a]	1.1±1.1[a]
α-amino-n-butyric acid	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.6±0.6[a]	0.4±0.4[a]	0.1±0.1[a]	0.2±0.2[a]	0.0±0.0[a]	0.0±0.0[a]
β-Alanine	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	3.5±3.5[a]	3.3±3.3[a]	3.5±3.5[a]	3.5±3.5[a]	3.7±3.7[a]	3.7±3.7[a]
Citrulline	1.2±1.0[a]	0.4±0.2[a]	0.8±0.2[a]	1.1±0.5[a]	4.8±3.8[a]	4.0±2.6[a]	2.9±2.8[a]	3.2±1.6[a]	3.1±1.2[a]	2.7±1.4[a]
SUM	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

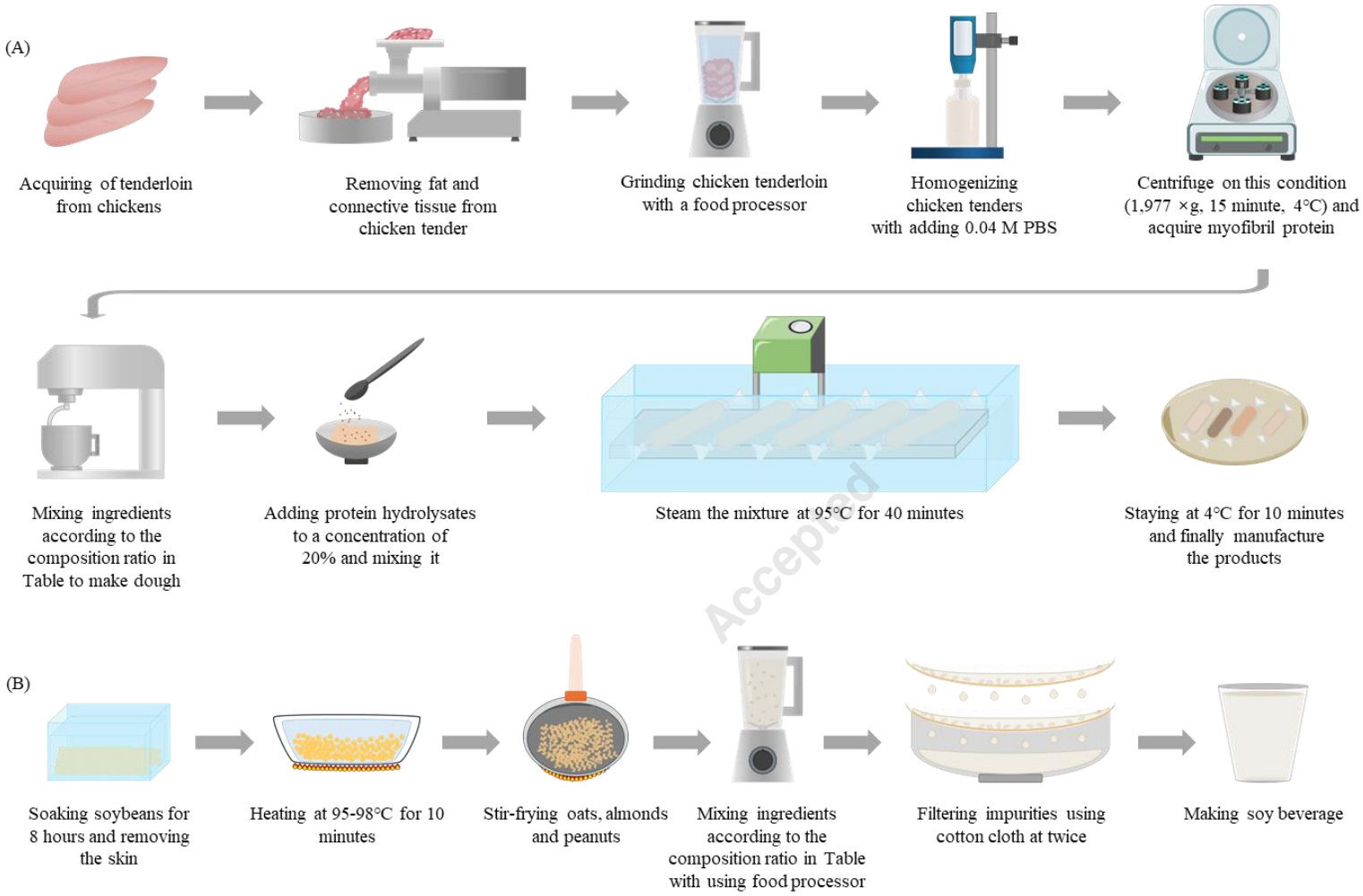
[a-c]Lowercase letters indicate significant differences after *in vitro* digestion ($p < 0.05$).

649 **Figures**



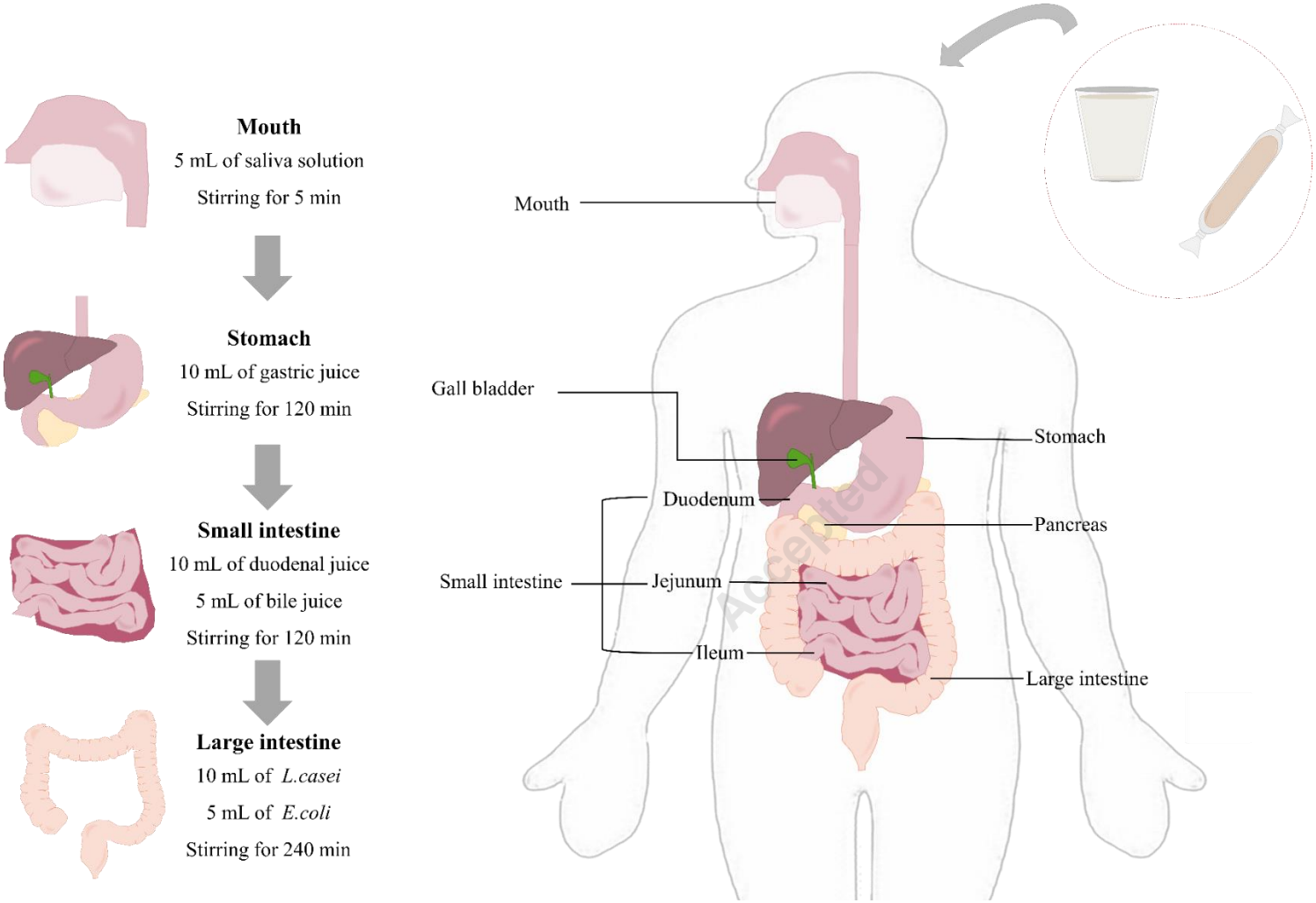
650

651 **Figure 1. Process for the preparation of protein hydrolysates (< 10 kDa) derived from the myofibrillar proteins of Korean native cattle and**
652 **Black Angus**



653

654 **Figure 2. Manufacturing procedure for chicken tenderloin protein bars and soy beverages. (A) Chicken tenderloin protein bar (B) Soy**
655 **beverage**



656

657 **Figure 3. *In vitro* digestion process for soy beverages and chicken tenderloin protein bars**

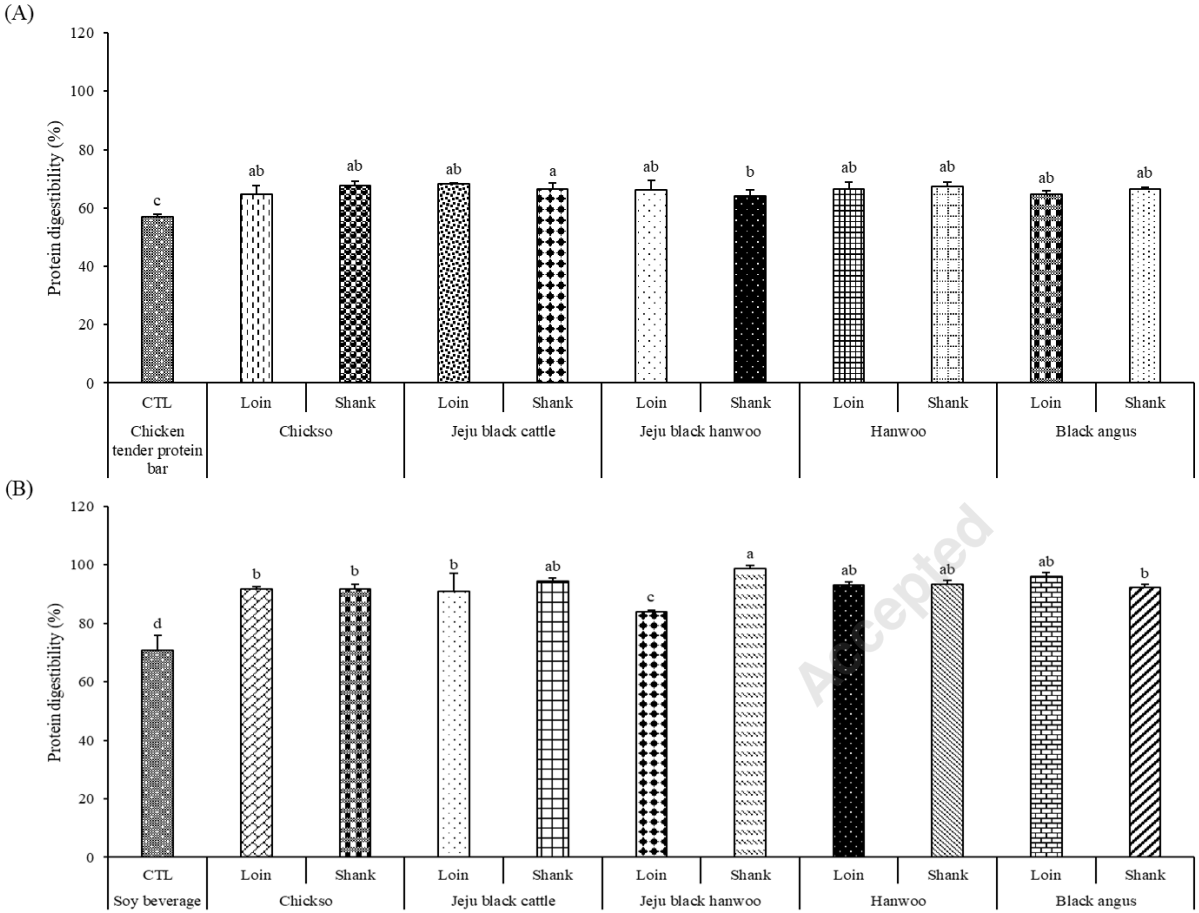


Figure 4. Protein digestibility of chicken tenderloin protein bars and soy beverages with varying percentages of protein hydrolysates following *in vitro* digestion (A) Protein digestibility of chicken tenderloin protein bars; (B) Protein digestibility of soy beverages. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$).

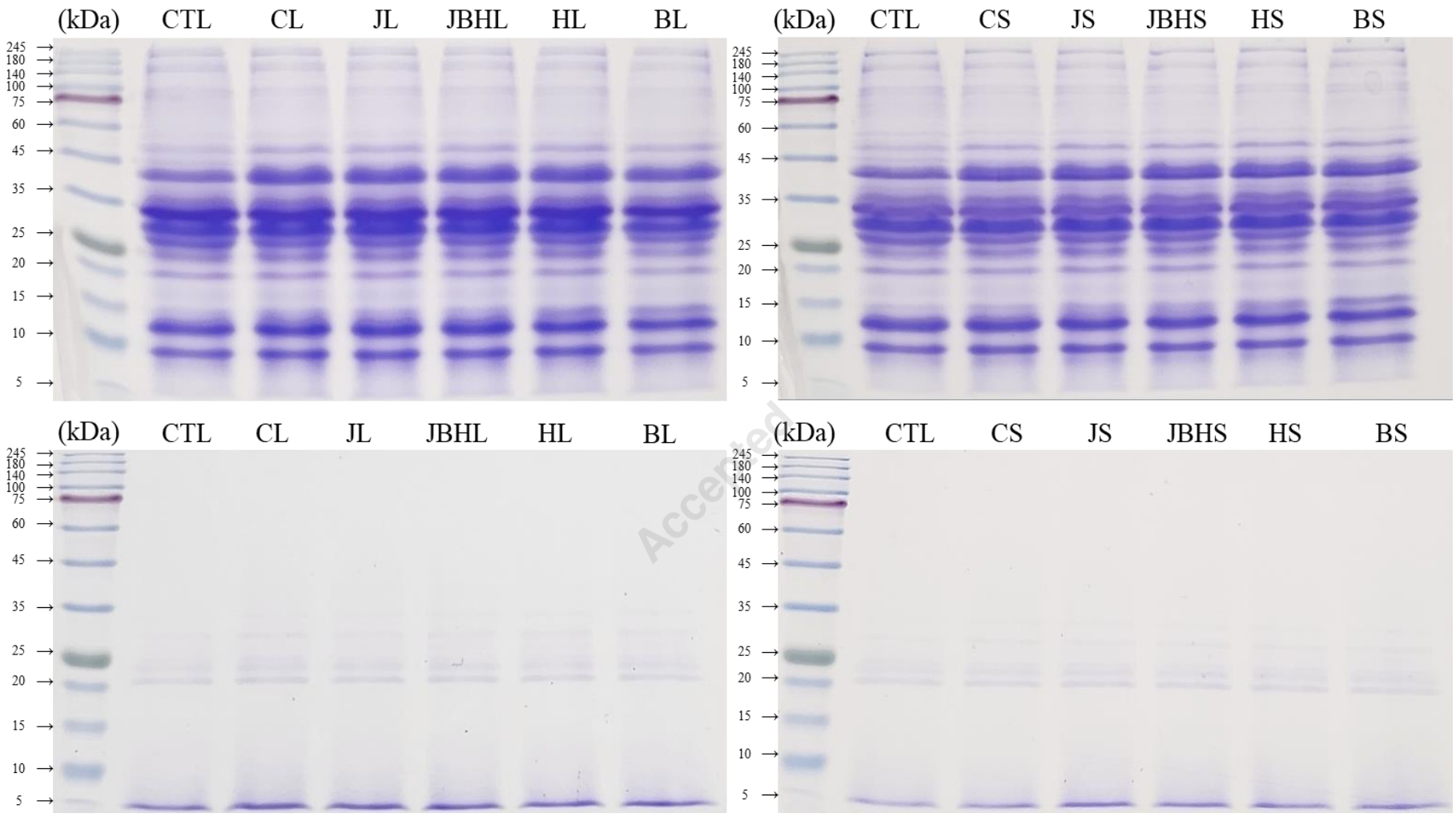
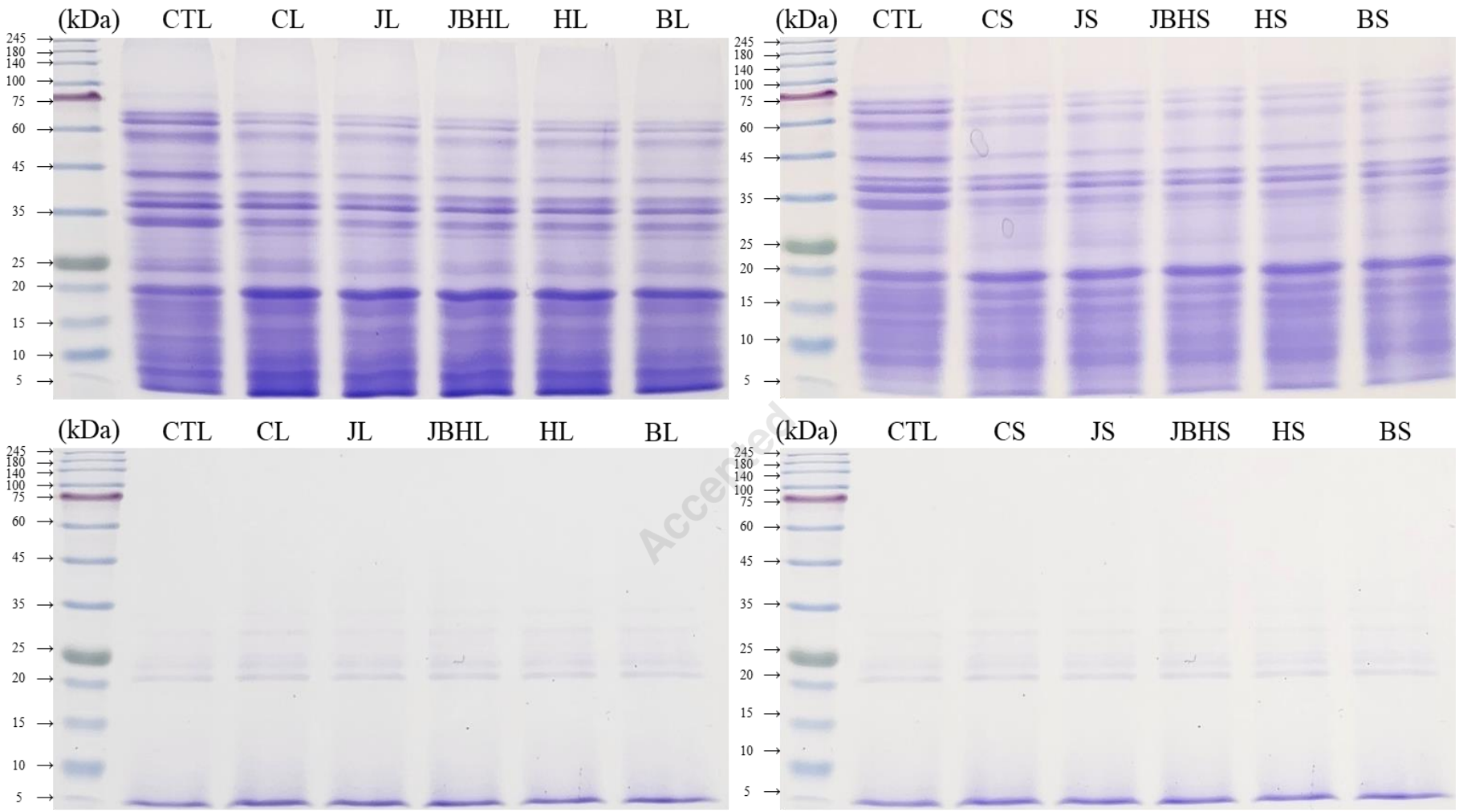


Figure 5. Changes in the protein molecular weights of chicken tenderloin protein bars before and after *in vitro* digestion. (A) Protein molecular weights before *in vitro* digestion; (B) Protein molecular weights after *in vitro* digestion. Protein amount per well: 20 μg/well.



665

666 **Figure 6. Changes in protein molecular weights of soy beverages before and after *in vitro* digestion.** A) Protein molecular weights before *in*
667 *vitro* digestion; (B) Protein molecular weights after *in vitro* digestion. Protein amount per well: 20 µg/well.

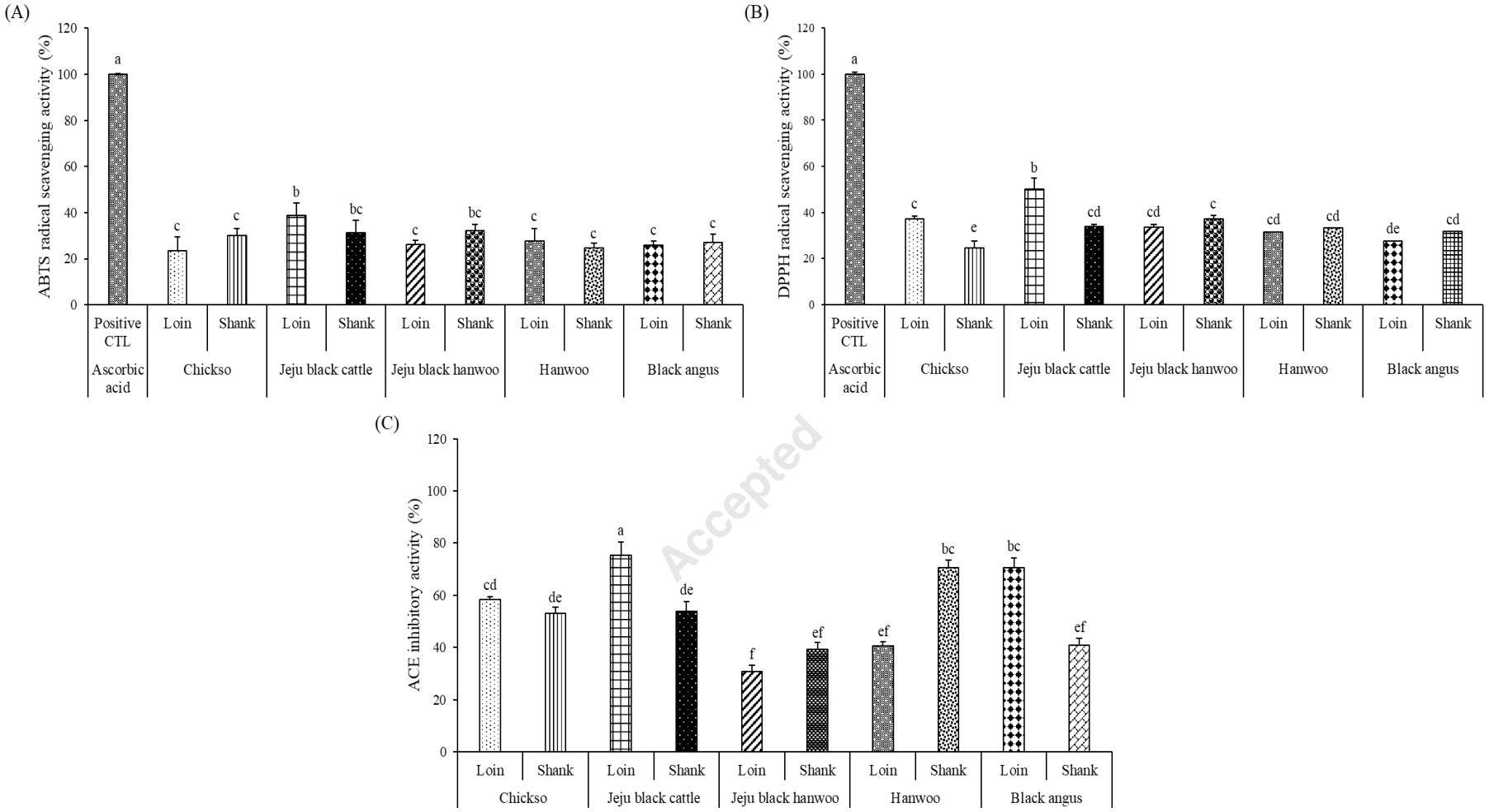
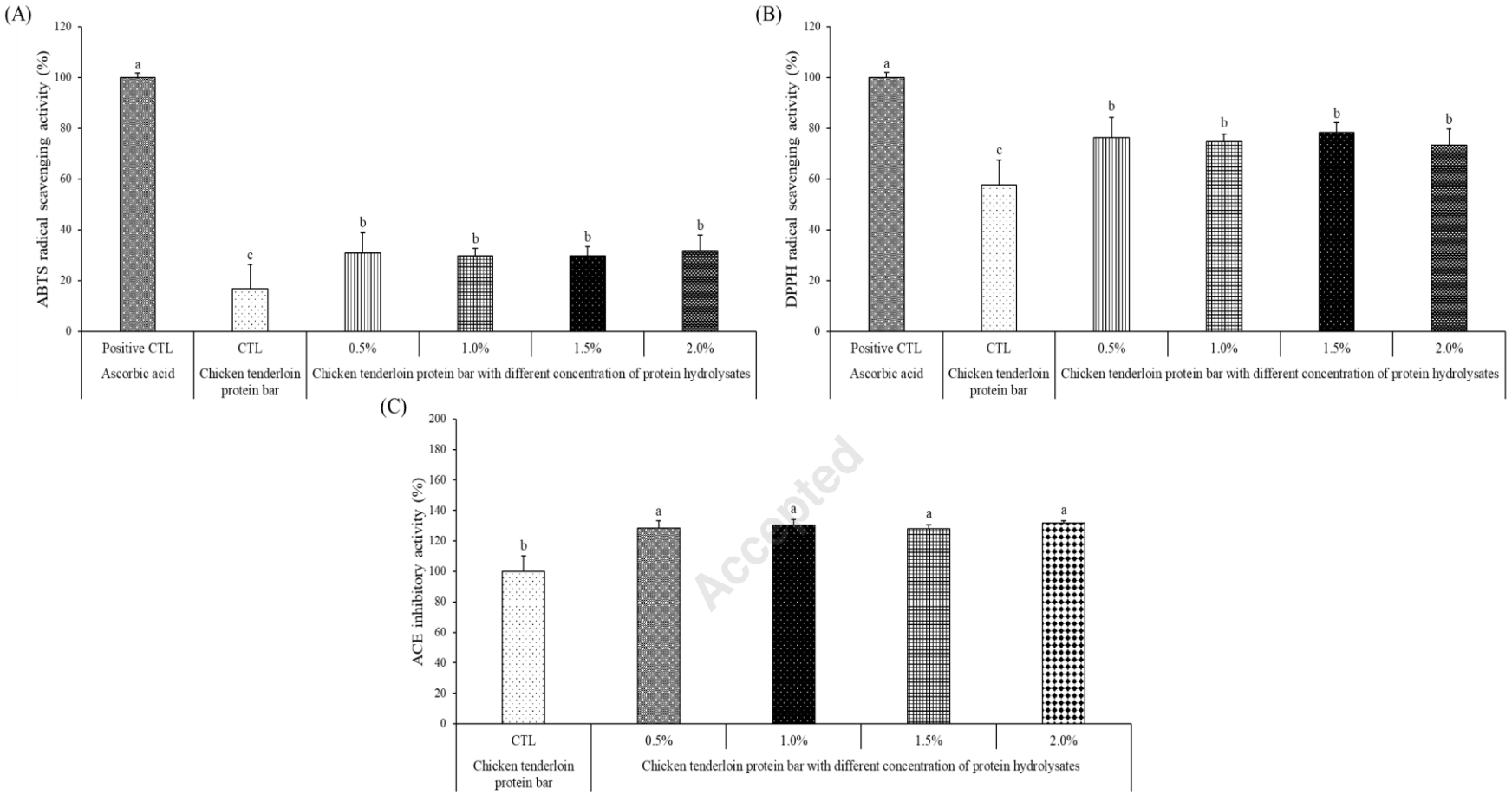
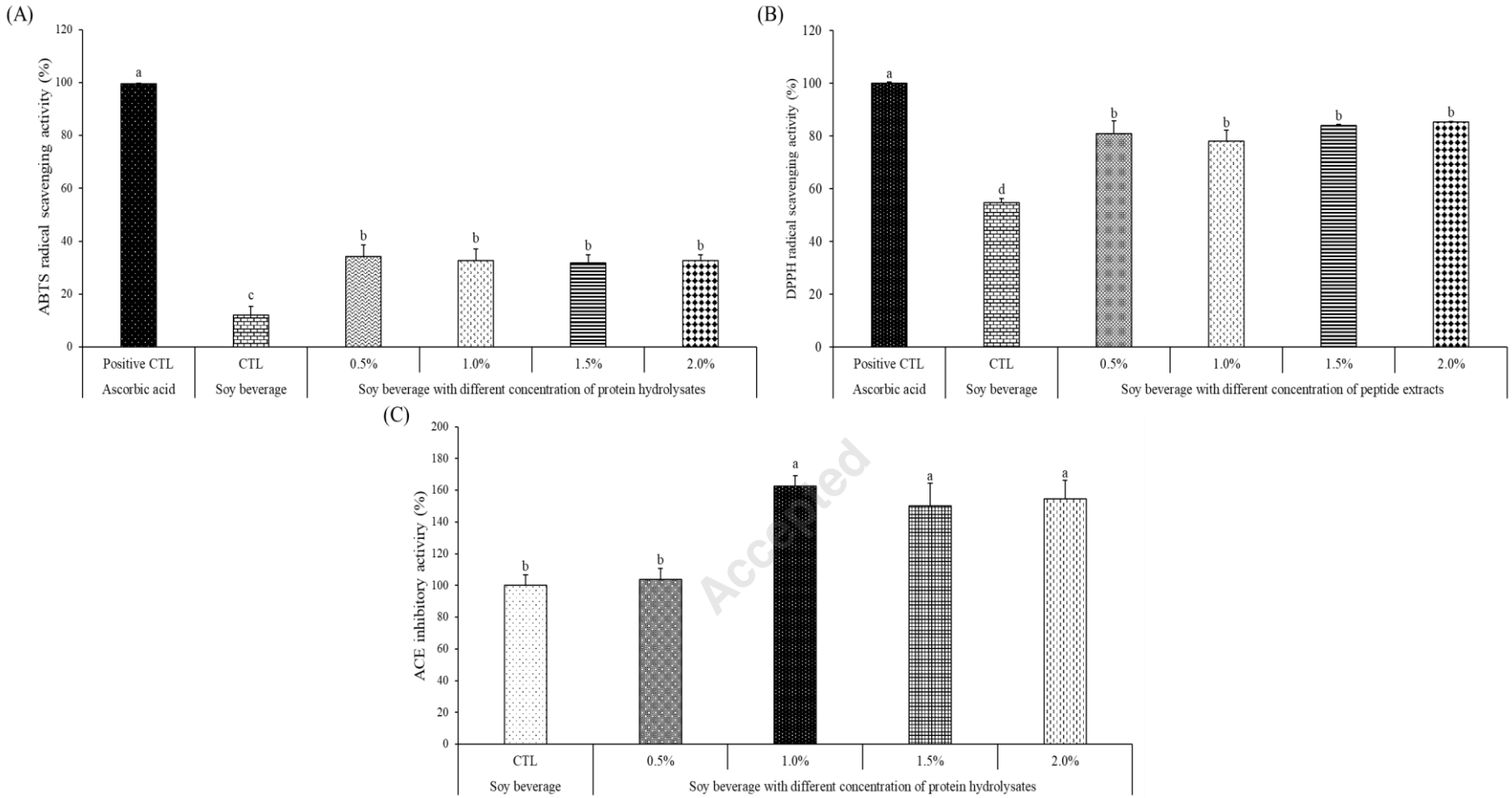


Figure 7. Comparison of DPPH and ABTS radical scavenging activities, as well as the ACE inhibitory activity of protein hydrolysates from the loin and shank of Korean native cattle. (A) ABTS radical scavenging activity; (B) DPPH radical scavenging activity; (C) ACE inhibitory assay. Lowercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$).



672

673 **Figure 8. Comparison of antioxidant and antihypertensive activities in chicken tenderloin protein bars with varying protein hydrolysate**
674 **concentrations.** (A) ABTS radical scavenging assay; (B) DPPH radical scavenging assay; (C) ACE inhibitory assay. Uppercase letters indicate
675 significant differences prior to *in vitro* digestion.



676

677 **Figure 9. Comparison of antioxidant and antihypertensive activities in soy beverages with varying protein hydrolysate concentrations.** (A)
678 ABTS radical scavenging assay; (B) DPPH radical scavenging assay; (C) ACE inhibitory assay. Uppercase letters indicate significant differences
679 prior to *in vitro* digestion, while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$).

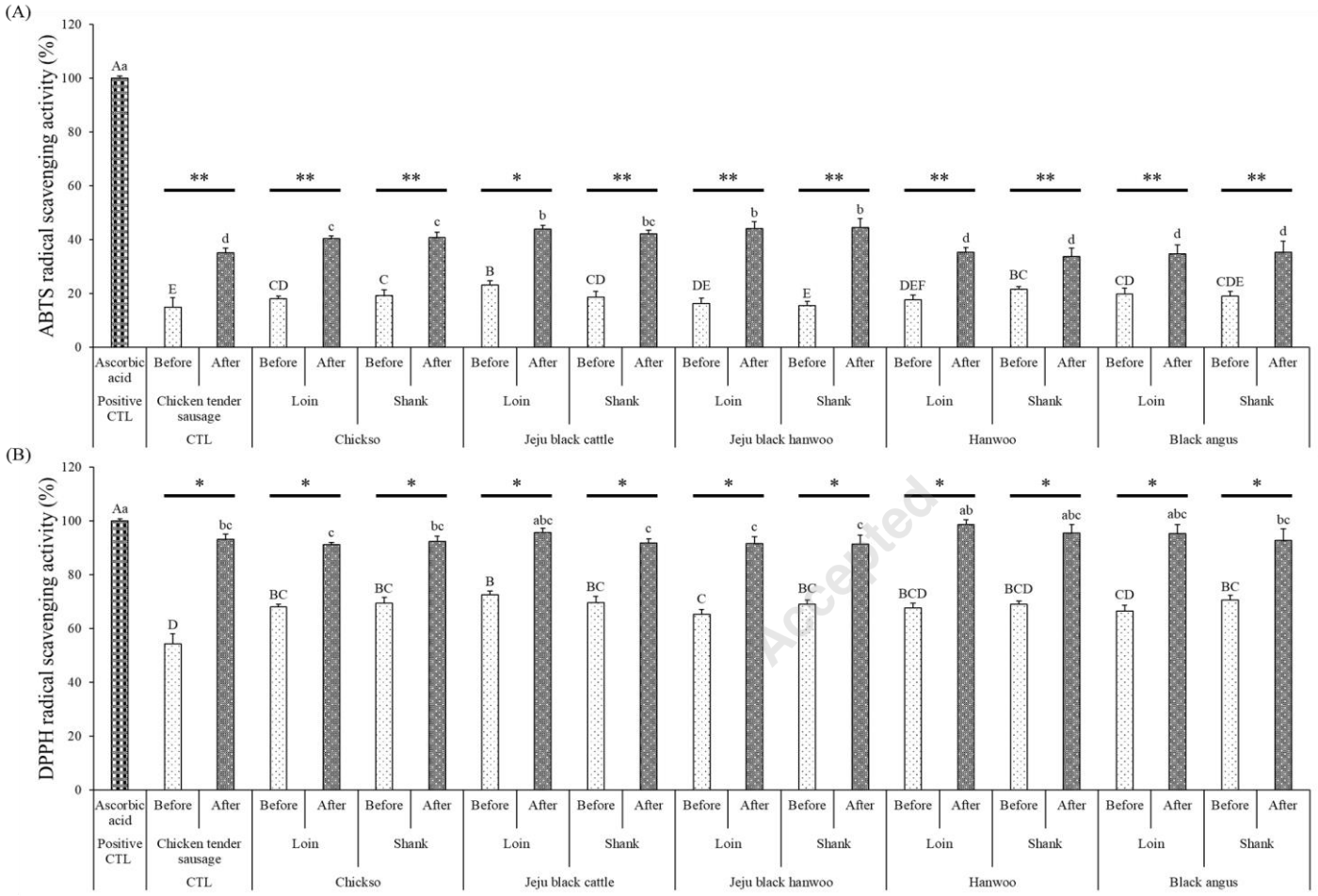
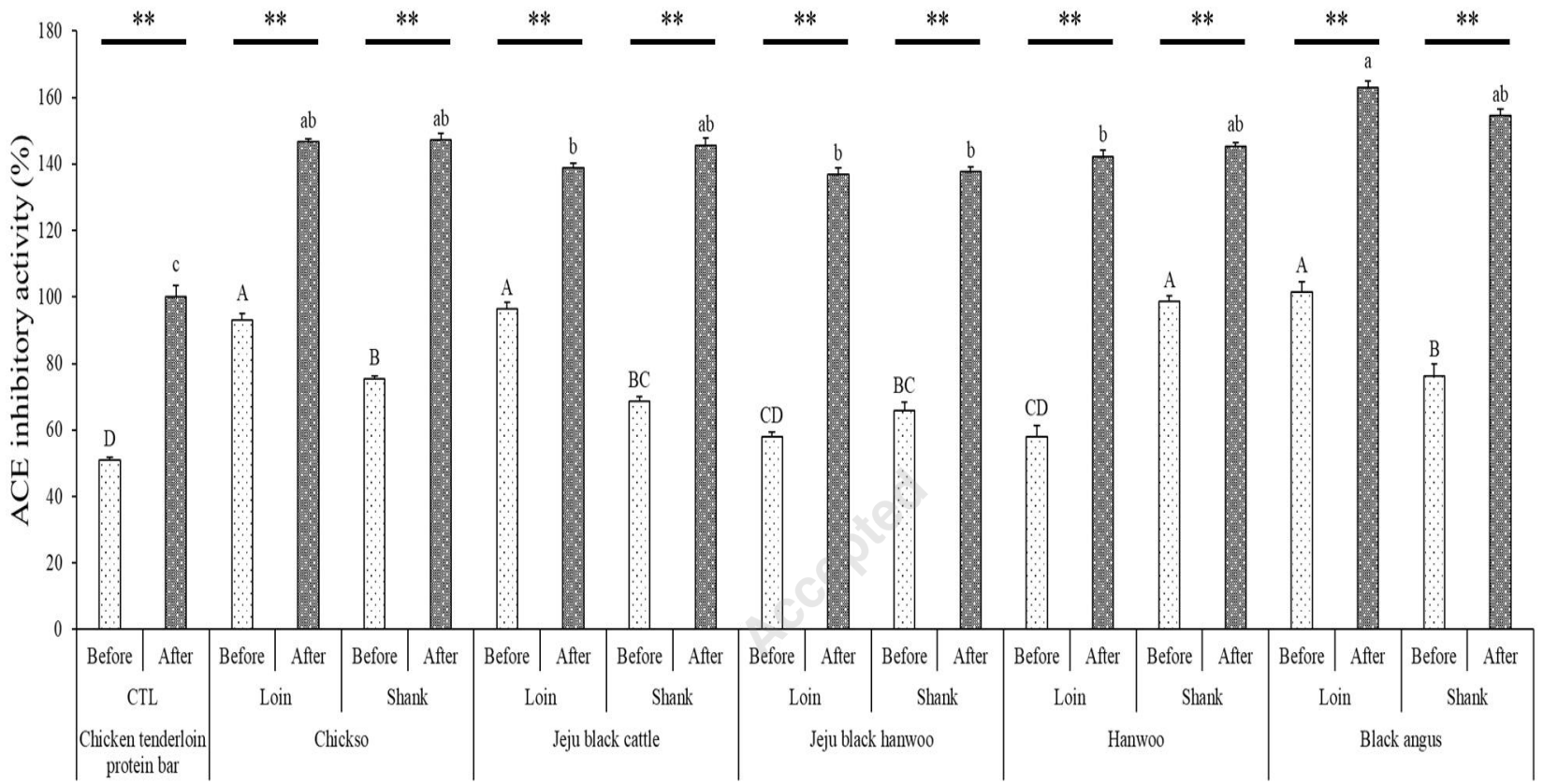
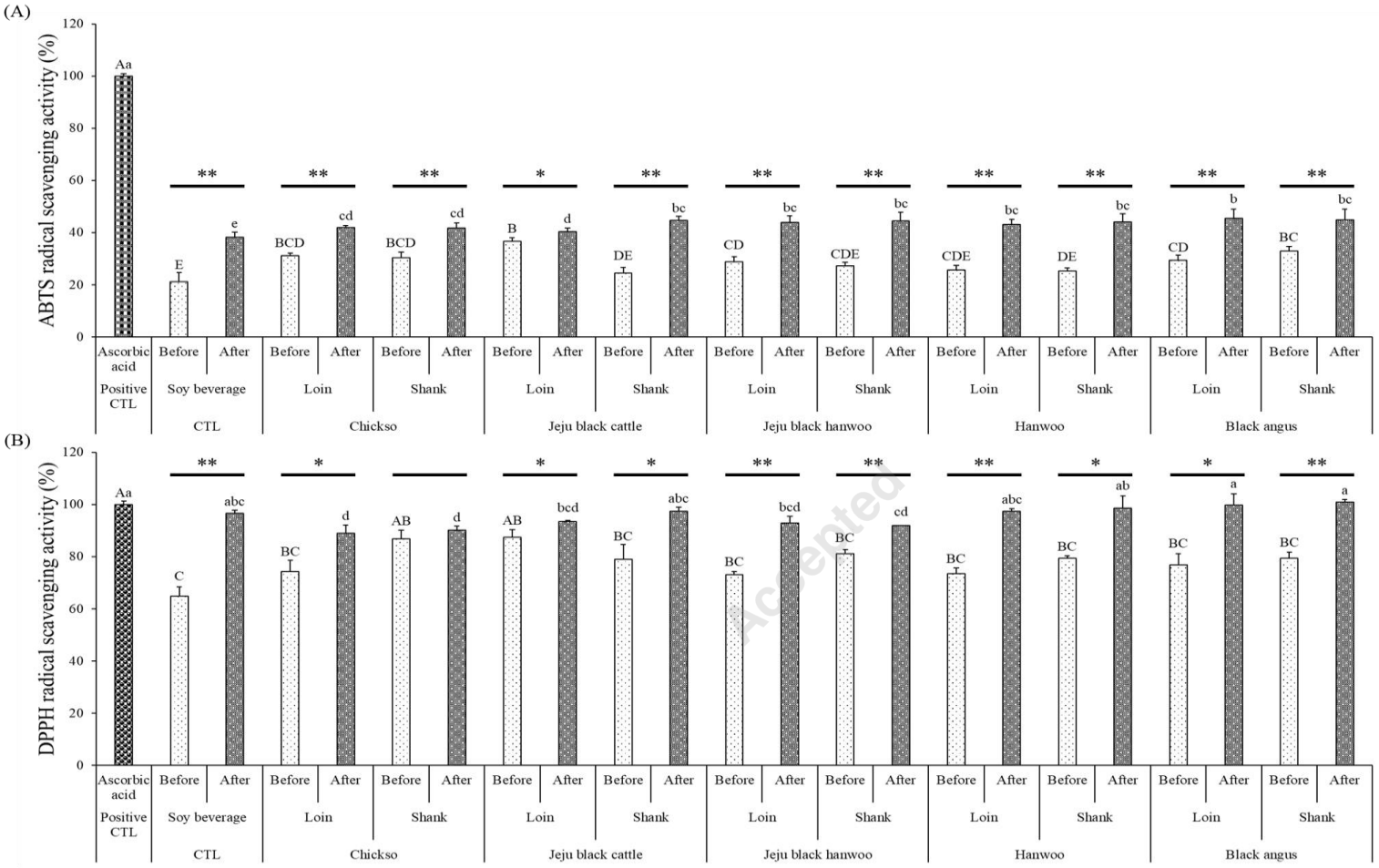


Figure 10. Comparison of antioxidant activity in chicken tenderloin protein bars before and after *in vitro* digestion. (A) ABTS radical scavenging assay; (B) DPPH radical scavenging assay. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$), while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$). A single asterisk ($p < 0.05$) and double asterisks ($p < 0.001$) denote significant differences before and after *in vitro* digestion.



685

686 **Figure 11. Comparison of antihypertensive activity in chicken tenderloin protein bars before and after *in vitro* digestion.** (A) Chicken
687 tenderloin protein bar; (B) Soy beverage. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$), while
688 lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$). A single asterisk ($p < 0.05$) and double asterisks ($p <$
689 0.001) denote significant differences before and after *in vitro* digestion.



690

691 **Figure 12. Comparison of angiotensin-converting enzyme inhibitory activity in soy beverages before and after *in vitro* digestion.** (A)
692 Chicken tenderloin protein bar; (B) Soy beverage. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$),
693 while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$). A single asterisk ($p < 0.05$) and double asterisks
694 ($p < 0.001$) denote significant differences before and after *in vitro* digestion.

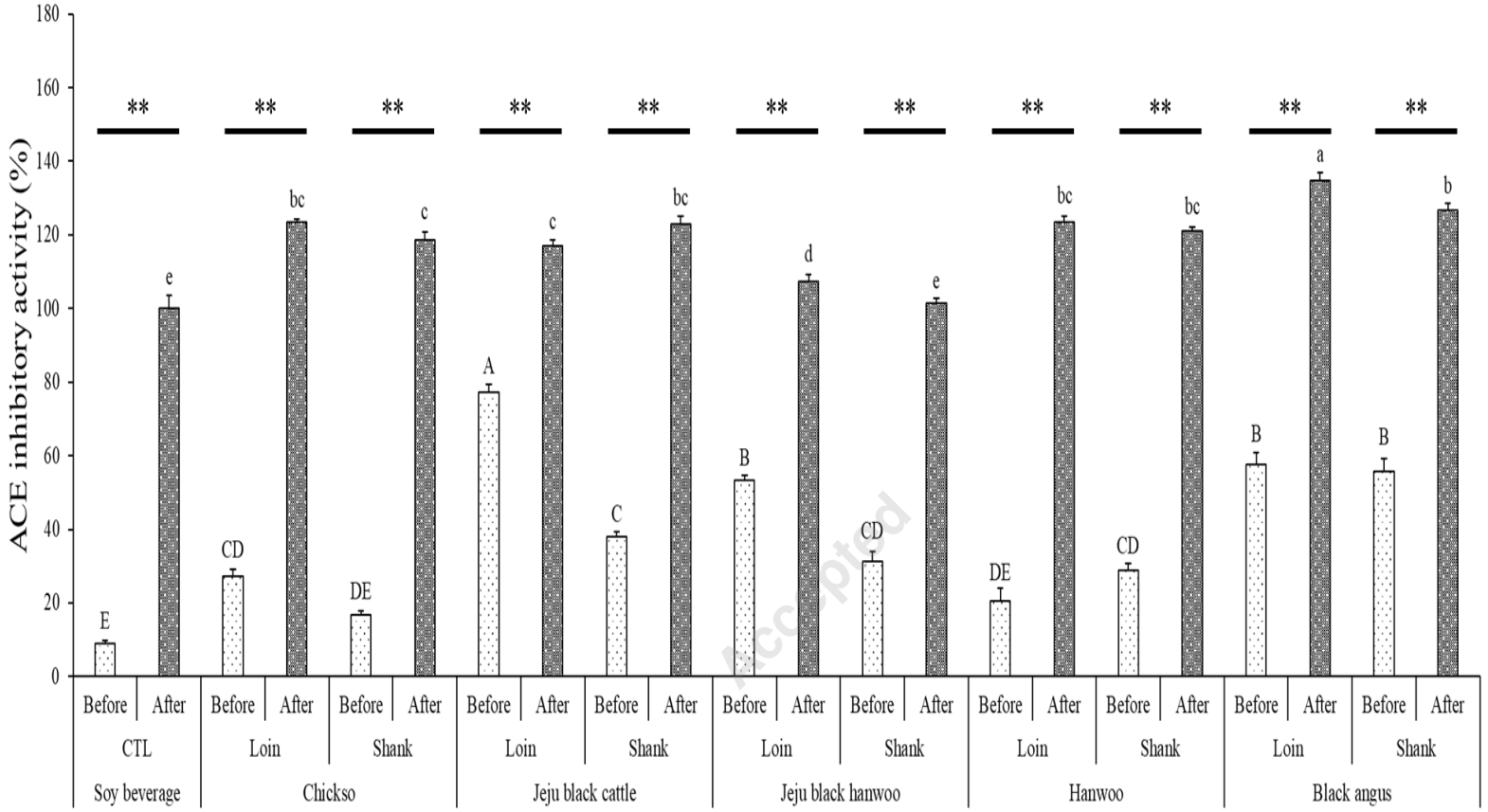


Figure 13. Comparison of antihypertensive activity in soy beverages before and after *in vitro* digestion. (A) Chicken tenderloin protein bar; (B) Soy beverage. Uppercase letters indicate significant differences prior to *in vitro* digestion ($p < 0.05$), while lowercase letters indicate significant differences following *in vitro* digestion ($p < 0.05$). A single asterisk ($p < 0.05$) and double asterisks ($p < 0.001$) denote significant differences before and after *in vitro* digestion.