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Assessment of the effect of marination with broccoli (*Brassica oleracea* var. *italica*) juice and balsamic vinegar on tenderness and quality of beefsteak

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Abstract

Due to the increasing demand for natural foods with fewer artificial additives, broccoli is considered by the consumers and researchers due to its taste, nutritional values and biochemical components. Therefore, this research was conducted to investigate the effect of broccoli juice and balsamic vinegar on the physicochemical and textural attributes of the beefsteak aimed at tenderizing it and decreasing the ageing period. Experiments were carried out through marinating the beefsteak with three different treatments containing 3.00 units protease g^{-1} beefsteak of broccoli juice and 10.00% (v/w) of balsamic vinegar during 48 hr storage at 4.00˚C. A significant reduction in pH and Warner-Bratzler shear force as well as the increase in myofibrillar fragmentation index and myofibrillar protein solubility were obtained showing that the beefsteak was tendered in the presence of broccoli juice and broccoli juice+balsamic vinegar. Scanning electron microscopy images showed the destruction of endomysium collagen and sarcolemma areas of muscle filaments in the above-mentioned treatments. The electrophoretic analysis of isolated myofibrillar proteins indicated that the rate of troponin-T breakdown was quicker in all treatments compared to the control and its degradation was progressively obvious with 20.00 and 28.00 kDa protein bands in mentioned treatments. Based on the results, marinades of broccoli juice and balsamic vinegar could be utilized as a beneficial additive for improving beefsteak tenderness.

Introduction

Quality, tenderness and taste of the meat are important factors for the customers. Hence, the absence of satisfaction can be the reason for complaints and reduced meat consumption.[1] Marinating by soaking, injection and vacuum tumbling is one of the most important and convenient procedures to improve meat quality and tenderness.[2] Mixtures containing water, salt, acidic marinades, vinegar and fruit juices were generally utilized for marinating the meat.[1] In terms of penetration and saving the ageing time, injection is one of the most well-known and widely utilized techniques for meat marinating. Meat tenderization through marinating occurs by two primary mechanisms: The first is through decreasing the pH leading to expanding and dissolving the connective tissues and muscle proteins.[3] The second is by providing appropriate conditions to increase the action of intra-cellular proteases or proteolytic catalysts extricated from the fruits and vegetables. Adding the salts such as sodium chloride, calcium salt and phosphate modifies the quality of meat due to the improved ability to maintain the water.[4]

Nowadays, consumption of healthy additives with controlled effectiveness and free of chemicals is increasingly considered.[5] Therefore, attention to the herbal compounds and their application in the food industry is of special importance. Alteration of the meat texture and tenderization is one of the significant applications of plant proteases. The properties of these plant-derived proteases (such as papain, bromelain, ficin, etc.) in hydrolyzing the myofibrillar proteins and connective tissues to modify the structure of contractile proteins i.e., actin and myosin as well as their activities in a wide range of temperature and pH have received a great deal of attention.[6] Broccoli (*Brassica oleracea* var. *Italica*) is one of the most significant and abundant vegetables which was considered in terms of its taste, anti-cancer and antioxidant components i.e., sulfurfan, indole-3-carbinol,

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and selenium. Kim *et al*. demonstrated that adding the broccoli juice in meat/meat products would be a wiser alternative way to improve organoleptic properties, to inhibit fat oxidation and to prevent cancer in comparison with chemical agents.[7] It was presumed that the metallo-protease, cysteine, serine and aspartic proteases present in the broccoli juice display proteolytic activity on the myofibrillar proteins and connective tissues. Four cysteine proteases and one aspartic protease were recognized in the broccoli juice which were similar to papain protease.[8] The most enzymatic activity of the broccoli juice was observed in low pH conditions. The proteolytic activity of the broccoli is equal to 16.90 U g^{-1} in acidic pH and the density of *Brassica oleracea* proteases is about 45.00 – 70.00 kDa.[9] Aktaş *et al*. claimed that the tenderizing and softning of meat can be achieved by applying an appropriate concentration of weak organic acid marinades, specially when they were distributed uniformly.[3]

Traditional balsamic vinegar is a conceivably healthy flavoring with a high level of cancer preventive constituents and is rich in phenolic acids and flavonoids.[10] It has been reported that the panelists accepted the meat samples marinated with balsamic vinegar. This could be due to the reduction of textural parameters and insignificant aroma of meat/vinegar that was disappeared during cold storage.[11] Nevertheless, there are limited investigations on the meat-softening strategies with balsamic vinegar.[12] Therefore, the role of beefsteak marinating with broccoli juice and balsamic vinegar and their effect on the meat quality attributes during 48 hr of tenderization period were investigated.

Materials and Methods

Materials. The beefsteak from tight muscle (*M. Semitendinosus*) of a one-year-old male cattle of Holstein breed was conveyed from the slaughterhouse to the laboratory 24 hr after slaughter under cold conditions. Fresh broccoli (*Brassica oleracea* var. *italica*) and 6.00% balsamic vinegar (pH 4.01; Bonini, Modena, Italy) were purchased from a local market. All chemicals used were purchased from Merck (Darmstadt, Germany).

Broccoli juice extraction. The extraction of broccoli crude juice was done based on the procedure proposed by Baker *et al*. with slight modifications.[13] Fresh broccoli was homogenized by a Ultra turrax homogenizer (T-25; IKA, Staufen, Germany). The blended material was compacted in a triple-layer cheesecloth and the juice was extracted and centrifuged at 16,000 *g* for 5 min at 4.00 ˚C. The resulting supernatant (pH 6.56; extraction efficiency: 10.40%) was poured into the topped microcentrifuge tubes and was kept at –18.00 ˚C until further testing.[14]

Determination of proteolytic activity. Protease activity of broccoli juice (U mL^{-1}) was determined according to the Anson method using casein as substrate solution and trichloroacetic acid (TCA) as reaction blocking reagent based on tyrosine liberation per minute under the standard assay conditions.[15]

Preparation of the samples. The fat-free beefsteaks with a weight of around 1.00 kg were cut into cubic pieces (10.00 × 10.00 × 10.00 cm). Based on the preliminary studies and minimal adverse organoleptic changes, three marinade solutions containing broccoli juice and balsamic vinegar were prepared and injected into the beefsteak cuts as follow: Control: Beefsteak without marinade, A: Broccoli juice (3.00 U protease g^{-1} of beefsteak), B: 10.00% (v/w) balsamic vinegar, C: Broccoli juice (3.00 U protease g^{-1} of beefsteak) + 10.00% (v/w) balsamic vinegar. For better distribution, the treated samples were gently massaged by hand for about 1 min. Then, all samples were transferred to the polyethylene sacks (Sedat Tahir Ltd., Ankara, Turkey) and stored at the refrigerated temperature (4.00 ˚C), and influence of meat marinating on designed tests at 1, 3, 24, and 48 hr intervals was evaluated.[16] Different samples were used to carry out the time-dependent analysis.

Measurement of pH value. First, the raw sample (5.00 g) was mixed with distilled water (20.00 mL) in a homogenizer and centrifuged at 8,000 *g* for 60 sec. Then, the estimation of pH values was carried out by a digital pH meter (inoLab™ pH 7110; WTW, Weilheim, Germany).[1]

Myofibrillar protein solubility (MPS). The solubility of myofibrillar protein was measured by the procedure introduced by He *et al*. with a slight alteration.[14] The sarcoplasmic protein solubility (SPS) was characterized through blending 2.00 g of raw sample and 20.00 mL of 0.025 M super cold potassium phosphate buffer (pH 7.40) by the Ultra-Turrax homogenizer. Next, the mixtures were kept at 4.00 ˚C for 24 hr and centrifuged for 15 min at 6,000 *g*. The protein grouping of the resulting supernatant was characterized through the Biuret method.[17] The total protein solubility (TPS) was characterized by blending 2.00 g of the crude sample in 20.00 mL of super cold 1.10 M potassium iodide into 0.10 M potassium phosphate buffer (pH 7.40). The solubility of myofibrillar protein was calculated as follows:

$$MPS\ (mL\ g^{-1}) = TPS\ (mL\ g^{-1}) - SPS\ (mL\ g^{-1}).$$

Warner-Bratzler shear force (WBSF). Beefsteaks were cooked in an air convection stove with an objective temperature of 75.00 ˚C to an inside temperature of 70.00 ˚C. Then, they were cooled in the chilled running water for 30 min and each steak was dried with a paper towel. Next, lengthwise to the direction of muscle fibers, eight-round cores were cut by a mechanical coring device (1.27 cm of width). Cores were sheared by a Warner-Bratzler shear blade (V-type device) connected to an Instron Universal Testing Machine (TA-XT Plus Texture Analyzer, Texture Technologies Corp., Tokyo, Japan) using a 50.00 kg

pressure load cell and at a 200 mm min^{-1} of cross-head speed. The WBSF was recorded in newton (N).[18]

Myofibrillar fragmentation index (MFI). The MFI estimation was done according to the method proposed by Kim *et al*. with slight changes.[4] The MFI buffer (20.00 mM K_2HPO_4/KH_2PO_4, pH 7.00, 100 mM KCl, 1.00 mM EDTA, 1.00 mM NaN_3) and the Biuret method were separately utilized for extracting the myofibrils and characterizing the protein content of the last suspension.[17] After diluting the last suspension to reach a protein content of 0.50 ± 0.05 mg mL^{-1}, the absorbance of the fragmented myofibril suspensions was determined using a UV-VIS spectrophotometer (Cecil, Cambridge, UK) at 540 nm of wavelength. The absorbance was multiplied by 200 to accomplish the MFI values.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The isolated myofibrillar protein fraction was surveyed by the MFI method as described before. The SDS-PAGE was conducted using the vertical electrophoresis cells (PowerPac™ Basic; Bio-Rad, Herculesm USA) based on the technique suggested by Kim *et al*. and applying 12.00% running gels and 5.00% stacking gels (20.00 µL loaded).[4] The stacked gel was recoloured by the Coomassie Brilliant Blue (Sigma, St. Louins, USA) and was destained in methanol, refined water, and acetic acid (50 : 40 : 10). Appeared protein bands were recognized in comparison with a standard protein marker (PageRuler perfect protein stepping stool 26614; ThermoFisher, Waltham, USA).

Scanning electron microscopy (SEM). The samples with 2.00- 3.00 mm of thickness were fixed in 2.50% glutaraldehyde in 0.10 M phosphate buffer (pH 7.20) at 4.00 ˚C for 24 hr. The 2.00% osmium tetroxide in H_2O (TAAB Laboratories Equipment Ltd., Aldermaston, UK) was used to re-fix them. Next, the samples were dehydrated in the serial dilutions of ethanol (50.00, 70.00, 90.00, and 100%) and were sectioned by a razor blade in liquid nitrogen and then, they were covered with gold and were analyzed utilizing the SEM (LEO 1450VP, Carl Zeiss Microscopy GmbH, Jena, Germany).[18] The magnification of 1000× at accelerating voltage of 20.00 kV was employed.

Statistical analysis. The Shapiro-Wilk test was utilized to check the normal distribution of the variables prior to analysis. The data were analyzed using the repeated measures analysis of variance (ANOVA) test in the SPSS software (version 25.00; IBM Corp., Armonk, USA). Different treatments were analyzed at 95.00% confidence level ($p < 0.05$) through ANOVA test and Duncan's numerous range tests. All the designed tests were conducted in triplicate and all the experiments were performed twice. Results were represented as mean ± standard deviation values. The Excel software (version 15.0; Microsoft Corporation, Redmond, USA) was used to depict the plots.

Results

pH of the beefsteaks subjected to marinating. Table 1 shows the mean values of pH for various treatments during tenderization. The effect of marinade variant, and tenderization period as well as their interaction on the pH values of all treated samples were significant ($p < 0.05$). During the storage, the pH value was lower than 6.00 for all the treatments except for variant A in 48 hr. The sample treated with the broccoli juice had the most elevated mean value during storage whereas the lowest pH mean value was observed in the sample containing the balsamic vinegar (Table 1; $p < 0.05$). A significant pH reduction was observed at 24 hr compared to 3 hr and 48 hr of ageing meaning that the pH was declined up to 24 hr, then it was increased at 48 hr in all treatments ($p < 0.05$). On 2nd day, the minimum pH was observed in the treatment containing the balsamic vinegar (pH 4.83) while the sample treated with the broccoli juice had a maximum pH (pH 6.32). Considering the pH, an important incremental pattern was observed in A and C samples which showed the most and least mean value changes within 24 - 48 hr, respectively ($p < 0.05$).

MPS of the beefsteaks subjected to marinating. Table 1 exhibits the solubility of myofibrillar proteins in different treatments. The dissolvability of myofibril proteins was continuously increased from 1 to 48 hr in all the treatments ($p < 0.05$). As indicated in Table 1, treatment C showed the highest dissolvability of myofibrillar proteins, A and B samples showed the intermediate dissolvability, while the control sample showed the lowest one ($p < 0.05$). Since the proteolytic activity of the broccoli enzymes was higher at low pH, more degradation of salt-soluble proteins and subsequently higher solubility of the proteins was obtained in the C sample containing broccoli juice+balsamic vinegar.[9]

The textural properties of the beefsteaks subjected to marinating. Table 1 presents the mean WBSF values of different treatments during the tenderization period. WBSF was effectively influenced by the marinade variant and storage time ($p < 0.05$). As shown in Table 1, the mean WBSF value followed a decremental trend during ageing period and all the treatments showed a lower WBSF mean value compared to the untreated sample ($p < 0.05$). Therefore, minimum WBSF (74.88 N) was related to treatment C ($p < 0.05$), whereas, no significant difference was observed in the shear force between two treatments of A (81.37 N) and C ($p > 0.05$, 48 hr). Furthermore, as the marinating time progressed, a noticeable difference was observed in the WBSF between different storage times (1, 3, 24, and 48 hr) in A, B, and C variants ($p < 0.05$) except for the control group ($p > 0.05$, 24 - 48 hr). Since these exogenous proteases mainly influence the myofibrillar proteins and connective tissues in acidic condition,[19] the lowest WBSF was observed in the C sample containing the

Table 1. Effect of marinating with broccoli juice and balsamic vinegar on physicochemical and textural properties of beefsteak cuts during the ageing period at 4.00 ˚C.

Variable	Treatment	Tenderization period (hr)			
		1	3	24	48
pH	Control	5.76 ± 0.07^{Ab}	5.67 ± 0.07^{Abc}	5.59 ± 0.03^{Ac}	5.88 ± 0.08^{Ba}
	A	5.74 ± 0.04^{Ab}	5.70 ± 0.02^{Ab}	5.58 ± 0.07^{Ac}	6.32 ± 0.07^{Aa}
	B	4.52 ± 0.05^{Cb}	4.50 ± 0.09^{Cb}	4.47 ± 0.10^{Cb}	4.83 ± 0.08^{Da}
	C	4.80 ± 0.05^{Bc}	4.72 ± 0.04^{Bbc}	4.64 ± 0.05^{Bb}	5.06 ± 0.08^{Ca}
MPS (mL g^{-1})	Control	83.50 ± 2.39^{Cd}	89.31 ± 1.19^{Bc}	105.89 ± 1.10^{Db}	112.53 ± 3.19^{Da}
	A	84.28 ± 4.27^{Bd}	90.57 ± 5.21^{Bc}	117.33 ± 1.34^{Bb}	123.58 ± 2.24^{Ba}
	B	83.83 ± 2.14^{Cd}	89.95 ± 3.17^{Bc}	112.45 ± 1.44^{Cb}	119.60 ± 3.10^{Ca}
	C	86.44 ± 4.32^{Ad}	98.56 ± 4.38^{Ac}	121.32 ± 3.49^{Ab}	134.30 ± 6.32^{Aa}
WBSF (N)	Control	215.22 ± 7.80^{Aa}	203.51 ± 6.34^{Ab}	168.23 ± 6.72^{Ac}	164.62 ± 5.96^{Ac}
	A	211.18 ± 9.89^{Aa}	185.27 ± 5.03^{Cb}	127.46 ± 7.60^{Cc}	81.37 ± 7.04^{Cd}
	B	212.90 ± 7.00^{Aa}	191.60 ± 0.97^{Bb}	145.93 ± 5.36^{Bc}	119.83 ± 3.36^{Bd}
	C	192.80 ± 3.07^{Aa}	172.58 ± 4.81^{Db}	111.77 ± 12.93^{Dc}	74.88 ± 4.52^{Cd}

MPS: Myofibrillar protein solubility, and WBSF: Warner-Bratzler shear force.

balsamic vinegar. Accordingly, less force was required to reach the shear point in this treatment.[20,21]

The MFI of the beefsteaks subjected to marinating. Figure 1 shows the mean MFI values for different beefsteak treatments during the tenderization period. The interaction effect of the tenderization period and marinade variant on the estimation of short myofibrillar segments was noticeable (p < 0.05). As depicted in Figure 1, the MFI mean estimations of all the treatments had a relatively upward pattern as the ageing time advanced, whereas, during 24 - 48 hr, the proteolysis rate of the myofibrils was noticeably greater in the treatment C compared to the others. On the second day of storage, the highest MFI was observed in sample C, while the intermediate value was determined in the A and B samples. Nonetheless, the untreated sample showed the least MFI value compared to the others throughout ageing period (p < 0.05, 1 - 48 hr).

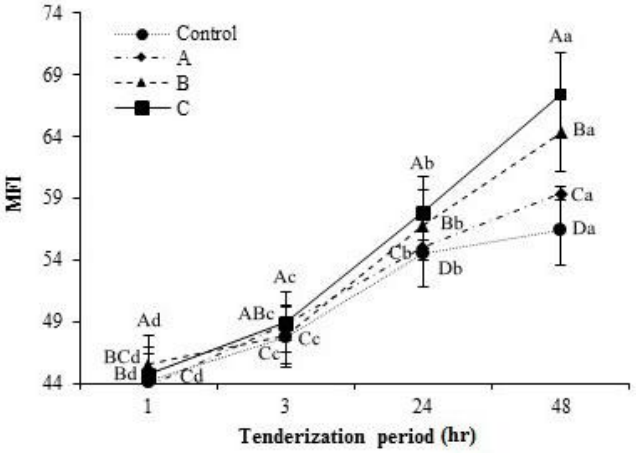

Fig. 1. The MFI of beefsteak cuts marinated with broccoli juice and balsamic vinegar during the ageing period. A-D different letters within marinade variant and a-d different letters within the tenderization period indicate statistically significant differences at 95.00% confidence level (p < 0.05). Treatments: Control: Without marinade, A: 3.00 U g^{-1} broccoli juice, B: 10.00% balsamic vinegar, C: 3.00 U g^{-1} broccoli juice+10.00% balsamic vinegar.

Electrophoretic analysis of the myofibrillar proteins isolated from the marinated beefsteak. Figure 2 shows the SDS-PAGE image acquired from the various treatments at 48 hr of ageing period. The pattern of the myofibrillar protein bands was remarkably influenced by the marinade variant (p < 0.05). A significant difference was observed in the normal seven diverse proteins between the A, B, C, and untreated samples as depicted in Figure 2.

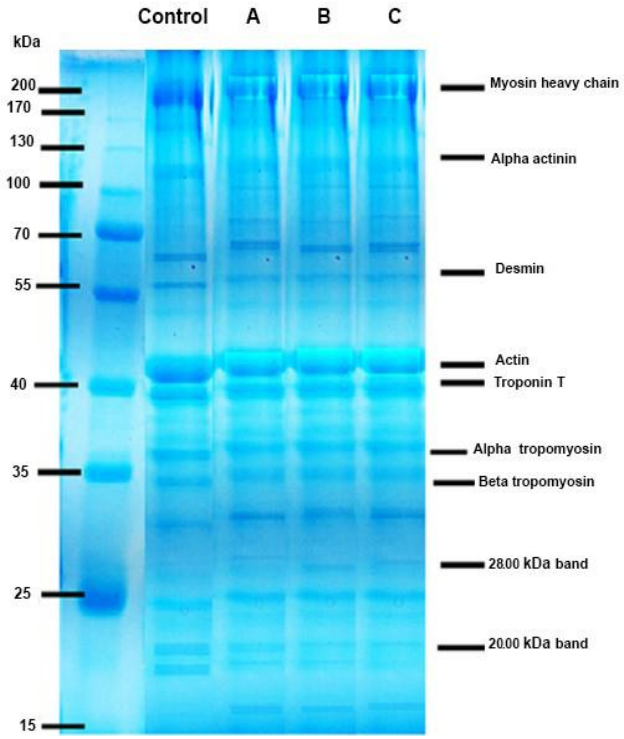

Fig. 2. Myofibrillar proteins pattern of beefsteak cuts marinated with broccoli juice and balsamic vinegar on the second day of storage at 4.00 ˚C. Treatments: Control: Without marinade, A: 3.00 U g^{-1} broccoli juice, B: 10.00% balsamic vinegar, C: 3.00 U g^{-1} broccoli juice + 10.00% balsamic vinegar

The main distinguished protein bands in the samples were probably the heavy chain of myosin (220 kDa), alpha-actinin (105 kDa), desmin (50.00 kDa), actin (45.00 kDa), troponin-T (40.00 kDa), alpha- and beta-tropomyosin (33.00 - 35.00 kDa).[22] Thus, on the second day of storage the intensity and number of high-density protein bands was decreased in all the treatments specifically in A and C samples. Meanwhile, elevated low-density protein bands were observed in all the treatments compared to the control group. The actin and myosin proteins mostly involved in the muscle contraction as well as actin-binding protein i.e., alpha-actinin underwent decomposition in all the treated samples particularly in the C treatment. The 50.00 kDa band, surrounding the Z disks, belonging to the intermediate filament desmin, was obviously degraded in the A and C treatments compared to the control group. Troponin-T is one of the most significant markers of the proteolysis involving in meat tenderization during ageing. It has been shown that the appearance of the small 28 and 30.00 kDa peptides demonstrates the breakdown of this protein.[16,23] As demonstrated in Figure 2, troponin-T, alpha- and beta-tropomyosin corruption as well as production of low-density 20.00 and 28.00 kDa bands at the bottom of the gel in the marinated samples occurred quicker than the control sample which was increasingly recognizable in the A and C treatments.

Muscle microstructure of the marinated beefsteak. Figure 3 presents the morphological structure of the A, B, C and control samples after 48 hr of ageing period. The control group had tight and non-spaced muscle filaments, while, the treated samples indicated expanded myofibrillar space with a lot of exudates. Furthermore, enormous gaps were observed between the muscle filaments compared to the untreated sample.

Discussion

The glycolysis process and the production of lactic acid as well as the influence of the marinade solution on the structure of proteins had a remarkable impact on pH. Furthermore, it is supposed that lower pH could also be caused by the increased microbiological activity enhanced by the storage of beefsteak cuts in the polyethylene sacks during ageing. On the other hand, this phenomenon could be due to the natural pH of marinade substances and their concentration.[1,4] On the second day of storage, the broccoli juice increased the pH of the samples probably due to the degradation of muscle structure and releasing free amino acids and non-protein nitrogen substances which can be caused by the proteolytic enzymes present in the broccoli juice i.e., metalloprotease, cysteine, serine, and aspartic proteases through collaboration of which, pH increment would take place over time.[16] Besides, balsamic vinegar

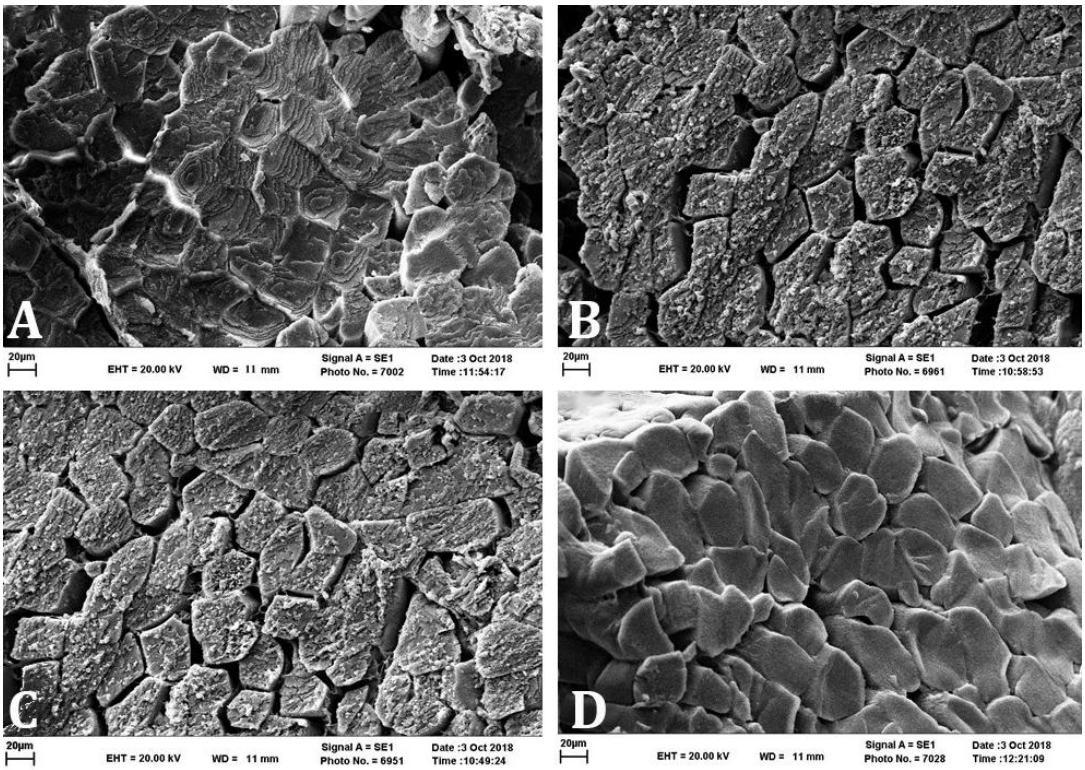

Fig. 3. Scanning electron micrographs of the transversal section of raw beefsteak cuts marinated with broccoli juice and balsamic vinegar after 48 hr storage at 4.00 ˚C (× 1,000). **A)** Control (without marinade), **B)** 3.00 U g^{-1} broccoli juice, **C)** 10.00% balsamic vinegar, and **D)** 3.00 U g^{-1} broccoli juice + 10.00% balsamic vinegar.

constituents i.e., acetic and benzoic acids could cause a decrease in the pH of the samples including B marinade. In the present research, the effect of marinating on the pH was similar to the results reported in the studies by Kim *et al.*[4] and Serdaroğlu *et al*.[24]

Protein solubility has been utilized as a factor to evaluate the alteration of meat texture during ageing.[25] Changes in the salt protein solubility take place under a variety of extracting conditions such as proteiṅ conformation degree, i.e., denaturation, pH level as well as the permeability of myofibrils influenced by the broccoli juice comprising of mixtures of the proteases.[26] The permeability of myofibrils was increased in the treated samples leading to easily breakdown of the proteins which affirms myofibrils dissolvability. The elevated MPS of all the treatments throughout ageing could be related to the decomposition and creation of the myofibrillar proteins of low-density peptides during the tenderization period as a result of the proteolytic enzymatic activity of the broccoli juice and pH reduction applied by the balsamic vinegar.[27,28] Such MPS increased the improved tenderness. These findings were consistent with the results acquired by Naveena *et al*.[19] in buffalo meat, Maqsood *et al*.[26] in camel meat and Rawdkuen *et al*.[28] in beef meat.

Acid marinating may destroy the hydrogen bonds of collagen fibers. Consequently, the connective tissues become swollen, ultimately leading to the increased solubility of the connective tissue and a decrease in the shear force.[29] These changes could be attributed to the activity of the proteolytic enzymes of the broccoli which were mostly the cysteine proteases. In addition, the synergistic effect of the broccoli juice proteases and meat endogenous proteases in reducing the WBSF could be mentioned which was well expressed in the marinade A and C variants. The WBSF results were well matched with the MFI estimations in the present research. Texture assessment is frequently utilized to describe the tenderness of the cooked meat. Many parameters influence the properties of the cooked meat such as breed, animal age, fat content, natural enzymes, pH, proteins' contraction state, cooking temperature and the behavior of proteins during the heating procedure. Hence, in the current research, the usefulness of evaluating the meat texture was investigated in predicting the tenderness of the cooked meat. Similar findings have been reported in the samples treated with cucumis protease and ginger powder,[29] bromelain and papain enzymes,[30] ficin protease[26] as well as organic acid solution.[3]

The MFI shows the quantity of myofibril pieces in the sarcoplasm of muscle cells. It would be the best strategy to follow the proteolysis intensity during ageing.[4] Generally, the action of endogenous proteases of meat as well as the broccoli juice proteases is better in acidic pH, thereby the elevation of MFI value in treatment C can be justified due to their cooperation in this condition. Also, the broccoli proteases (as previously mentioned) and endogenous meat proteases i.e., calpains, lysosomal cathepsins, proteasome, and caspases are activated in acidic conditions.[16] Therefore, synergistic effects of exogenous/ endogenous proteases and low pH conditions caused further degradation of the myofibrillar proteins[12] which was well specified in the MFI values in the A and C treatments. MFI was found to identify the pattern of myofibrillar fragmentation, cytoskeletal proteins and band-I breakdown during storage, which was strongly correlated with the results of the textural evaluation.[4] As expected, the greater MFI caused more tenderness of the meat. In this study, increased levels of salt soluble proteins and lower values of WBSF in treatments A and C were well expressed in the MFI values over time. Similar reports have also been represented after marinating with natural enzymes and low-pH marinades.[16,4]

The changes in SDS-PAGE of the specimens can be attributed to the action of different proteases of the broccoli juice specifically cysteine proteases and meat endogenous proteases i.e., lysosomal D- and L-cathepsins activated at low pH indicating superior breakdown of the myofibrillar proteins. The rigor bonds produced from the actin-myosin interaction might be modified by the deterioration of heavy chains of myosin. These changes could be associated with further tenderness.[16] Moreover, the desmin decomposition as well as the elevation of MFI value in the marinated beefsteak samples throughout ageing period indicated the more intense proteolysis of the samples as revealed by the SDS-PAGE images. Maqsood *et al*. [26] and Mazaheri Kalahrodi *et al*.[12] declared that the bands between 15.00 and 25.00 kDa generated from the degradation of high-density proteins in all the samples treated with the plant-derived proteases were associated with the tenderized meat. Possibly, the increased low-density protein molecules are associated with the increased solubility of the myofibrillar proteins and elevated fragmented myofibrils.[31]

Increased collagen solubility, swelling and deterioration of the connective tissues such as perimysium and endomysium would occur in acidic conditions causing the development of distinct holes between the filaments which are progressively perceptible during the extended storage.[4] Enzymatic hydrolysis of the beefsteak proteins by the metalloprotease, cysteine, serine and aspartic proteases present in the broccoli juice was proved through the increase in the solubilization of collagen which may lead to the muscle disruption as well as shear force and reduction of insoluble collagen.[29,31] Similar results have also been reported by Kim *et al*. and Naveena *et al*.[4,29] The results of this research indicated that the broccoli juice contained efficient proteases that were better activated under acidic conditions. Hence, the broccoli juice as well as its combination with the balsamic vinegar could be utilized as a powerful marinade to improve the tenderness of the

beefsteak. Improvement of the MFI and MPS as well as the reduction of pH value, and WBSF were found as the beneficial outcomes of meat marinating with the aforementioned treatments. The electrophoretic analysis and SEM images showed the extensive degradation of troponin-T, destruction of endomysium collagen and sarcolemma areas of muscle filaments in the above-mentioned treatments, respectively.

The results of this research indicated that the broccoli juice contained efficient proteases that were better activated under acidic conditions. Thus, it could be concluded that by optimizing the formulation of the broccoli juice and broccoli juice + balsamic vinegar marinades they could be used as natural meat additives to improve the physicochemical and structural attributes of the beefsteak.

Conflict of interest

The authors declare that they have no conflict of interest.

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