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ARTICLE

Antioxidative Changes of Blueberry Leaf Extracts in Emulsion-Type Sausage during *In Vitro* Digestion

Sun-Jin Hur, Doo-Hwan Kim, Se-Chul Chun and Si-Kyung Lee*
Department of Bioresources and Food Science, Konkuk University, Seoul 143-701, Korea

Abstract

This study was conducted to investigate the effects of *in vitro* human digestion on the antioxidant activity of blueberry leaf extracts (BLE) in emulsion-type sausages (ETS). Leaves from four cultivars of blueberries (Bluecrop, Bluegold, Duke, and Northland) collected from a wild blueberry farm were extracted with 80% ethanol. ETS were prepared with 0.2% BLE. The samples were then passed through an *in vitro* human digestion system which simulates the composition of the mouth, stomach, and small intestine juice. Only one phenolic compound (chlorogenic acid) was detected in the BLE. Northland BLE had appreciably higher amounts of chlorogenic acid than that of other BLE, both before and after *in vitro* human digestion. Antioxidant activity of any BLE was not influenced by *in vitro* human digestion, whereas the antioxidant activity of chlorogenic acid standard increased in response to *in vitro* human digestion in both 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric-reducing ability of plasma (FRAP). In the present study, the antioxidant activities of the BLE were not strongly influenced by *in vitro* human digestion, and the antioxidant activity depended on the chlorogenic acid content of ETS. Thus, compounds from blueberry leaves may have important applications in the future as natural antioxidants for meat products.

Key words: antioxidant activity, blueberry leaf, emulsion-type sausage, *in vitro* human digestion

Introduction

Emulsion-type sausage (ETS) is usually made from ground meat with high fat content (20-50%), which renders it prone to lipid oxidation during storage. Thus, numerous studies have been conducted to develop natural antioxidants such as flavonoids, phenolic compounds, or vitamins for meat products. In general, fruits and vegetables contain many different phyto-nutrients, many of which have antioxidant properties (Ehlenfeldt and Prior, 2001). In particular, among the berry fruits, blueberries (*Vaccinium corymbosum* L.) are considered to be a good source of phenolic compounds and are well known for their high antioxidant activity scores (Prior *et al*., 1998). Chlorogenic acids are family of esters formed between certain *trans*-cinnamic acids and quinic acid which is the major phenolic compounds found in numerous plant species. Chlorogenic acids have the phenolic groups enabling them to act as the natural antioxidant.

*Corresponding author: Si-Kyung Lee, Department of Bioresources and Food Science, Konkuk Univeristy, Seoul 143-701, Korea. Tel: 82-2-450-3759, Fax: 82-2-450-3726, E-mail: lesikyung@konkuk.ac.kr

Significant differences in the phenolic content and antioxidant activities occur between individual blueberry species, as well as between varieties (Prior *et al*., 1998), and the changes in phenolic composition and content are influenced by postharvest storage, handling, and processing (Prior *et al*., 1998). Therefore, individual species of blueberry may harbor different phytochemical contents and display different antioxidant effects.

Furthermore, the absorption of certain micronutrients by the human body may be influenced by the concomitant ingestion of other substances. The bioavailability of polyphenols to peripheral tissues can be diminished by high intestinal and biliary secretion of their conjugates (Bermúdez-Soto *et al.*, 2007; Silberberg *et al*., 2006). Previous studies (Bermúdez-Soto *et al*., 2007) have shown that dietary polyphenols are highly sensitive to the mild alkaline conditions in the small intestine and these previous findings suggest that, during duodenal digestion, a proportion of these compounds may be transformed into different structural forms with different chemical properties. Although several studies have been conducted to develop new ingredients as antioxidants for meat processing, the changes in antioxidant activities upon ingestion have not been extensively determined in ETS. Thus, the

present study aims to determine the effect of *in vitro* human digestion on the antioxidant activity of blueberry leaf extracts in ETS.

Materials and Methods

Sausage formulation and processing

Lean pork and back fat were purchased from a local meat processing plant. Excess fat was trimmed from the meat and the lean muscle was diced into approximately 2 cubes (8×4×2 cm) and ground through a 7-mm diameter orifice using a mincer. Ground meat was cured with phosphate and NaCl using a meat mixer for 24 h at 4°C. Cured meat was placed in a bowel cutter along with sausage seasoning (salt 73.5%, dextrose 11.4%, sage powder 7.3%, monosodium glutamate 1.4%, red pepper 3%, nutmeg 3% and nitrite 0.4%) water and different kind of 0.2% BLE stipulated in the experiment design. Chopping was continued until the batter temperature reached to 10°C. The emulsified meat batters were stuffed into PVDC casings (30 mm diameter) and placed in a cooking chamber pre-programmed to operate at 65°C for 30 min. Next, the temperature was increased to 75°C and maintained for 30 min. Finally, the temperature was increased further to 80°C and this was maintained for 20 min. After cooling in ice water for 20 min, the ETS were stored at 5°C until further use.

In vitro human digestion

The *in vitro* human digestion model used was a modified version of a previously described one (Hur *et al*., 2009; Versantvoort *et al*., 2005). The model contained the following components:

I. *Pre-ingestion*: The ETS containing BLE.

II. *Mouth*: About 3 g of sample was mixed with 6 mL of simulated saliva fluid (pH 6.8) and then stirred for 5 min at 37°C.

III. *Stomach*: Approximately 12 mL of simulated gastric fluid (pH 2) was added, and then the mixture was stirred for 2 h at 37°C.

IV. *Small Intestine*: Approximately 12 mL of duodenal juice, 6 mL of bile juice, and 2 mL of HCO_3 solution (pH 6.5-7) were added, and the mixture was stirred for 2 h at 37°C.

The compositions of the simulated saliva, gastric, duodenal, and bile fluids are presented in Table 1. During the digestion, the samples were swirled (60 rpm) on a shaking water bath (Model HB-205SW, Hanbaek Scientific Co., Korea) to simulate the motility of the gastrointestinal tract.

Plant materials

Leaves from four blueberry cultivars were collected from a wild blueberry farm. Bluecrop blueberry has a firm light blue medium-sized fruit and bluecrop is generally considered the best all around variety for adaptability, long production period, good fruit yield and disease resistance (Anonym, 2013). Bluegold is a mid-season variety that is highly productive, with superior quality berries for midseason (Anonym, 2013). Bluegold has medium-

Table 1. Constituents and concentrations of the various synthetic juices of the *in vitro* human digestion model representing fed conditions

	Saliva	Gastric juice	Duodenal juice	Bile juice
Inorganic components	10 ml KCl 89.6 g/L 10 ml KSCN 20 g/L 10 ml NaH_2PO_4 88.8 g/L 10 ml $NaSO_4$ 57 g/L 1.7 ml NaCl 175.3 g/L 20 ml $NaHCO_3$ 84.7 g/L	15.7 ml NaCl 175.3 g/L 3.0 ml NaH_2PO_4 88.8 g/L 9.2 ml KCl 89.6 g/L 18 ml $CaCl_2{\cdot}2H_2O$ 22.2 g/L 10 ml NH_4Cl 30.6 g/L 6.5 ml HCl 37%g/g	40 ml NaCl 175.3 g/L 40 ml $NaHCO_3$ 84.7 g/L 10 ml KH_2PO_4 8 g/L 6.3 ml KCl 89.6 g/L 10 ml $MgCl_2$ 5 g/L 180 µl HCl 37%g/g	30 ml NaCl 175.3 g/L 68.3 ml $NaHCO_3$ 84.7 g/L 4.2 ml KCl 89.6 g/L 150 µl HCl 37%g/g
Organic components	8 ml urea 25 g/L	10 ml glucose 65 g/L 10 ml glucuronic acid 2 g/L 3.4 ml urea 25 g/L 10 ml glucosamine hydrochloride 33 g/L	4 ml urea 25 g/L	10 ml urea 25 g/L
Add to mixture of organic+inorganic components	290 mg α-amylase 15 mg uric acid 25 mg mucin	1 g BSA 2.5 g pepsin 3 g mucin	9 ml $CaCl_2{\cdot}2H_2O$ 22.2 g/L 1 g BSA 9 g pancreatin 1.5 g lipase	10 ml $CaCl_2{\cdot}2H_2O$ 22.2 g/L 1.8 g BSA 30 g bile
pH	6.8±0.2	1.30±0.02	8.1±0.2	8.2±0.2

The inorganic and organic components are augmented to 500 mL with distilled water.
If necessary, the pH of the juices would be adjusted to the appropriate value.

sized fruit with small, and dry blossom scars. It has good flavor and firmness (Weber, 2012). Duke blueberry is considered the best early season cultivar available. The fruit size and quality is very good but the flavor can be bland if picked late (Weber, 2012). Northland blueberry is an extremely productive half-high type with medium sized, dark and soft fruit (Weber, 2012). The leaves were separated from other debris by hand, dried for 10 d at room temperature, and then stored in sealed polyethylene bags at –20°C.

Preparation of blueberry leaf extracts

Each phenolic extract of freeze-dried blueberry leaves (particle size, 60 mesh) was obtained as follows. Powdered blueberry leaves (50 g) were suspended for 2 h in and extracted with 500 mL of 80% ethanol at 70°C. The extracts were then filtered through Whatman No. 2 filter paper (Whatman International Limited, England), and the filtrate was evaporated to dryness. Subsequently, the filtrate was frozen and lyophilized. The extracts were then placed in a glass bottle and stored at –20°C until use.

Determination of total phenolics

The total phenolics were determined by spectrophotometry (Jeong *et al*., 2010). The standard curve for total phenolics was obtained from gallic acid standard solution (0-100 mg/L). The total phenolics in extract from the four cultivars of blueberry leaves were expressed as milligrams of gallic acid equivalents (GAE) per gram of sample.

Quantification of chlorogenic acid using HPLC

The content of chlorogenic acid in the phenolic extracts obtained from the leaves of each of the four cultivars was determined at 280 nm using a diode array UV-visible detector (Agilent 1100 series, Agilent Co., USA) by using chlorogenic acid standard solution. The components of the extracts were separated on a Shiseido C18 column (250 mm × 4.6 mm id, 5 μm, Shiseido Co., Japan). The elution solvents were (A) 0.01 M potassium phosphate buffer adjusted to pH 3.0 by phosphoric acid and (B) methanol. The solvent gradient elution program used was as follows: initial 90% (A), hold for 9.5 min; linear gradient to 68% (A) in 3.5 min; linear gradient to 67% (A) 17 min; linear gradient to 20% (A) in 1 min; linear gradient to 90% (A) in 1 min, hold for 10 min. The flow rate was constant at 1.5 mL/min. Chlorogenic acid was identified by comparing retention times and ultraviolet spectra with those of known standards and was quantified using the relevant peak areas in the chromatograms. All analyses were run in triplicates and mean values were calculated. Content of chlorogenic acid was expressed in mg/g extract.

ABTS radical-scavenging activity

2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was dissolved in water to attain a concentration of 7 mM/L ABTS reagent stock was produced by reacting the ABTS stock solution with 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The working ABTS reagent solution was prepared by diluting the ABTS reagent stock with phosphate-buffered saline (5 mmol/L, pH 7.4) adjusted to an absorbance of 0.70 at 734 nm. For testing the samples, 1.0 mL of ABTS working reagent standard was added to 0.5 g of the sample and absorbance was determined at 5 min after the initial mixing (Jeong *et al*., 2010). The ABTS-scavenging activity was expressed as percent ABTS scavenging and was calculated from the following formula:

$$\%\text{ABTS scavenging activity} = [(\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance})] \times 100$$

Ferric-reducing ability of plasma (FRAP) assay

The FRAP assay was performed according to the protocol developed in a previous study (Jeong *et al*., 2010). Briefly, 1.5 mL of working FRAP reagent (10 vol of 300 mmol/l acetate buffer, pH 3.6 + 1 vol of 10 mmol/L 2,4, 6-tripyridyl-*S*-triazine in 40 mmol/L HCl + 1 vol of 20 mmol/L $FeCl_3$) that had been pre-warmed to 37°C was added to 50 μl of test samples and standards. This was mixed by vortexing and maintained at 37°C. Then, the absorbance at 593 nm was determined against a reagent blank at a predetermined time within a time window of 0-4 min after mixing of the sample and reagent.

Statistical analysis

Statistical analyses were done for 3 batches for emulsion-type sausage. Data for each batch of emulsion-type sausage collected for total phenolic content, chlorogenic acid content, ABTS and FRAP were analyzed using ANOVA with SAS software (SAS Inst. Inc., USA). Significant differences ($p<0.05$) between mean values of quintuplicate samples were determined for total phenolic content, chlorogenic acid content, ABTS and FRAP.

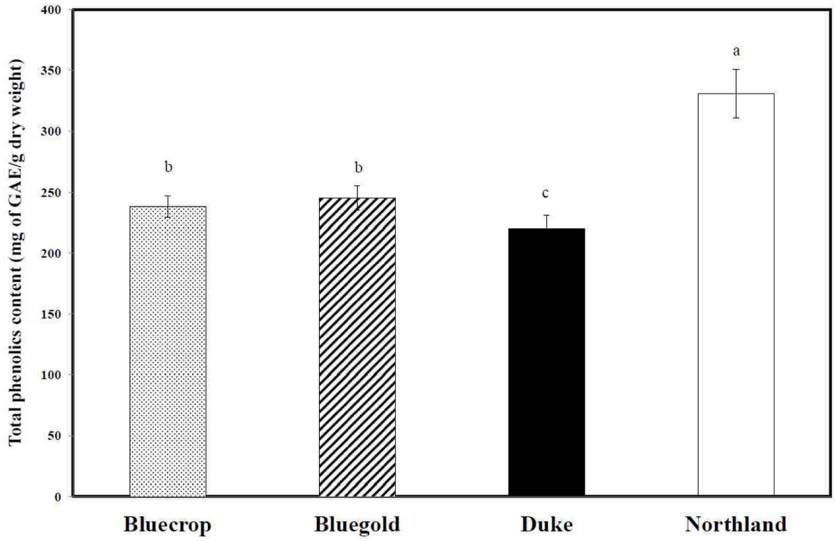

Fig. 1. Total phenolic content of extracts from the leaves of four cultivars of blueberry. Different letters indicate significant difference at $p<0.05$.

Results and Discussion

The total phenolic contents of BLE from the four cultivars (Fig. 1) were 268.50-331.17 mg GAE/g. The phenolic extract from the Northland cultivar showed the highest total phenolic content (331.17 mg GAE/g), whereas that from the Duke cultivar (268.50 mg GAE/g) contained the least total phenolic content.

The chlorogenic acid content of ETS containing BLE during *in vitro* human digestion is presented in Fig. 2. Specifically, the ETS containing Northland BLE had higher content of chlorogenic acid than the ETS containing BLE from other cultivars before and after *in vitro* human digestion. The fruit of blueberries is known to be a rich source of phenolic acids, catechins, flavonols, anthocyanins, and condensed tannins (Naczk *et al*., 2006). Blueberry extract is largely composed of anthocyanins, chlorogenic acid, epicatechin, and glycosides of ferulic acid and quercetin (Stevenson *et al*., 2007). However, in the present study, only one phenolic compound (chlorogenic acid) was detected in ETS containing BLE. This difference could be due to the difference in the sources of phenolic compounds, namely, the blueberry fruit and blueberry leaf (Sellappan *et al*., 2002; Kim *et al*., 2009).

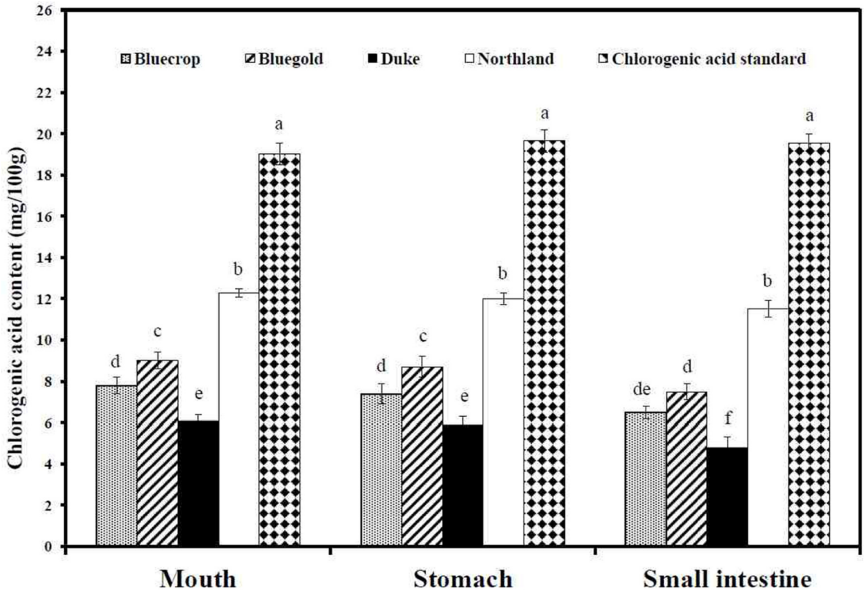

Fig. 2. Chlorogenic acid content of emulsion-type sausage containing four cultivars of blueberry leaf extracts as they pass through an *in vitro* human digestion model. Different letters indicate significant difference at $p<0.05$.

The phenolic contents in the BLE from the cultivars tested also differed because of the differences in the seasonal and geographic origins of the various cultivars, which are known to contribute to the characteristics of the phenolic compounds they contain (Sellappan *et al*., 2002). Following digestion, the amount of chlorogenic acid in all ETS decreased. This decrease was particularly pronounced when the samples moved from the simulated stomach to the small intestine (Fig. 2). Moreover, unknown compounds appeared in all ETS containing BLE after digestion in the small intestine (data not shown). Our results disagreed with those of a previous study (Bermúdez-Soto *et al*., 2007), indicating that most dietary polyphenols remain quite stable during gastric digestion. Conversely, dietary polyphenols display high sensitivity to the mild alkaline conditions in the small intestine, which may result in their transformation to different structural forms with altered chemical properties during duodenal digestion (Bermúdez-Soto *et al*., 2007). Another study (McDougall *et al*., 2005) also reported that the pH shift to > 7.5 during pancreatic and/or small intestinal digestion was the main factor responsible for the irreversible breakdown of the anthocyanins. In the present study, the content of chlorogenic acid in ETS containing BLE changed upon entry of the sample into the small intestine. This change may be attributed to the differing pH values between the stomach and small intestine during *in vitro* human digestion, as well as to chlorogenic acid-bile acid

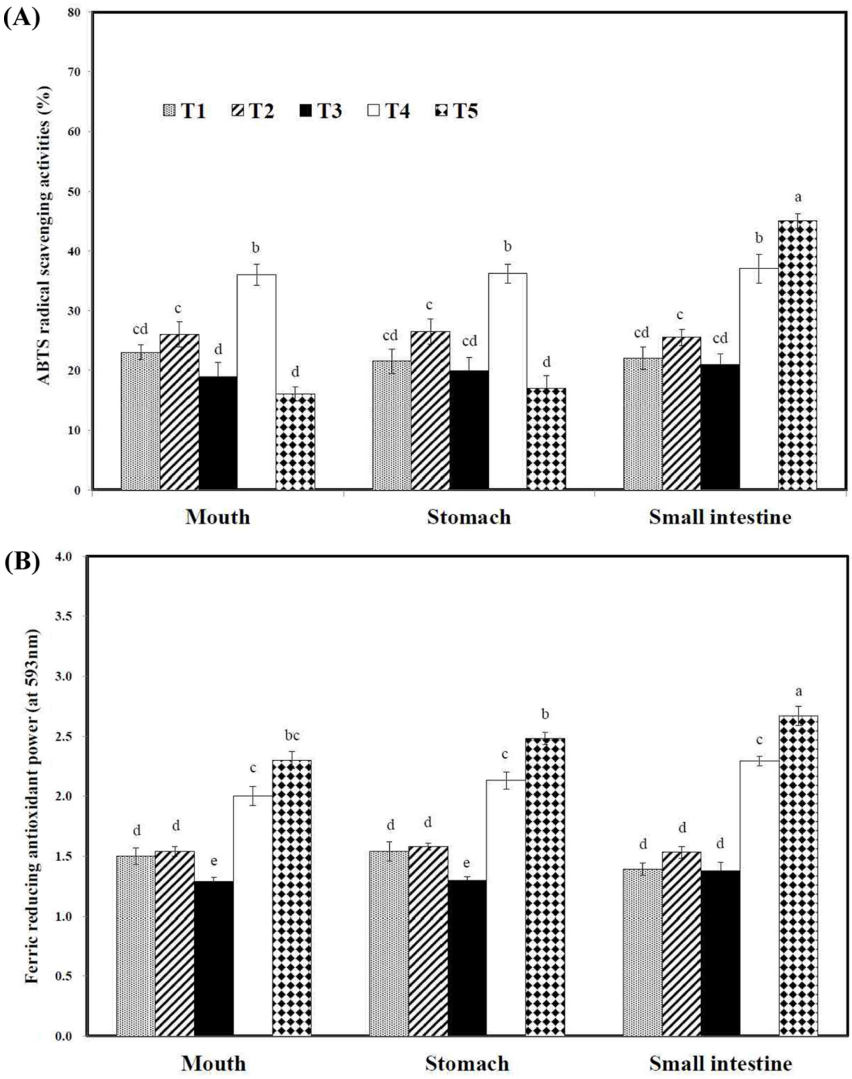

Fig. 3. ABTS (A) FRAP (B) of emulsion-type sausage containing four cultivars of blueberry leaf extracts and chlorogenic acid standard as they pass through an *in vitro* human digestion model. T1: sausage with 0.2% Bluecrop blueberry leaf extract, T2: sausage with 0.2% Bluegold blueberry leaf extract, T3: sausage with 0.2% Duke blueberry leaf extract, T4: sausage with 0.2% Northland blueberry leaf extract, T5: sausage with 0.2% chlorogenic acid standard. Different letters indicate significant difference at $p<0.05$.

interactions. Presumably, the change in the chlorogenic acid content in ETS during *in vitro* human digestion may also have influenced various bioactive properties. However, the nature of the alterations in the chemical structures, polymer chain lengths, and molecular weight of phenolics during *in vitro* human digestion are hitherto unknown, which necessitates further detailed studies.

The antioxidant activity in ETS containing chlorogenic acid standard (T5) was higher than that in ETS containing BLE from the different cultivars (T1-T4) during *in vitro* human digestion (Fig. 3). The antioxidant activity differed among the ETS containing BLE from the different cultivars, with ETS containing Northland BLE displaying the highest activity and ETS containing Duke BLE showing the least activity. In general, the high antioxidant activity of blueberry extracts may be related to the high levels of polyphenolic compounds (Molan *et al*., 2008). In the present study, we also found that during *in vitro* human digestion, the antioxidant activity of ETS containing blueberry extract depended on the amount of phenolic compound (chlorogenic acid).

Previous work (Marques and Farah, 2009) has shown that chlorogenic acids are antioxidant components produced by plants in response to environmental stress conditions such as infections by microbial pathogens, mechanical wounding, and excessive levels/intensities of UV or visible light. The results from the present study also showed that the antioxidant activity increased with increasing chlorogenic acid content in ETS containing BLE. These findings suggest that the antioxidant activity of all ETS containing BLE may be less influenced by *in vitro* human digestion and that the antioxidant activity of ETS depended on the chlorogenic acid content.

According to an earlier study (Su and Chien, 2007), other factors such as oxygen, enzymes, and temperature also influence phenolic compositions and antioxidant activities of blueberry products. The bioavailability of polyphenols to peripheral tissues may be lowered by high intestinal and biliary secretion of their conjugates (Bermúdez-Soto *et al*., 2007; Silberberg *et al*., 2006). In the present study, the chlorogenic acid content decreased slightly when the ETS containing BLE moved from the simulated stomach to the small intestine, which may be due to variation in the enzymes, pH, or oxygen levels, and this change in the chlorogenic acid content can influence the antioxidant activity in ETS containing BLE during *in vitro* human digestion. Zheng and Wang (2002) reported that chlorogenic acid was the major contributor to antioxidant activity owing to its high concentration in blueberry, and the antioxidant activity of chlorogenic acid is associated to some extent with the number of hydroxyl groups in their molecular structure (Zheng and Wang, 2002). Increased ability to donate a hydrogen atom from the hydroxyl group to a free radical is the factor responsible for the antioxidative activity of chlorogenic acid in the current investigation. Thus, compounds from blueberry leaves may have important applications in the future as natural antioxidants for meat products.

Conclusions

In the present study, simulated mouth, stomach, and small intestine digestion of ETS containing BLE was carried out to determine the stability of phytochemicals and antioxidant activities under *in vitro* human digestion conditions. A single phenolic compound (chlorogenic acid) was detected in the BLE. A change in the amount of chlorogenic acid occurred when the ETS containing BLE moved from the simulated stomach to the small intestine. Antioxidant activities were not influenced by *in vitro* human digestion in any of the tested ETS containing BLE, whereas the antioxidant activity of the chlorogenic acid standard increased in response to *in vitro* human digestion. Moreover, the results indicated that the antioxidant activity increased with increased chlorogenic acid content in the ETS containing BLE, suggesting that the antioxidant activities of all the BLE were not strongly influenced by *in vitro* human digestion and that the antioxidant activity depended on the chlorogenic acid content of ETS.

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