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ARTICLE

Effect of Calamansi Pulp Ethanol Extracts on the Meat Quality and Biogenic Amine Formation of Pork Patty during Refrigerated Storage

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Abstract This study evaluated the antibacterial and antioxidant activities of ethanol extract of calamansi pulp (CPE) and its effect on quality and biogenic amine (BAs) formation in pork patties during storage. The CPE were prepared in various conditions (ethanol concentrations of 50%, 70%, and 90% with extraction periods of 3 and 6 days). The extract with potent antibacterial and antioxidant activities (90%, 6 days) was selected for addition to pork patties. Three groups were tested: Control (without extract addition), CPE addition at 0.2% w/w (0.2PCPE), and 0.4% w/w (0.4PCPE). The addition of CPE inhibited the formation of BAs, mainly cadaverine, histamine, and tyramine, in pork patties during storage. The pH and bacterial count of pork patties decreased significantly in a concentration-dependent manner following the addition of CPE. The instrumental color (CIE L*, CIE a*, and CIE b*) tended to be higher in 0.4PCPE than in the control during storage. The thiobarbituric acid reactive substances and volatile basic nitrogen (VBN) values of pork patties were affected by CPE, showing a reduction toward lipid oxidation at any storage period, and maintaining the lowest VBN value in 0.4PCPE at the final storage day. Similarly, the reduction of total BAs in pork patties was observed ranged between 3.4%–38.1% under treatment with 0.2% CPE, whereas 18.4%–51.4% under 0.4% CPE addition, suggesting significant effect of CPE to improve meat quality. These novel findings demonstrate the efficacy of 0.4% CPE as a natural compound to preserve the quality and reduce BAs formation in pork patties during storage.

Keywords antioxidant activities, calamansi pulp, biogenic amine, pork patty, meat quality

Introduction

Several studies have been conducted to address and understand the mechanisms underlying the formation of toxic and hazardous compounds derived from foods (Lee et al., 2020). In red and processed meat categorized as group 2A status of 'probably carcinogenic' and group 1 status of 'carcinogenic' (WHO and IARC, 2006), the complex interaction of potentially carcinogenic substances that might be formed during storage and processing is enormously dictating the safety aspect for consumption. One of which is formed during storage is the biogenic amines (BAs). It is a low molecular biogenic substance equipped with mono- or poly- amine groups. Although serving essential function at low concentrations of BAs serve as neurotransmitters in the brain signaling system of mammals (Burchett and Hicks, 2006), exaggerated ingestion of BAs has been reported to cause health problems such as migraine, digestive disorders, hypotension, and food intoxication (Bodmer et al., 1999; Drabik-Markiewicz et al., 2011). Furthermore, the interaction between nitrites with paticular BAs, putrescine (PUT), and cadaverine (CAD) produces the highly carcinogenic substance N-nitrosamine (Drabik-Markiewicz et al., 2011; Eerola et al., 1997).

The rapid formation of BAs is mostly observed in foods with high protein content, such as poultry and red meat (Vinci and Antonelli, 2002). Its formation results mainly from enzymatic decarboxylation of amino acids by microbiomes (Halász et al., 1994). Histamine (HIM), serotonin, and phenethylamines are decarboxylation products of histidine, tyramine (TYM), and phenylalanine, respectively. In addition, CAD, PUT, TYM, spermine, and spermidine (SPD) polyamines are generated as a result of the decarboxylation of lysine, ornithine, PUT, and SPM, respectively (Bodmer et al., 1999; Halász et al., 1994; Min et al., 2007). Min et al. (2007) inferred that the production of individual BAs during storage is strongly correlated with the concentration of volatile basic nitrogen (VBN) in various types of meat. Furthermore, the distinct form of the individual BAs generated from diverse types of meat implied the potent role of the existing bacterial microflora in utilizing available sources in determining the proportion of BAs. Enterobacteriaceae and *Pseudomonas* spp. of gram-negative bacteria, *Lactobacillus* of gram-positive bacteria, and aerobic bacteria were reported to be capable of producing PUT, CAD, TYM, and PUT, respectively (Halász et al., 1994; Triki et al., 2018). Therefore, in addition to its efficacy as an indicator of bacterial contamination, the quantification of BAs in meat and meat products is important to measure their hazardous level upon consumption.

To date, studies involving natural extracts have been widely conducted to control the excessive formation of BAs in meat, in which antimicrobial compounds, mainly polyphenols, are thought to be the major contributors to the growth of BA-producing bacteria (Lee et al., 2020; Wang et al., 2015). The lowering effect on BAs formation has been reported in luncheon rolls containing green tea extract and thyme oil (Abu-Salem et al., 2011), pork belly marinated with black currant juice (Cho et al., 2021), lamb patties with ginger, ginseng, jatropha, and jojoba (Ibrahim et al., 2011) and dry fermented sausages prepared with *Thymbra spicata* oil (Bozkurt, 2007). The underlying mechanism was explained by the possibility of small fractures of polyphenol to infiltrate into the microbial cell, thus impairing the homeostatic state of the cell through interference of nutrient uptake, electron transport, and nucleic and amino acid biosynthesis (Cueva et al., 2010; Kim et al., 2020).

Based on the the aforementioned elaborations, efforts to find potential natural extracts with robust antioxidative and antimicrobial properties that strongly limit the formation of BAs in meat products, such as pork patties, are necessary. One of these is calamansi (*Citrus microcarpa*). It is an exotic fruit from the family of *rutaceae* that widely cultivated in Southeast Asia, China, Taiwan, and some parts of the USA, with a high content of phenolic acids, mainly coumaric, sinapic, and caffeic acid (Cheong et al., 2012). The calamansi are widely utilized in native foods as seasoning to provide sweet, acidic, and peel-like aroma of orange. Besides, the iron absorbing properties owned by calamansi is harnessed to extend the storage period of

various foods (Cheong et al., 2012). Previous studies concluded that the presence of ferulic, p-coumaric, and sinapic acid synergically contributed for the inhibition of the bacterial growth and maintenance of the desirable physical quality properties in chili bird paste (Hussain et al., 2021). In addition, study by Husni et al. (2021) revealed that the bioactive compounds and essential oils from calamansi strongly inhibited *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus mutans* bacteria. Further, Wang and Tang (2018) reported the efficacy of potent organic acid to provide tenderization effect for muscle protein through the denaturation of the intramuscular connective tissue. Considering its potential, however, studies involving the utilization of calamansi extract to lower the formation of BAs in pork patties during storage are scarce. Therefore, this study aimed to investigate the effect of calamansi pulp extract (CPE) on BAs formation and meat quality of pork patties during refrigerated storage.

Materials and Methods

Preparation of calamansi pulp extracts (CPEs)

Calamansi (*Citrofortunella microcarpa*) was purchased from a local market (Vietnam) and washed with running tap water before extraction. The calamansi was divided and used as part of the pulp. After that, it was lyophilized, ground, passed through a 20 mesh sieve and stored at –20°C until extraction. The sample powder was macerated with 50%, 70%, or 90% ethanol (1:50 w/v) for 3 or 6 days at 25°C. The obtained extracts were filtered through Whatman No. 4 paper, and filtrates were collected. Thereafter, the filtrates were concentrated using a rotary evaporator at 40°C. The concentrated extracts were lyophilized and stored at –20°C until analysis.

Antibacterial activities

Bacterial strain

The antibacterial activity of calamansi pulp ethanol extracts was assessed against five bacterial species: *E. coli* (KCCM 11234), *Listeria monocytogenes* (KCCM 40307), *P. aeruginosa* (ATCC 27853), *S. aureus* (KCCM 12256), and *Salmonella* Enteritidis (*S.* Enteritidis, CCARM 8260). Four bacterial strains (*E. coli*, *P. aeruginosa*, *S. aureus*, *S.* Enteritidis) were streaked on Mueller-Hinton agar (MHA, MB Cell, Seoul, Korea) and incubated at 37°C for 24 h. *L. monocytogenes* was streaked on MHA and incubated at 30°C for 24 h. A single colony of each test organism from the culture plates was inoculated into 10 mL sterile Mueller Hinton broth (MHB, MB Cell) and incubated at each incubation temperature. Subsequently, the cells were subcultured three times and used for paper disc analysis.

Paper disc diffusion assay

Paper disc diffusion was used to assess antibacterial activity using the method described by Ramos et al. (2006), with slight modifications. Each ethanol extract was dissolved in dimethyl sulfoxide (DMSO) at concentrations of 1.25, 2.5, 5, or 10 mg/disc. The extracts in DMSO were filter sterilized using a 0.45 μm hydrophobic membrane filter (Rephile Bioscience, Shanghai, China). The test organisms were inoculated by transferring a loopful of culture into 10 mL of sterile MHB (MB Cell) and incubating at 30°C or 37°C for 24 h, after which the culture was adjusted to 5–6 Log CFU/mL and inoculated in MHA (MB Cell). Sterile 8 mm paper discs (ADVANTEC; Toyo Roshi Kaisha, Tokyo, Japan) were aseptically placed on the MHA surfaces, and each extract was immediately added to disc in volumes of 50 μL. A negative control was DMSO (50 μL) added to a sterile paper disc, and a positive control was used for disc containing 0.01 mg/disc of streptomycin for *E. coli*, *L.*

monocytogenes, and *S. aureus*, whereas for *S.* Enteritidis and *P. aeruginosa* disc containing 0.2 and 0.05 mg/disc of streptomycin were loaded in the paper disc, respectively. Thereafter, the plates were incubated at 30℃ or 37℃ for 24 h. After incubation, the diameter of the inhibition zone (mm) was measured using a digital caliper. The antibacterial activity of the ethanol extracts was compared according to ethanol concentration and extract period. The sample with the highest antibacterial activity was selected and used in the BAs inhibition test.

Antioxidant activity analysis

1,1-Diphenyl-2-pricrylhydrazyl (DPPH)

The DPPH radical scavenging activity was analyzed following the method of Blois (1958), with slight modifications. One hundred microliters of extract solution (1 mg/mL) was placed in 100 μL of methanolic solution containing DPPH radicals (0.2 mM) in a 96-well microplate. The mixture was allowed to react for 30 min at 25℃ in the dark. The absorbance of each extract solution was measured at 517 nm using a spectrophotometer (SpectraMax M2, Molecular Devices, San Jose, CA, USA). The standard curve was established using Trolox, and the DPPH values were expressed as mmol Trolox equivalent (TE)/g dry matter (DM).

Ferric reducing antioxidant power (FRAP)

The FRAP assay was performed as described by Kim et al. (2019), with slight modifications. The FRAP working solution was prepared with 300 mM acetate buffer, 10 mM 2,4,6-tripyridyl-S-triazine in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution mixed at a ratio of 10:1:1 (v/v/v). Twenty-five microliters of the extracted sample (1 mg/mL) were reacted with 175 μL of FRAP working solution for 30 min at 37℃ in the dark. The absorbance of the reacted solution was determined at 590 nm using a spectrophotometer (Spectra Max M2, Molecular Devices). The FRAP activity was expressed as mmol TE/g DM.

Oxygen radical absorption capacity (ORAC)

The ORAC assay was performed as described by Gillespie et al. (2007), with slight modifications. To measure the ORAC, the mixture composed of extract sample of 25 μL (60 μg/mL) and 80 nM fluorescein of 150 μL was mixed and incubated for 15 min at 37℃. After incubation, 150 mM 2,2'-azobis (2-amidinopropane) hydrochloride (25 μL) was added to generate peroxyl radicals, and each well contained a final volume of 200 μL. The change in the absorbance of the reacted extract sample was recorded every minute at an excitation wavelength of 480 nm and an emission wavelength of 520 nm at 37℃. The ORAC assay was performed using a spectrophotometer (Spectra Max M2, Molecular Devices). Trolox was used as the standard, and the results are expressed as mmol TE/g.

Total phenolic content (TPC)

TPC was measured using the Folin-Ciocalteu colorimetric method described by Singleton (1966), with slight modifications. The 70% and 90% ethanol extracts were dissolved in 70% ethanol, and the 50% ethanol extract was dissolved in 50% ethanol. Each extract solution (2 mg/mL) was diluted with methanol. The diluted extract solution (0.5 mL) was mixed with 5 mL distilled water and Folin-Ciocalteu phenol reagent (Sigma -Aldrich, St. Louis, MO, USA) and kept for 3 min, after which mixture was added with 1 N Na_2CO_3 and reacted for 90 min at 25℃ in the dark. The absorbance of the reacted samples was measured at 760 nm using a spectrophotometer (Spectra Max M2, Molecular Devices). A standard curve was established

using gallic acid, and the TPC was expressed as mg gallic acid equivalent (GAE)/g.

Preparation of pork patty

Frozen lean pork legs and pork back fat were purchased from a local supermarket in Chuncheon, Korea. The visible fat on the pork legs was trimmed. The defatted pork leg and back fat were minced through the first 8 mm plate and then through the second 4 mm plate a meat chopper (M-12S, Fujee, Hwaseong, Korea). After mincing, the defatted pork leg and back fat were mixed with salt, sterilized water, and lyophilized calamansi pulp ethanol extract using a mixer (5 KPM50, KitchenAid, Benton Harbour, MI, USA). The formulations of the pork patties are presented in Table 1. Approximately 80 g of the mixture was formed into pork patties using a Petri dish (15 mm thick×90 mm diameter). The patties were placed on a plastic foam meat tray, wrapped with polyethylene film, and stored in an incubator at 4℃ for seven days. Each sample was analyzed on days 1, 3, 5, and 7 of storage.

Proximate composition and pH value

The proximate composition was measured using the methods of the Association of Official Agricultural Chemists (AOAC, 2012). The moisture content of the pork patties was measured by weight loss after oven drying at 105℃ for 12 h. The crude protein content was measured using the Kjeldahl method. Crude fat content was measured by solvent extraction using ether. The burned pork patties in the furnace at 550℃ were analyzed for crude ash. The pH was determined using a pH meter (Orion 230A, Thermo Fisher Scientific, Waltham, MA, USA). Ten grams of pork patty were homogenized with 90 mL distilled water using homogenizer (PolyTron® PT-2500E, Kinematica AG, Malters, Switzerland).

Instrumental color

The instrumental color of the pork patties was determined using a colorimeter (CR-400 Minolta colorimeter, Minolta, Osaka, Japan) with an aperture of 8 mm and illuminant-C. The color values of CIE L*, CIE a*, and CIE b* were measured after 10 min of removing the polyethylene films of patties on days 1, 3, 5, and 7 of storage.

Bacterial cell counts

Ten grams of each pork patty sample was aseptically placed into sterile stomacher bags (Interscience, Saint Nom la Bretêche, France) and homogenized with 90 mL sterile saline using a stomacher (BagMixer 400 VW, Interscience) for 40 s.

Table 1. Formulation of pork patties

Ingredients (%)	Treatment		
	Control	0.2PCPE	0.4PCPE
Lean pork leg	73.5	73.5	73.5
Pork back fat	21.0	21.0	21.0
Salt	0.5	0.5	0.5
Water	5.0	5.0	5.0
Plant extract	0.0	0.2	0.4

0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition.

The homogenate was serially 10-fold diluted in sterile saline, and microorganism populations were evaluated by the pour plate method in Petri dishes as follows: The total aerobic bacteria (TAB) counts were measured on Plate Count Agar (PCA, MB Cell), incubated at 37℃ for 48 h; lactic acid bacteria (LAB) counts were measured on MRS agar (MB Cell), incubated under anaerobic conditions at 37℃ for 48 h, *Pseudomonas* spp. and Enterobacteriaceae counts were measured on Cetrimide Agar (CN, MB Cell) and Violet Red Bile Glucose Agar (VRBG, MB Cell), respectively, incubated at 37℃ for 24 h.

Volatile basic nitrogen (VBN)

The VBN content was analyzed using the micro-diffusion method described by Kim et al. (2019), with slight modifications. Ten grams of each pork patty was homogenized for 30 min in 50 mL of distilled water using a magnetic stirrer, and the homogenate was then filtered through filter paper (Whatman No. 1). One milliliter of the filtrate was added to 1 mL of saturated K_2CO_3 in the outer chamber of the Conway unit, 1 mL of 0.01 N H_2SO_4 was added to the inner chamber, immediately covered and then incubated for 1 h at 25℃. After incubation, Brunswik regent of 20 μL was added to the inner chamber of the Conway unit and titrated against 0.01 N NaOH. The VBN value was expressed in mg/100 g.

$$\text{VBN (mg / 100 g)} = 0.14 \times (\text{b} - \text{a}) \times \text{F} / \text{W} \times 100 \times 50$$

where a is the volume of 0.01 N NaOH was added to the sample (mL), b is the volume of 0.01 N NaOH added to the blank (mL), F is the standard factor for 0.01 N NaOH, and W is the sample weight (g).

2-Thiobarbituric acid reactive substances (TBARS)

TBARS content was analyzed using the method described by Buege and Aust (1978). Pork patties (5 g) were added to 50 μL of 7.2% *tert*-butyl-4-hydroxyanisole and 15 mL of distilled water and then homogenized for 30 s using a homogenizer (Polytron PT-2500E, Kinematica AG). One milliliter of homogenate was transferred to a test tube, and 2 mL of thiobarbituric acid (TBA)/trichloroacetic acid (TCA) solution (20 mM TBA/15% TCA) was added to the test tube. A blank (2 mL of each patty homogenate) was added to 2 mL of 15% TCA solution. The sample mixture was incubated in a water bath at 90℃ for 15 min to develop color. After incubation, the samples were cooled in ice water for 10 min and centrifuged at 2,000×g at 4℃ for 15 min. The absorbance of the supernatant solution was measured at 531 nm using a spectrophotometer (Spectra Max M2, Molecular Devices). The TBARS content was expressed as mg of malondialdehyde (MDA)/kg of patty, as follows:

$$\text{TBARS (mg MDA/kg of patty)} = (\text{Absorbance of sample} - \text{Absorbance of blank sample}) \times 5.88.$$

Biogenic amines (BAs)

The BAs content was analyzed using the method described by Eerola et al. (1993). Pork patties (10 g) were homogenized in 10 mL of 0.4 M perchloric acid (PCA) and centrifuged (1,763×g, 4℃, 10 min). After centrifugation, the homogenate was filtered using filter paper (Whatman No. 1), and the remaining pellet was re-extracted using 10 mL of 0.4 M PCA. The filtrated solution was collected and filled up to 25 mL using a 0.4 M PCA. The extracted solution (0.2 mL) was mixed with 2 N NaOH (40 μL), saturated $NaHCO_3$ (60 μL), and dansyl chloride (10 mg/mL in acetone, 0.4 mL) and then incubated at 45℃ for 40 min. After incubation, the solution was mixed with 20 μL of ammonium hydroxide and kept in the dark for 30 min at ambient temperature to remove dansyl chloride. The solution was made up to 1 mL with acetonitrile (ACN). The mixture was

centrifuged at 589×g at 4℃ for 10 min and filtered using a 0.22 μm hydrophobic membrane filter (Rephile Bioscience).

Quantification of BAs was performed using an Agilent 1260 HPLC (Agilent, Santa Clara, CA, USA) with a Poroshell 120 EC-C18 (4 μm, 4.6×150 mm) column (Agilent). The HPLC analysis used a gradient elution program with 0.1 M ammonium acetate as solvent A and ACN as solvent B. The gradient started with a solvent A-solvent B mixture (50:50, v/v) and then proceeded linearly for 19 min in a solvent A-solvent B mixture (10:90, v/v). This ratio was changed linearly over 5 min to a solvent A: solvent B mixture (50:50, v/v). This composition was maintained for 5 min until the end of the program. A waiting time of was necessary before the next analysis for equilibrium (the total run time with equilibration was 29 min). The column temperature was set to 40℃. The sample of 20 μL volume was injected, and the amounts of BAs were quantified by UV absorption at 254 nm and fluorescence at 550 nm. The content of the BAs (PUT, CAD, HIM, TYM, and SPD) was determined with reference to the amine standards. BAs content was expressed as μg/g of patties.

Sensory evaluations

Sensory evaluation of the pork patties was performed by 15 panelists from the College of Animal Life Sciences, Kangwon National University. The sensory properties of each pork patty were evaluated on days 1, 3, 5, and 7 of storage and scored for color, aroma, off-odor, drip loss, and overall acceptability using a 9-point scale system as follows: Color, aroma, and overall acceptability (1=extremely undesirable, 9=extremely desirable) and off-odor (1=extremely weak, 9=extremely strong) and drip loss (1=extremely low, 1=extremely high). Sensory evaluation was approved by the Kangwon National University Institutional Review Board (KWNUIRB-2020-09-005-002).

Statistical analysis

All data were analyzed using the general linear model procedure of the SAS program (ver. 9.2; SAS Institute, Cary, NC, USA). Tukey's test was used to determine the significance of the differences in the mean values for the different extract samples. Differences were considered significant at $p<0.05$.

Results and Discussion

Antimicrobial activity of calamansi pulp extracts (CPEs)

In this study, a paper disc diffusion assay was used to measure the efficacy of CPE against foodborne pathogens, represented by *E. coli*, *S.* Enteritidis, and *P. aeruginosa* of the gram-negative, and *S. aureus* and *L. monocytogenes* of the gram-positive bacteria, respectively. Using this assay, the inhibition zones of CPE under different extraction conditions and periods at any given concentration were recorded, and the results were compared to those of *Streptomycin*. As shown in Table 2, the inhibition zones of CPE against *E. coli* were between 8.50–15.05 mm, with the highest inhibition zones observed in 90CPE for 6 days at 10 mg/disc concentration ($p<0.05$). The treatment with CPE at a concentration of below than 2.5 mg/disc did not sufficiently inhibit the growth of *E. coli* and had no inhibition zones. Similarly, *S.* Enteritidis treatment at 1.25 mg/disc did not significantly contribute to antimicrobial activity compared to the higher concentration treatments.

In addition, the highest inhibition zone against *S.* Enteritidis was observed in the sample group treated with 90CPE for 6 days at 10 mg/disc (17.49 mm), surpassing that *Streptomycin* at 0.20 mg/disc of 11.06 mm. In addition, with respect to the *P. aeruginosa*, *L. monocytogenes*, and *S. aureus*, the inhibition zone by CPE was strating to be seen at a concentration of 5 mg/disc, with no effect at 1.5 and 2.5 mg/disc, unless for *S. aureus* that treated with 90CPE at 2.5 mg/disc (10.05 mm).

Table 2. Antimicrobial effect of calamansi pulp extracts against five food pathogens by paper disc diffusion assay (unit: mm)

Plant extract	Concentration (mg/disc)											Streptomycin
	1.25		SEM	2.5		SEM	5		SEM	10		SEM	
	Day 3	Day 6		Day 3	Day 6		Day 3	Day 6		Day 3	Day 6		
Escherichia coli													17.14 (0.01 mg/disc)
50	ND	ND	-	9.39^{a}	8.50^{Ca}	0.314	12.05^{Ba}	11.67^{a}	0.158	14.48^{a}	13.83^{Bb}	0.121	
70	ND	ND	-	8.50^{b}	9.00^{Ba}	0.000	12.50^{Aa}	12.00^{b}	0.000	14.83^{a}	14.17^{Bb}	0.167	
90	ND	ND	-	9.50^{b}	10.06^{Aa}	0.008	11.50^{Cb}	11.63^{a}	0.005	14.33^{b}	15.05^{Aa}	0.137	
SEM	-	-		0.256	0.007		0.086	0.096		0.138	0.148		
Staphylococcus aureus													14.00 (0.01 mg/disc)
50	ND	ND	-	ND	ND	-	10.00^{Aa}	9.67^{Ba}	0.118	13.33^{a}	13.50^{Aba}	0.118	
70	ND	ND	-	ND	ND	-	9.17^{Ba}	9.33^{Ba}	0.264	13.00^{a}	12.83^{Ba}	0.118	
90	ND	ND	-	ND^{b}	10.05^{a}	0.002	10.00^{Ab}	11.17^{Aa}	0.026	13.17^{b}	14.15^{Aa}	0.193	
SEM	-	-		-	0.002		0.096	0.216		0.136	0.157		
Salmonella Enteritidis													11.06 (0.2 mg/disc)
50	ND	ND	-	9.50^{Bb}	10.00^{Ba}	0.000	11.17^{a}	11.50^{Ba}	0.118	14.00^{Ba}	13.00^{Bb}	0.000	
70	ND	ND	-	10.17^{Aa}	9.00^{Cb}	0.118	11.33^{a}	10.50^{Cb}	0.118	15.00^{Aa}	12.50^{Cb}	0.000	
90	ND	ND	-	9.67^{ABb}	10.37^{Aa}	0.118	11.83^{b}	12.90^{Aa}	0.128	15.00^{Ab}	17.49^{Aa}	0.067	
SEM	-	-		0.136	0.009		0.167	0.042		0.000	0.055		
Pseudomonas aeruginosa													19.69 (0.05 mg/disc)
50	ND	ND	-	ND	ND	-	10.16^{Ba}	8.99^{Cb}	0.043	12.88^{Ba}	12.23^{Cb}	0.140	
70	ND	ND	-	ND	ND	-	9.53^{Cb}	9.77^{Ba}	0.053	12.91^{ABa}	12.61^{Ba}	0.079	
90	ND	ND	-	ND	ND	-	10.38^{Aa}	10.45^{Aa}	0.012	13.43^{Aa}	13.52^{Aa}	0.042	
SEM	-	-		-	-		0.028	0.049		0.125	0.053		
Listeria monocytogenes													16.16 (0.01 mg/disc)
50	ND	ND	-	ND	ND	-	12.00^{Aa}	11.17^{Bb}	0.118	18.50^{Aa}	16.17^{Cb}	0.118	
70	ND	ND	-	ND	ND	-	10.67^{Bb}	11.50^{Ba}	0.118	14.00^{Cb}	16.83^{Ba}	0.118	
90	ND	ND	-	ND	ND	-	11.00^{Bb}	12.65^{Aa}	0.053	17.17^{Bb}	18.53^{Aa}	0.136	
SEM	-	-		-	-		0.096	0.106		0.096	0.147		

The diameter of paper disc (8 mm) is included.
$^{A–C}$ Means within a column with different superscript differ significantly at $p<0.05$.
^{a,b} Means within extraction period with different superscript differ significantly at $p<0.05$.
ND, not detected.

Furthermore, this study revealed that CPE tended to have stronger antimicrobial activity against gram-negative bacteria than gram-positive bacteria, as indicated by the lower concentration needed to impart strong inhibitory zones, which agrees with a previous study (Husni et al., 2021). This might be due to a thinner cell wall possessed by gram-negative bacteria (1.5–10 nm), which is believed to be more easily damaged by the actions of phenolic acids than in gram-positive bacteria with a thicker cell wall (20–80 nm; Mai-Prochnow et al., 2016).

The antimicrobial activity of 90CPE in this study was categorized as strong, with an inhibition zone of >10 mm (Vollmer et al., 2008) at a minimum concentration of 5 mg/disc. In addition, based on the results of this study, extending the extraction period to 6 days with 90% ethanol toward calamansi pulp resulted in significantly stronger inhibition zones against all bacteria at any given concentration ($p<0.05$). This study also indicated that the antimicrobial activity of 90CPE was likely dose-dependent, with the strongest effect being well-documented for the sample group treated with 90CPE at 10 mg/disc ($p<0.05$). Cheong et al. (2012) reported that the robust antimicrobial activity of calamansi is due to the abundance of phenolic acids, including coumaric, sinapic, and caffeic acids. When exposed to these compounds, the main component of the bacterial cell wall, peptidoglycan, experiences extreme stress, leading to the loss of cell integrity and promotion of cell lysis. In addition, the pH value of CPE in this study (2.01 is assumed to initiate the hyperacidification of phenolic acid, which affects the membrane permeability of bacteria. Hyperacidification is caused by disruption of ATP synthesis and cell death (Barido et al., 2022; Cueva et al., 2010).

Antioxidant activity of calamansi pulp extracts (CPEs)

Table 3 shows the antioxidant activity of the calamansi pulp extracted with different ethanol concentrations (50%, 70%, and 90%) at different extraction periods (3 and 6 days). Three antioxidant assays (DPPH, FRAP, and ORAC) and TPC were employed to determine the appropriate conditions for extracting the calamansi pulp. As for the result, the antioxidant activity of the CPE were significantly influenced by both percentage of ethanol and duration of extraction ($p<0.05$), unless for FRAP assay. The fundamental differences in the mechanisms of antioxidant assays are thought to be the main reason for these differences (Sun and Ho, 2005). Compared to other antioxidant assays that are capable of measuring various antioxidant activities based on the electron or hydrogen donor, the FRAP assay determines the antioxidant activity of certain compounds based on their ability to donate an electron that converts ferric (Fe^{3+}) to ferrous (Fe^{2+}). According to the DPPH result, 90CPE exhibited the highest scavenging percentage toward DPPH radicals compared to that of 50CPE and 70CPE, equivalent to 19.00 and 13.89 μmol TE/g DM for day 3 and day 6, respectively ($p<0.05$). In addition, extending the extraction period tended to decrease the antioxidant activity of CPE, as indicated by a lower value on day 6 compared to that on day 3 in 70CPE and 90CPE ($p<0.05$). Accordingly, extending the duration of extraction to 6 days resulted in a significant decrease in TPC, as seen for 50CPE and 90CPE ($p<0.05$). In contrast, the TPC of CPE was at the highest concentration under extraction using 90% ethanol when compared to that of 50% and 70%, exhibited 12.62 and 12.12 mg GAE/g DM for day 3 and day 6,

Table 3. Antioxidant activity of calamansi pulp extract

Ethanol concentration (%)	DPPH (μmol TE/g DM)		SEM	FRAP (mmol TE/g DM)		SEM	ORAC (mmol TE/g DM)		SEM	TPC (mg GAE/g DM)		SEM
	Day 3	Day 6		Day 3	Day 6		Day 3	Day 6		Day 3	Day 6	
50	11.45^{Ba}	10.94^{Ba}	0.323	0.03	0.03	0.000	0.45^{Ba}	0.44^{Ba}	0.011	12.11^{Ba}	11.31^{Bb}	0.127
70	12.08^{Ba}	10.59^{Bb}	0.276	0.03	0.03	0.000	0.44^{Ba}	0.45^{Ba}	0.012	12.06^{Ba}	11.92^{Aa}	0.042
90	19.00^{Aa}	13.89^{Ab}	0.176	0.03	0.03	0.000	0.52^{Ab}	0.56^{Aa}	0.009	12.62^{Aa}	12.12^{Ab}	0.069
SEM	0.365	0.085		0.000	0.000		0.011	0.011		0.075	0.098	

^{A,B} Means within a column with different superscript differ significantly at $p<0.05$.
^{a,b} Means within a row with different superscript differ significantly at $p<0.05$.
DPPH, 1,1-diphenyl-2-pricrylhydrazyl; TE, Trolox equivalent; DM, dry matter; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorption capacity; TPC, total phenolic content.

respectively. Similarly, the antioxidant activity of the calamansi pulp under ORAC assay reached the highest score after extraction with 90% ethanol, possessed equivalent score of 0.52 and 0.56 mmol TE/g DM, wherein extracting CPE for 6 days had significantly higher score than that of 3 days ($p<0.05$). The polarity of the extracting solution is the first essential factor to concentrate the antioxidant compounds from natural plants (Zhu et al., 2014), and previous studies have proven that the phenolic acid contents, which are strongly correlated with the antioxidant capacity of natural extracts, were in higher concentrations in organic solutions than in aqueous solutions (Barido et al., 2021a; Bera et al., 2006; Zhu et al., 2014). In accordance with these results, Gong et al. (2018) showed that the major phenolic acids, particularly caffeic, chlorogenic, isovanillic, sinapic, and gallic acid, were strongly extracted in high polarity solutions.

Proximate composition and pH value of pork patty

In this study, CPE treatment did not significantly affect the proximate composition of the pork patties ($p>0.05$). As shown in Table 4, the moisture content was ranging between 62.54%–63.70%, and the crude fat percentage was between 18.71%–19.29%. In addition, as expected, the incorporation of CPE at various concentrations into pork patties did not cause significant changes in either crude protein or ash content ($p>0.05$). The protein content was 15.52%–15.85%, and the crude ash content was approximately between 1.20%–1.27%. This finding on proximate composition agreed with previous reports of acceptable pork patties (Bellucci et al., 2022; Overholt et al., 2016). Insignificant changes in proximate composition following the addition of natural extracts to meat products were previously reported by Bellucci et al. (2022) after adding açaí (*Euterpe oleracea*) extract to prok patties and de Carvalho et al. (2020) following the addition of the tumeric extract to lamb sausages.

Table 5 shows the pH values of the pork patties during refrigerated storage for 7 days in the control and CPE-treated samples. The incorporation of CPE into pork patties resulted in significantly lower pH values throughout the storage period compared to the control ($p<0.05$), in which the highest addition percentage resulted in the lowest pH value on any storage day ($p<0.05$). The pH value of pork patties in this study ranged between 4.99–6.18, within the range of our previous report on marinated black currant juice pork patties (4.71–5.82; Cho et al., 2021), and slightly lower than that of Bellucci et al. (2022) after treatment with açaí extract that was stored for 10 days (5.69–5.88). The pH of meat products may increase or decrease during refrigerated storage due to the accumulation of lactic acid or the formation of alkaline substances by microorganisms, the state of raw materials, types of additives, formulation, or storage conditions affect (Park et al., 2011). Calamansi, which belongs to the genus *citrus* included as an organic acid with the possibility of lowering the pH value of meat. A previous study reported that marination with tamarind, calamansi, lemon, and lime extracts significantly reduced the pH of grilled chicken (Jinap et al., 2018). In contrast, the low pH value of the extracts was thought to contribute to antimicrobial activity through the mechanism of hyperacidification (Cueva et al., 2010; Tan et al., 2014).

Table 4. Effect of calamansi pulp extract on proximate composition of pork patty

Treatment	Proximate composition (%)			
	Moisture	Crude protein	Crude fat	Crude ash
Control	63.70	15.85	19.29	1.21
0.2PCPE	62.54	15.80	19.28	1.27
0.4PCPE	62.92	15.52	18.71	1.20
SEM	0.271	0.176	0.229	0.033

0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition.

Table 5. Effect of calamansi pulp extract on pH values of pork patty during storage at 4℃

Treatment	Storage days (d)				SEM
	1	3	5	7	
Control	6.01^{Ab}	5.89^{Ad}	5.96^{Ac}	6.18^{Aa}	0.004
0.2PCPE	5.48^{Ba}	5.39^{Bb}	5.38^{Bc}	5.39^{Bbc}	0.002
0.4PCPE	5.14^{Ca}	5.06^{Cb}	5.04^{Cc}	4.99^{Cd}	0.002
SEM	0.003	0.003	0.004	0.002	

$^{A–C}$ Means within a column with different superscript differ significantly at $p<0.05$.
$^{a–d}$ Means within a row with different superscript differ significantly at $p<0.05$.
0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition.

Instrumental color of pork patty

In this study, both CPE addition and storage period significantly influenced the instrumental color of the pork patties ($p<0.05$; Table 6). With respect to the CIE L*, a markedly higher score was observed following treatment with CPE at the highest percentage (0.4%) at any storage period, with no effect at 0.2% compared to the control. This might be due to the basic color of the phenolic extract, mainly anthocyanin, which is capable of permeating into the muscle, thus altering the light color of the meat products (Barido et al., 2022; Lee et al., 2016). In addition, in terms of CIE a*, CPE-treated groups differed significantly from that of the control ($p<0.05$) and produced a lesser red color on day 1. During the storage period, an inconsistent effect of CPE at 0.40% was observed when compared to the control, whereas treatment with CPE at 0.20% exhibited the highest score among treatments. Furthermore, CPE treatment did not change the CIE b* of pork patties on day 1 ($p>0.05$). However, the effect was observed as the storage period increased, with the CIE b* of the CPE treated group having a markedly higher score on days 3 and 5 compared to the control, whereas on the ultimate storage day, 0.4PCPE alone

Table 6. Effect of calamansi pulp extract on the instrumental color of pork patty during storage at 4℃

Traits	Treatment	Storage days (d)				SEM
		1	3	5	7	
CIE L*	Control	68.37^{Ba}	68.20^{Ba}	67.67^{Bab}	67.01^{Bc}	0.231
	0.2PCPE	68.00^{Ba}	68.04^{Ba}	67.85^{Ba}	66.69^{Bb}	0.072
	0.4PCPE	72.15^{Aa}	71.25^{Ab}	71.22^{Ab}	72.24^{Aa}	0.078
	SEM	0.252	0.084	0.088	0.090	
CIE a*	Control	12.75^{Aa}	11.82^{Ab}	7.53^{Cc}	7.52^{Bc}	0.087
	0.2PCPE	12.28^{Ba}	11.66^{Ab}	10.09^{Ac}	7.94^{Ad}	0.014
	0.4PCPE	10.24^{Ca}	9.09^{Bb}	7.92^{Bc}	7.19^{Bd}	0.042
	SEM	0.031	0.050	0.013	0.095	
CIE b*	Control	15.95^{a}	15.23^{Bb}	14.46^{Bc}	14.26^{Cc}	0.059
	0.2PCPE	15.81^{a}	15.70^{Aa}	15.11^{Ab}	14.84^{Bc}	0.052
	0.4PCPE	15.95^{a}	15.61^{Ab}	15.21^{Ac}	15.12^{Ac}	0.036
	SEM	0.043	0.070	0.023	0.052	

$^{A–C}$ Means within a column with different superscript differ significantly at $p<0.05$.
$^{a–d}$ Means within a row with different superscript differ significantly at $p<0.05$.
0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition.

produced pork patties with the highest CIE b* ($p<0.05$). The increase in storage period significantly affected all instrumental color variables in this study, which is in agreement with previous studies (Bellucci et al., 2022; Lorenzo et al., 2018). The inevitable onset of lipid and meat pigment (myoglobin) oxidation during storage is the main factor responsible for color changes in meat and meat products. Oxidized myoglobin results in excessive conversion of myoglobin to metmyoglobin, thus imparting a brown perception. However, the oxidation of lipids and proteins leads to increased formation of free radicals, affecting myoglobin redox stability, thus causing deterioration of meat color (Barido et al., 2021b; Young et al., 1996).

Bacterial cell counts of pork patty

With respect to its strong correlation with the production of meat BAs, quantification of bacterial colonies is an essential factor in determining the efficacy of natural products in suppressing the formation of BAs (Lee et al., 2020). In this study, the antimicrobial activity of the CPE at various concentration are shown in Table 7. The incorporation of CPE into pork patties significantly inhibited the growth of spoilage bacteria (Enterobacteriaceae and *Pseudomonas* spp.), LAB, and TAB. The Enterobacteriaceae count in all treatment groups significantly decreased as the storage period increased ($p<0.05$). In additiom, the CPE effect was observed on the Enterobacteriaceae counts from the beginning until the end of the storage period, with the higher addition percentage imparting a significantly stronger inhibitory effect ($p<0.05$). Therefore, the growth of *Pseudomonas* spp. In pork patty was significantly suppressed after day 3 and was maintained until the end of the storage period ($p<0.05$). In addition, this study revealed that the addition of 0.40% CPE had a stronger inhibitory effect against

Table 7. Effect of calamansi pulp extract on bacterial cell counts of pork patty during storage at 4℃

Bacteria (Log CFU/g)	Treatment	Storage days (d)				SEM
		1	3	5	7	
Total aerobic bacteria	Control	5.71^{Ad}	6.10^{Ac}	7.13^{Ab}	7.37^{Aa}	0.017
	0.2PCPE	4.88^{Bc}	4.94^{Bc}	6.13^{Bb}	6.53^{Ba}	0.050
	0.4PCPE	4.54^{Cb}	3.66^{Cc}	4.49^{Cb}	4.96^{Ca}	0.034
	SEM	0.030	0.038	0.048	0.024	
Lactic acid bacteria	Control	3.53^{Ad}	4.07^{Ac}	4.75^{Ab}	4.92^{Aa}	0.020
	0.2PCPE	2.80^{Bc}	3.59^{Bb}	4.51^{Ba}	4.69^{Ba}	0.056
	0.4PCPE	2.62^{Bd}	3.22^{Cc}	4.54^{Bb}	4.88^{Aa}	0.048
	SEM	0.080	0.022	0.024	0.020	
Enterobacteriaceae	Control	3.32^{Ad}	5.29^{Ac}	5.95^{Ab}	6.34^{Aa}	0.080
	0.2PCPE	3.03^{Bd}	4.36^{Bc}	4.87^{Bb}	5.50^{Ba}	0.079
	0.4PCPE	2.79^{Cd}	3.04^{Cc}	3.54^{Cb}	3.76^{Ca}	0.041
	SEM	0.029	0.039	0.087	0.095	
Pseudomonas spp.	Control	2.90^{d}	4.07^{Ac}	4.92^{Ab}	5.22^{Aa}	0.043
	0.2PCPE	2.99^{c}	3.50^{Bb}	4.25^{Ba}	4.66^{Ba}	0.104
	0.4PCPE	2.97^{d}	3.23^{Cc}	3.57^{Cb}	3.89^{Ca}	0.031
	SEM	0.118	0.044	0.046	0.018	

$^{A–C}$ Means within a column with different superscript differ significantly at $p<0.05$.
$^{a–d}$ Means within a row with different superscript differ significantly at $p<0.05$.
0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition.

Pseudomonas spp. than 0.20% CPE on days 3, 5, and 7 ($p<0.05$). In addition, with regard to the LAB counts in pork patties, the addition of CPE, regardless of the concentration, showed a significantly lower total number of LAB when compared to that of the control group on any storage day, except on day 7. On day 7, significantly lower LAB counts were only observed in pork patties supplemented with 0.20% CPE (4.69 Log CFU/g), with no significant difference in 0.40% CPE (4.88 Log CFU/g) in comparison to that of the control group (4.92 Log CFU/g). This may be due to the tolerance of the LAB strain to extremely acidic conditions, thus maintaining a stable population in a low pH environment. This is in agreement with Xiao et al. (2018), who elucidated that the impedance of most microbial populations occurred at low pH conditions or during the later stage of storage or fermentation period, unless the LAB showed resistance to dropped pH conditions. Furthermore, TAB counts decreased significantly after the addition of CPE, and as the percentage addition increased, a lower amount of TAB was observed across the storage period ($p<0.05$). By utilizing the available source of nutrients, mainly free amino acids, these pacticular bacteria act to deplete the carboxyl group from the free amino acid chain via enzymatic decarboxylation reactions, resulting in BA formation. The Enterobacteriaceae and *Pseudomonas* spp. of gram-negative bacteria, *Lactobacillus* of gram-positive bacteria, and aerobic bacteria were reported to be capable of producing PUT, CAD, TYM, and PUT, respectively (Halász et al., 1994; Min et al., 2007; Triki et al., 2018). Furthermore, this study describes the ability of CPE to inhibit the growth of various bacterial populations in pork patties, which may be attributed to the action of phenolic acids. Apart from the disruption of ATP synthesis in bacteria caused by the insertion of a small fracture of phenolic acid, the low pH of CPE stimulates the onset of hyperacidification by phenolic acid, causing disruption of the bacterial membrane and cell lysis (Barido et al., 2022; Cueva et al., 2010).

Volatile basic nitrogen (VBN) of pork patty

The effects of CPE incorporation on the VBN values of pork patties during storage are shown in Table 8. Its value did not differ between the control and CPE-treated groups until storage day 3 ($p>0.05$), when the value was significantly lower on days 5 and 7 ($p<0.05$). At the final storage period, the order of VBN value from the highest to the lowest were control, 0.2PCPE, and 0.4PCPE with 11.94, 7.98, and 7.60 mg/100 g respectively ($p<0.05$). In addition, with respect to the storage period, the VBN value of control group experienced significant increased as the storage period extended ($p<0.05$). However, the VBN value did not differ significantly in CPE-treated samples until day 5, irrespective of the addition percentage. Moreover, in this study, the VBN value of the pork patties was regarded as acceptable (< 20 mg/100 g), with values ranged from 6.98–13.31 mg/100 g during a storage period of 7 days. VBN has been used as an indicator of meat freshness and is

Table 8. Effect of calamansi pulp extract on VBN value of pork patty during storage at 4℃ (unit: mg/100 g)

Treatment	Storage days (d)				SEM
	1	3	5	7	
Control	6.98^{d}	7.77^{c}	11.94^{Ab}	13.31^{Aa}	0.170
0.2PCPE	7.21^{b}	7.77^{b}	7.98^{Bb}	9.80^{Ba}	0.273
0.4PCPE	7.61^{b}	7.97^{b}	7.60^{Bb}	8.76^{Ca}	0.175
SEM	0.183	0.146	0.303	0.181	

$^{A–C}$ Means within a column with different superscript differ significantly at $p<0.05$.
$^{a–d}$ Means within a row with different superscript differ significantly at $p<0.05$.
VBN, volatile basic nitrogen; 0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition.

mainly produced by the enzymatic decarboxylation of specific amino acids by bacteria. Min et al. (2007) proposed the measurement of VBN as a good index for certain BAs formations in pork, beef, and chicken due to its high correlation score. Furthermore, as the VBN value is also associated with Enterobacteriaceae and *Pseudomonas* spp. (Li et al., 2019), the ability of the CPE extract to inhibit the formation of these bacterial strains was regarded as the reason for the lower VBN value in CPE-treated patties.

Lipid oxidation of pork patty

The TBARS values of pork patties during cold storage are presented in Table 9. In control sample without any CPE addition, the TBARS value ranged between 0.28–0.33 mg MDA/kg, in which at day 7, its score was significantly higher than that of the remaining storage days ($p<0.05$). This study observed that the inclusion of CPE, irrespective of the concentration, resulted in a significantly lower TBARS value at any storage day when compared to the control, except for day 7. At the final storage day, the TBARS value of the pork patty treated with the addition of 0.40% CPE (0.87 mg MDA/kg) had remarkably higher score than that of control (0.33 mg MDA/kg) and 0.2PCPE (0.28 mg MDA/kg; $p<0.05$). This might be related to the extremely low pH of the calamansi extract, thus upregulating the excessive rate of lipid oxidation. Thiansilakul et al. (2011) revealed that the occurrence of myoglobin and lipid oxidation was higher in an extremely acidic environment, wherein under this condition, the onset of autoxidation occurs, especially on hemoglobin, which is further converted into methemoglobin. This conversion results in overproduction of superoxide anion radicals (Richards and Hultin, 2000). The capacity of CPE to inhibit lipid oxidation is related to the abundance of phenolic acids. Ascorbic acid, which naturally exists at high concentrations within the calamansi, serves as a sequestrant to remove the highly reactive metal ions and free radicals, which agrees with a previous report (Hussain et al., 2021).

Biogenic amines of pork patty

BAs are essential in the mammalian brain and function as neurotransmitters at low concentrations (Burchett and Hicks, 2006). However, it is present in a highly abundant portion, which causes quality deterioration of meat and health problems upon ingestion. In this study, five major BAs (PUT, CAD, HIM, TYM, and SPD), which are considered hazardous materials in meat, were recorded in pork patties during storage, wherein CPE at various concentrations was employed to inhibit its formation (Table 10). The content of PUT at 0.4PCPE was the highest on any storage day among the remaining treatments ($p<0.05$), while 0.2PCPE shared no differences with the control group, except at day 5. In contrast, the addition of CPE at

Table 9. Effect of calamansi pulp extract on TBARS value of pork patty during storage at 4℃ (unit: mg MDA/kg)

Treatment	Storage days (d)				SEM
	1	3	5	7	
Control	0.28^{Ab}	0.30^{Ab}	0.29^{Ab}	0.33^{Ba}	0.005
0.2PCPE	0.19^{Bc}	0.23^{Bb}	0.26^{ABa}	0.28^{Ba}	0.005
0.4PCPE	0.20^{Bb}	0.23^{Bb}	0.26^{Bb}	0.87^{Aa}	0.015
SEM	0.004	0.008	0.008	0.014	

^{A,B} Means within a column with different superscript differ significantly at $p<0.05$.
$^{a–c}$ Means within a row with different superscript differ significantly at $p<0.05$.
TBARS, 2-thiobarbituric acid reactive substances; 0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition; MDA, malondialdehyde.

Table 10. Effect of calamansi pulp extract on biogenic amines of pork patty during storage at 4℃

BAs (μg/g)	Treatment	Storage days (d)				SEM
		1	3	5	7	
PUT	Control	4.80^{Ca}	4.47^{Cbc}	5.98^{Ba}	4.05^{Cc}	0.132
	0.2PCPE	6.85^{Bb}	7.01^{Bb}	9.73^{Aa}	7.50^{Bb}	0.271
	0.4PCPE	10.91^{Aa}	9.82^{Aa}	10.75^{Aa}	10.85^{Aa}	0.444
	SEM	0.190	0.181	0.506	0.242	
CAD	Control	3.82^{Aa}	3.09^{ABa}	2.28^{b}	3.71^{Aa}	0.166
	0.2PCPE	4.01^{Aa}	3.26^{Ab}	2.83^{b}	2.01^{Bc}	0.139
	0.4PCPE	3.39^{Ba}	2.44^{Bb}	2.62^{b}	2.25^{Bb}	0.141
	SEM	0.065	0.176	0.167	0.161	
HIM	Control	6.30^{Ac}	10.52^{Ab}	14.04^{Aa}	10.99^{Cb}	0.383
	0.2PCPE	3.90^{Cd}	7.71^{Bc}	9.87^{Bb}	16.26^{Aa}	0.369
	0.4PCPE	5.66^{Bc}	8.81^{ABb}	9.93^{Bb}	14.64^{Ba}	0.490
	SEM	0.066	0.519	0.571	0.314	
TYM	Control	4.49^{c}	14.25^{Ab}	41.24^{Aa}	43.18^{Aa}	0.546
	0.2PCPE	3.82^{d}	5.13^{Bc}	16.21^{Bb}	32.29^{Ba}	0.251
	0.4PCPE	4.29^{b}	4.85^{Bb}	6.53^{Ca}	6.85^{Ca}	0.167
	SEM	0.189	0.228	0.269	0.599	
SPD	Control	1.23^{a}	1.08^{Bb}	0.76^{Bc}	1.14^{ABab}	0.026
	0.2PCPE	1.34^{a}	1.24^{Aa}	1.17^{Aa}	0.95^{Bb}	0.041
	0.4PCPE	1.50^{a}	1.34^{Aa}	1.45^{Aa}	1.26^{Aa}	0.097
	SEM	0.075	0.025	0.086	0.046	
Total BAs	Control	20.64^{Bc}	33.40^{Ab}	64.30^{Aa}	63.06^{Aa}	0.894
	0.2PCPE	19.94^{Bd}	24.35^{Cc}	39.80^{Bb}	59.00^{Ba}	0.425
	0.4PCPE	25.75^{Ac}	27.26^{Bc}	31.27^{Cb}	35.85^{Ca}	0.481
	SEM	0.227	0.649	0.393	0.994	

$^{A–C}$ Means within a column with different superscript differ significantly at $p<0.05$.
$^{a–d}$ Means within a row with different superscript differ significantly at $p<0.05$.
BAs, biogenic amines; PUT, putrescine; CAD, cadaverine; HIM, histamine; TYM, tyramine; SPD, spermidine; 0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition.

0.40% notably suppressed CAD formation on days 1, 3, and 7 in comparison to the control group, while maintaining lower formation at days 1 and 3 when compared to that of addition at 0.20% ($p<0.05$). Furthermore, regarding the concentration of HIM, 0.2PCPE shared no differences with 0.4PCPE, whereas it was significantly lower when compared to the control group until storage day 5, whereas on the final storage day, its concentration was the highest in pork patties added at this concentration, followed by 0.40% CPE and control, respectively ($p<0.05$). Moreover, the concentration of TYM did not differ between the control and CPE treated groups on the initial storage day, while its formation was significantly inhibited by CPE from day 3 until the end of the storage day, with the highest added concentration showing a stronger inhibitory effect ($p<0.05$). In addition, with respect to the SPD content in pork patties, the inhibitory effect of CPE was not clearly observed on any storage day in comparison to the control samples. Its concentration was even higher until storage day 5 in CPE-treated

samples ($p<0.05$). Eventually, the concentration of the total BAs in pork patties was significantly reduced by CPE regardless of the percentage from day 3 of storage and remained until the end of the storage period. At day 3, the order of total BAs from the lowest to the highest were 0.2PCPE, 0.4PCPE, and control group with 24.35, 27.26, and 33.40 μg/g respectively ($p<0.05$). Meanwhile, on days 5 and 7 of storage, the higher addition of CPE resulted in a significantly stronger capacity to reduce the total BAs content in pork patties ($p<0.05$).

The formation of individual BAs is strongly determined by the type of raw material and bacterial population. As previously mentioned, decarboxylation occurs in food commodities by certain bacterial colonies that utilize the available source of FAAs (Halász et al., 1994). Therefore, the increase in BAs during storage is species specific. Min et al. (2007) reported that CAD, PUT, and TYM increased greatly during storage in pork loin, which was also observed in the present study. Meanwhile, the increase in HIM and SPD found in this study during storage might be due to the large portion of fat used to make the pork patties, thus allowing a wider range of bacterial colonies and their consequence in generating BAs, which is consistent with a previous study (Cho et al., 2021). In contrast, compared to the control, the reduction of total BAs in pork patties was observed to range between 3.4%–38.1% under treatment with 0.20% CPE and 18.4%–51.4% under treatment with 0.40% CPE. Its strong inhibition rate toward total BAs might be related to the potent antimicrobial activity of calamansi, which agrees with previous studies (Cheong et al., 2012; Husni et al., 2021; Jinap et al., 2018). However, although they have robust antimicrobial activity, calamansi have been reported to contain considerable amounts of PUT. Cipolla et al. (2007) reported that the concentration of calamansi could reach as much 1,047.7 nmol/g, which might underline our findings regarding the high PUT concentration following CPE inclusions. Furthermore, this study demonstrated the possibility of CPE strongly inhibiting the formation of CAD, TYM, and HIM during storage, wherein a higher addition percentage tended to show a greater reduction effect. This is thought to be caused by the strong antimicrobial activity of CPE against Enterobacteriaceae and *Pseudomonas* spp., which act as CAD-producing bacteria (Halász et al., 1994; Triki et al., 2018). In addition, the decarboxylation of tyrosine and histidine by a particular strain of bacteria tended to be hindered by CPE, which lowered the formation of TYM and HIM in pork patties. The regulations issued by the United States Food and Drug Administration (USFDA) state that the threshold for HIM to be safely consumed by humans should be lower than 500 μg/g, should the limit of TYM to cause cell death should not exceed 301.80 μg/g (Linares et al., 2016).

Sensory evaluation of pork patty

Table 11 shows the effects of CPE addition on the sensory perception of pork patties during storage. Color perception differed significantly on days 3 and 5, in which the highest addition percentage tended to receive lower scores from panelists ($p<0.05$). However, the 0.2PCPE samples showed no significant differences from the control samples during the storage period ($p>0.05$). Accordingly, the aroma profile of 0.2PCPE received a similar score to that of the control samples at the beginning of the storage period (days 1 and 3), whereas 0.4PCPE had a significantly lower score ($p<0.05$). However, as the storage period was extended, 0.4PCPE shared a similarly higher aroma score with that of 0.2PCPE when compared to that of control samples on day 5 ($p<0.05$). In addition, in terms of off-odor, significantly different perceptions were observed only on day 3, wherein the addition of CPE at 0.20% did not differ from that of the control samples, while the addition of CPE at 0.40% received a significantly higher score for detected off-odor. The reason might be due to a tendency of higher VBN value at day 3 of storage that owned by 0.4PCPE (7.97 mg/100 g) than that of 0.2PCPE (7.77 mg/100 g) and control (7.77 mg/100 g). According to previous studies, in addition to the oxidation of lipids, the products of protein degradation by microorganisms are another factor for the intensification of off-odor in meat (Barido et al., 2022; Bellucci et al., 2022).

Table 11. Effect of calamansi pulp extract on sensory properties of pork patty during storage at 4℃

Traits	Treatment	Storage days (d)				SEM
		1	3	5	7	
Color	Control	8.47^{Aa}	8.07^{Aa}	6.27^{ABb}	6.07^{Ab}	0.259
	0.2PCPE	8.33^{Aa}	8.20^{Aa}	6.73^{Ab}	5.80^{Ac}	0.226
	0.4PCPE	7.33^{Aa}	7.00^{Ba}	5.87^{Bb}	5.27^{Ab}	0.218
	SEM	0.151	0.237	0.219	0.314	
Aroma	Control	8.27^{Aa}	7.73^{Aa}	4.27^{Bb}	4.07^{Ab}	0.292
	0.2PCPE	7.40^{ABa}	7.33^{Aa}	5.53^{Ab}	4.47^{Ac}	0.273
	0.4PCPE	6.80^{Ba}	6.20^{Ba}	5.67^{Aab}	4.60^{Ab}	0.341
	SEM	0.279	0.295	0.275	0.343	
Drip loss	Control	1.07^{Aa}	1.00^{Aa}	1.07^{Aa}	1.20^{Aa}	0.086
	0.2PCPE	1.23^{Aa}	1.07^{Aa}	1.07^{Aa}	1.13^{Aa}	0.085
	0.4PCPE	1.20^{Aa}	1.07^{Aa}	1.20^{Aa}	1.33^{Aa}	0.125
	SEM	0.096	0.054	0.202	0.146	
Off-odor	Control	1.27^{Ab}	1.73^{Bb}	4.53^{Aa}	5.53^{Aa}	0.299
	0.2PCPE	1.73^{Ac}	1.73^{Bc}	3.80^{Ab}	5.40^{Aa}	0.304
	0.4PCPE	1.80^{Ab}	2.60^{Ab}	4.13^{Aa}	4.80^{Aa}	0.301
	SEM	0.219	0.244	0.286	0.421	
Overall acceptability	Control	8.40^{Aa}	7.80^{Aa}	5.00^{Ab}	4.80^{Ab}	0.264
	0.2PCPE	7.67^{ABa}	7.80^{Aa}	5.53^{Ab}	4.80^{Ab}	0.229
	0.4PCPE	6.93^{Ba}	6.67^{Ba}	5.60^{Ab}	5.07^{Ab}	0.282
	SEM	0.239	0.271	0.184	0.323	

^{A,B} Means within a column with different superscript differ significantly at $p<0.05$.
$^{a–c}$ Means within a row with different superscript differ significantly at $p<0.05$.
0.2PCPE, pork patty with 0.2% calamansi pulp extract addition; 0.4PCPE, pork patty with 0.4% calamansi pulp extract addition.

Conclusion

Food, including meat, can produce harmful substances during storage. In addition, these harmful substances can adversely affect the human body. In this study, CPE was utilized as a natural additive, and its antioxidant and antibacterial activities can be used to enhance the nutritional value and safety of meat. CPE exhibited superior antibacterial activity against pork spoilage and pathogenic bacteria. In addition, the use of this extract maintained quality and prevented the formation of BAs in pork patties, particularly total BAs. In conclusion, this study suggests the potential of CPE as a novel natural food additive to maintain the quality of meat products and inhibit the formation of harmful substances. However, further studies are needed to confirm the irregular fluctuations of PUT and CAD in refrigerated pork patties following CPE addition.

Conflicts of Interest

The authors declare no potential conflicts of interest.

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Ethics Approval

Sensory evaluation was approved by the Kangwon National University Institutional Review Board (KWNUIRB-2020-09-005-002).

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