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Effects of natural extract mixtures on the microbiological and quality characteristics of sausages during refrigerated storage

Abstract

Owing to the residual toxicity and adverse health effects of chemical preservatives, there is an increasing demand for using natural preservatives in food. Although many natural extracts have been evaluated, research on their antibacterial effects remains insufficient. Therefore, this study aimed to explore the possibility of developing *Psidium guajava*, *Ecklonia cava*, and *Paeonia japonica* (Makino) *Miyabe & Takeda* extracts as natural food preservatives. Further, the effect of mixing these extracts on microbial growth and quality was evaluated during the refrigeration of sausages. The antibacterial activity was evaluated against three pathogenic bacteria (*Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli*). The optimal mixing ratios were determined based on the minimum inhibitory and bactericidal concentrations of each mixed extract. D-optimal mixing design optimization tool was further used to obtain an optimum mixing ratio of Formulation 1 (F1). The antibacterial activity of F1 increased with increasing concentration, with similar activities at 0.5 and 1%. On the other hand, sorbic acid added in sausage production did not exhibit antibacterial activity, and grapefruit seed extract demonstrated the antibacterial activity in the shortest time. The sausages with synthetic or natural preservatives showed significantly lower lipid oxidation than those of the control and grapefruit extract-treated sausages after 4 weeks of storage. Total plate counts were observed only in the control and treatment groups stored for 3 weeks, and no significant effect of ascorbic acid was observed. Compared to the other samples, sausages with added natural extracts showed the highest overall acceptability scores initially and after 4 weeks. Therefore, similar amounts of grapefruit seed and natural extracts had the same effect on microbiological analysis and lipid rancidity during sausage storage. Hence, this mixture can serve as a potential natural preservative in meat products.

Keywords: preservative, natural extract, sausage, microorganism, antimicrobial activity, antioxidant

Introduction

Food additives are intentionally added to foods during manufacturing and processing to preserve their flavor or improve their taste, appearance, or other qualities (Yu et al., 2020). Unsaturated fatty acids and high protein concentrations in foods, particularly meat products, are exposed to light during storage, which oxidizes lipids and proteins. To prevent this, various food additives are used in sausage manufacturing (Lee et al., 2020; Yong et al., 2020). Synthetic additives are used effectively in meat products owing to their low cost, high stability, and high efficiency (Alirezalu et al., 2016). Chemical preservatives and antioxidants, including butylated hydroxyanisole and butylated hydroxytoluene (BHT), reduce lipid oxidation and enhance antibacterial activity, extending the product's shelf life (Lee et al., 2016). However, some chemical preservatives are carcinogenic and teratogenic; therefore, their use is restricted in each country.

Sorbate has received global regulatory approval for use as an antibacterial preservative in food, animal feed, pharmaceuticals, and cosmetics (Stopforth et al., 2005). Sorbate is effective against many bacteria, molds, and yeasts and has primarily been used as an antifungal agent in food (Robach et al., 1982). It is a weak acid (pKa = 4.76) in its undissociated form and shows maximum antibacterial activity at low pH. Therefore, it is consistently effective as an antimicrobial agent in foods with pH < 5.0–5.5 (Robach et al., 1982). Potassium sorbate or sorbic acid slows the growth and toxin production of the spoilage microorganisms *Clostridium* in products such as cooked and cured red meat and poultry sausages (Robach et al., 1982). It also extends the shelf life of several meat products, including bacon, and retards the growth of other pathogenic and spoilage microorganisms (Liewen et al., 1985).

Despite sorbates being registered as GRAS (Generally Recognized as Safe), consumers express concerns about their use because sorbates fall into the category of chemical preservatives. Therefore, the development of natural preservatives which can replace chemical

preservatives is required to address consumer and market demands for clean-label foods. The shelf life of meat products is primarily determined by microbial spoilage and lipid peroxidation (Lee et al., 2013). In general, fruits and vegetables contain various phytonutrients with antioxidant properties (Ehlenfeldt et al., 2001). Researchers continue to utilize fruit and vegetable extracts since the antioxidant properties effectively minimize or prevent lipid oxidation in foods, delaying the formation of toxic oxidation products, and extending their shelf life (Aziz et al., 2018).

Grapefruit seed extract has been reported to exhibit highly effective antibacterial activity when applied directly to food (Reagor et al., 2002). It can prevent the growth of foodborne pathogens present in various fruit, vegetable, meat and fish products, and this effect is thought to be caused by quaternary ammonium compounds (QACs) (Kim et al., 2021). Natural antioxidants, including grapeseed and green tea extracts, used to improve the quality of various meat products, have been evaluated on raw beef patties and cooked pork meatballs and successfully retard microbial growth in meat products (Banon et al., 2007). Among the components of berry extract, phenolic acids inhibit the growth of gram-negative bacteria. In particular, ellagitannin, a common ingredient in cloudberry, raspberry, and strawberry extracts, has a strong inhibitory effect on *salmonella* (Puupponen-Pimiä et al., 2001). Porcine and bovine ground meat stored in the raw and cooked state, show reduced lipid oxidation in the presence of oregano and sage essential oils (3% w/w) during storage at 4℃ for 12 days. Oregano essential oil consists of 20 ingredients, including thymol, *p*-cymene, γ-terpinene and carvacrol, and sage essential oil consists of 37 visible ingredients, including eucalyptol, camphor and α-pinene (Fasseas et al., 2008). Rosemary extract (main active ingredient is carnosic acid) was evaluated in various meat products, including chicken Frankfurt sausages, turkey products, and cooked ground beef. All samples showed lower thiobarbituric acid reactive substances (TBARS), a lipid oxidation values, indicating better oxidative stability than those of the control

samples (Rižnar et al., 2006; Yu et al., 2002; Ahn et al., 2007). In summary, these studies provide sufficient evidence for the use of natural antimicrobials as alternatives to chemical antimicrobials.

Psidium guajava is widely distributed in subtropical climates, and guava leaves possess higher antioxidant activity than that of its fruits (Lestari et al., 2022). Several functional ingredients such as terpenoid flavonoids, tannins, and quercetin are present in *Psidium guajava* leaves (Biswas et al., 2013). *Ecklonia cava* contains various compounds, including carotenoids, fucoidans, and phlorotannins, and has various physiological functions, including antioxidant, anticancer, and antihypertensive properties (Wijesinghe et al., 2012). *Paeonia japonica* (Makino) *Miyabe & Takeda* is used as a medicinal plant and is valuable as a functional food, owing to its antioxidant and antibacterial activities. Although many natural extracts have been studied primarily for their physiological functions, research on their antibacterial effects remains insufficient.

Therefore, the purpose of this study is to determine the optimal mixing ratio of extracts (*Psidium guajava*, *Ecklonia cava*, and *Paeonia japonica* (Makino) *Miyabe & Takeda*), investigate the antibacterial activity against pathogenic bacteria, and prepare a natural extract mixture that can be universally used against various types of pathogenic bacteria. And then, we aimed to determine its potential as a potential natural preservative for meat products by applying the natural extract mixture to sausages and investigating its effect on microbial growth and quality of the products during refrigerated storage.

Materials and Methods

Part 1. Effect of natural extract mixtures

Preparation of natural extracts

The plants used in this study were *Psidium guajava* (Youngchon, Korea), *Ecklonia cava* (Youngchon, Korea), *Paeonia japonica* (Makino) *Miyabe & Takeda* (Jechon, Korea), which were procured from the domestic market. Each sample was pulverized using a grinder (Cgoldenwall, China), mixed with 50% (v/v) ethanol at a ratio of 1:10, and stirred (120 rpm) at room temperature for 24 h. Supernatants of the extracts were separated using centrifugation (1,519 × g, 5 min), and impurities were removed using Whatman filter paper. Finally, the solvent was removed under reduced pressure using a rotary evaporator (Eyela N-3000, Shanghai Eyela, Shanghai, China), lyophilized, and stored in a quick freezer at -70℃. The stored extract was prepared at a concentration of 160 mg/mL by dissolving in tryptic soy broth (TSB, Becton, Dickinson and Company, Sparks, Philadelphia, PA, USA) containing 20% dimethyl sulfoxide.

Bacterial strains and growth conditions

Antibacterial activity was determined against three bacterial species, *Listeria monocytogenes* (LM, gram-positive), *Salmonella* spp. (SAL, gram-negative), and *Escherichia coli* (EC, gram-negative), which are associated with foodborne illnesses in meat products. *L. monocytogenes* strains (NCCP 10920, NCCP 10943, ATCC 13932, ATCC 51774, and ATCC BAA 839) were activated in 10 mL TSB containing 0.6% yeast extract (TSBYE) and incubated at 30℃ for 24 h. *Salmonella* spp. (*Enteritidis* NCCP 14645, *Typhimurium* NCCP 12219, *Typhimurium* NCCP 16207, Montevideo NCCP 10140, Kentucky NCCP 11686) and *E. coli* (NCCP 13717, NCCP 13718, NCCP 13719, NCCP 13720, and NCCP 13721) were activated in 10 mL TSB and incubated at 37℃ for 24 h.

Aliquots of bacterial cultures were sub-cultured in the same medium under the same conditions. The cultures were then centrifuged (1,912 × g, 15 min) and washed twice with 0.85% sterile saline (Cleancer; JW Pharmaceutical, Dangjin, Republic of Korea). A mixture of the same strains was used as inoculum for the experiments.

Evaluating antimicrobial activity of the natural extracts

The minimum inhibitory concentrations (MIC), defined as the lowest concentration of natural extracts and mixtures with no visible growth, were determined using the serial dilution method. Here, samples were 2-fold serially diluted and 90 μL aliquots of each sample was placed in individual wells of a 96-well microplate using TSB for EC and SAL or TSBYE for LM. Then, the bacterial samples were inoculated in each well at a concentration of 6–7 log colony forming units (CFU)/mL and incubated for 24 h at 37℃ (TSB) or 30℃ (TSBYE). Microbial growth was evaluated by measuring the turbidity of each well at 600 nm using a microplate reader (BioTek, Winooski, VT, USA).

The minimum bactericidal concentrations (MBC) of the plant extracts and mixtures were determined based on bacterial growth by streaking the samples on agar plates. After analyzing the MIC of the 96-well microplates, the contents of all microplates were streaked on TSA or TSAYE and incubated at 37℃ or 30℃, respectively, for 24 h. The lowest concentration in the plate with no growth was considered as the MBC. In both MIC and MBC experiments, sorbic acid and grapefruit seed extract were used as controls.

Experimental design for natural extract mixtures

Design Expert software 7.0 (Stat-Ease Inc, Minneapolis, MN, USA) was used to determine the optimum ratio of natural extracts, and D-optimal design was used as the mixture design. The independent variables selected were *Ecklonia cava* (A), *Psidium guajava* (B), and *Paeonia*

157 *japonica* (Makino) *Miyabe & Takeda* (C), and MIC and MBC were set as the dependent
158 variables. The total concentrations of *Psidium guajava*, *Ecklonia cava*, and *Paeonia japonica*
159 (Makino) *Miyabe & Takeda* were set to 160 mg/mL, and the minimum and maximum ranges
160 were set through preliminary experiments as follows: 2.34 mg/mL < *Ecklonia cava* <150
161 mg/mL, 2.34 mg/mL < *Psidium guajava* < 150 mg/mL, 4.68 mg/mL < *Paeonia japonica*
162 (Makino) *Miyabe & Takeda* < 150 mg/mL. The mixing ratios of the materials for each setting
163 range are listed in Table 1. Six repetition points were observed among the fourteen experimental
164 points.
165
166 **Optimization method of natural extracts mixture ratio**
167 The optimization method is suitable for increasing the process efficiency and does not require
168 an increase in cost. In this study, the material mixing ratio was optimized through numerical
169 optimization of the standard model and model optimization of the mixing components (Park et
170 al., 2017). The goals of the experimental items were minimized and in range, and the
171 optimization was determined using a multiple-response method called desirability.
172
173 **Effect of natural extracts mixture on the foodborne pathogen growth curve**
174 The effects of inhibitory concentrations of the natural extract mixtures on the growth of EC,
175 SAL, and LM within 2 days were evaluated. Concentrations of the natural extract mixtures used
176 were 0.2% (F0.2), 0.5% (F0.5), and 1% (F1.0); TSB (CON) was used as a negative control, and
177 sorbic acid (SOR) and grapefruit seed extract (GFS) were used as positive controls. Fifty
178 microliters of the solutions (at different concentrations) were divided into bottles to which, 500
179 μL of the bacterial suspension was added (maximum bacterial concentration of 6–7 log
180 CFU/mL) and incubated at 20˚C. The dilutions of interest were made on 0, 1, and 2-day and

cultured in TSA or TSAYE at 37℃ or 30℃, respectively, for 24 h. Following this, colony count and bacteria number (log CFU/mL) were determined.

Part 2. Experiment of sausages during storages

Preparation of sausages

Fresh pork ham muscles and pork back fat were ground using a chopper (3 mm plate). The sausages were formulated as described by Lee et al. (2021), using ground pork ham (50%), pork back fat (25%), and ice water (25%). Ground pork was homogenized using a silent cutter; salt (1.5%) and phosphate (0.3%) were then added. Sausage batter was prepared by adding fat (25%) and ice (25%); The natural extracts were then added and combined using a silent cutter. following this, the batter was filled with a collagen casing (approximate diameter: 25 mm). The control contained no preservatives, while T1 and T2 contained 0.2% sorbic acid and 0.2% grapefruit seed extract, respectively. The natural extracts were treated as T3 (0.2% natural extract), T4 (0.2% natural extract + 0.01% ascorbic acid), T5 (0.5% natural extract), and T6 (0.5% natural extract + 0.01% ascorbic acid). Cooking was performed at 85℃ for 30 min in a smoke chamber (MAXi3501 Chamber, Kerres, Postfach, Germany). Each portion of the sausage was vacuum packed as described previously and used for storage analysis after 0–28 days (Woo et al., 2023).

pH

The pH values of the sausages containing the natural extract mixtures were determined in a homogenate prepared with sausage sample (5 g) and DW (20 mL) using a pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland).

Color

CIE (Commission internationale de l'Eclairage) L*a*b* color analysis was conducted on sausages containing a mixture of natural extracts. The CIE L* (lightness), CIE a* (redness), and CIE b* (yellowness) values of the sausages containing the natural extract mixtures were determined using a CR-410 colorimeter (Minolta Ltd., Tokyo, Japan) calibrated with a white plate (Illuminate C Observer 2°).

Thiobarbituric acid reactive substances (TBARS)

Lipid oxidation in sausages containing the natural extract mixtures was determined using the TBARS method, described by Tarladgis et al. (1960). Sausages (10 g) were blended with distilled water (50 mL) and 0.3% BHT (200 µL) at 10,000 rpm for 60 s. Distilled water (47.5 mL), 4 N HCl (2.5 mL), and an antifoam agent (1 mL) were added to the flask. The distilled mixture (5 mL) and 0.02 M 2-thiobarbituric acid (5 mL) were added to a test tube. The sample solutions were mixed and heated in a water bath at 100 ℃ for 30 min. Absorbance was measured at 538 nm using a UV/Vis spectrophotometer. TBARS value corresponding to the malonaldehyde content was calculated using the formula of a previous research (Tarladgis et al., 1960), and it expressed as mg per kg of meat.

Total Plate Counts (TPC)

Microbiological analysis was conducted at 1, 14, 21, and 28 days during storage at 4℃. The samples were suspended in sterile saline (0.85%) and homogenized in a stomacher (MiniMix® 100, Interscience, St Nom, France) for 1 min. Aliquots of the homogenates were serially diluted, and 1 mL of each dilution was dispensed into a 3M Petrifilm Plate (3M, St. Paul, MN, USA) for total plate counts, coliform, and EC. The plates for total plate counts were incubated for 24-48 h at 37℃. Coliform and EC plates were incubated for 24 h at 37℃. Colonies were counted, and the results were expressed as log CFU/g of the sample.

Sensory evaluation

A total of 69 adults were selected from the Korea Food Research Institute (KFRI, Wanju, Korea). The panelists were aged 20–50 years (37 women and 32 men). Before the evaluation, the samples were boiled at 100℃ for 5 min and cooled at room temperature. They were cut into 2 cm-thick slices and placed in plastic cups covered with plastic lids. The samples were coded using 3-digit random numbers and presented according to the Williams–Latin square design. Spring water and unsalted crackers were provided to clean the mouth between different samples. The panelists evaluated the overall sausage samples using a 9-point hedonic scale (from 1 point = "extremely dislike" to 9 point = "extremely like") and other sensory properties using the RATA (Rate-All-That-Apply) method. Assessors were asked to select all the terms that described the samples and then rate the intensity of the selected terms on (3-point scale). The intensity was evaluated on a 3-point scale with guiding value labels (i.e. 1 = "low," 2 = "medium," 3 = "high"). This study was approved by the Institutional Review Board of KFRI (KFRI-2023-05-002-001).

Statistical analyses

The quantified results are shown as means±standard deviation. One-way and two-way analysis of variance were performed for statistical analyses using the IBM SPSS statistical software (SPSS Ver. 20.0, IBM, IL, USA). The significance of variations among the mean values was assessed using Duncan's multiple range test, with a confidence level of $p < 0.05$. An independent sample t-test ($p < 0.05$) was performed to determine significant differences in the sensory preference scores. A principal component analysis (PCA) biplot was constructed using the SIMCA 17 software (Umetrics, Umea, Sweden).

Results and Discussion

Part 1. Effect of natural extract mixtures

Design and antimicrobial activity of the natural extract mixtures

Table 1 lists the mixing ratios of the natural extract blends prepared at the 14 test points. The antibacterial effects of the mixed extracts were confirmed by measuring the MIC and MBC values for the three types of bacteria (EC, SAL, and LM) (Table 2). The MIC of the 14 mixed extracts ranged from 2.5–20.0 mg/mL, while the MBC values ranged from 2.5–40.0 mg/mL. The main components of *Psidium guajava*, *Ecklonia cava,* and *Paeonia japonica* (Makino) *Miyabe & Takeda* extracts are flavonoids, phenols, steroids, tannins, phlorotannins, and eckol. These extracts exhibit antibacterial properties against EM, SAL (gram-negative bacteria), *Saccharomyces cerevisiae* (yeast strain), and *Aspergillus niger* (fungal strain) (Das et al., 2019; Choi et al., 2014; Choi et al., 2010). Mixing the extracts may alter their active ingredients and concentrations, which can affect antibacterial activity (Ouedrhiri et al., 2016). The mixtures of 14 natural extracts showed antibacterial effects against EC at both MIC and MBC of 2.5–10.0 mg/mL. For SAL, the MIC and MBC were 2.5–10 mg/mL and 2.5–20 mg/mL, respectively. For LM, the MIC and MBC were 5–20 mg/mL and 10–40 mg/mL, respectively, showing relatively lower antibacterial activity than those of EC and SAL. The sorbic acid showed higher MBC values than those of the mixed extracts, with MIC and MBC values ranging from 2.5–5.0 mg/mL and 10.0–160.0 mg/mL, respectively. In contrast, grapefruit seeds exhibited antibacterial effects at 0.16–0.31 mg/mL for, both, the MIC and MBC. Grapefruit seeds are known natural antimicrobial agents with strong antibacterial and antifungal properties (Kim et al., 2021).

Fig 1 shows ternary diagrams (3D images and contour plots) illustrating the interactions between each independent variable used for analyzing antibacterial activity against the three pathogens (EC, SAL, and LM). In the contour plot and 3D image, dark blue areas represent low

MIC and MBC values (high antibacterial efficacy), while green to red areas represent medium to high MIC and MBC values (low antibacterial efficacy). The natural extract mixtures prepared according to the mixture design combinations showed stronger antibacterial effects against EC and SAL (gram-negative bacteria) than that against compared to Gram-positive bacteria, LM. The type of bacteria exhibiting an antibacterial effect may vary depending on the type of compound included in the natural extract mixture and the reaction expression method (Mau et al., 2001; Chakraborty et al., 2007). According to Table 2 and Fig 1, the antibacterial effect against pathogens in the central mixture design was found to weaken as the ratio of white peony increased. Consequently, a blend containing 49% *Ecklonia cava*, 48% *Psidium guajava*, and 3% *Paeonia japonica* (Makino) *Miyabe & Takeda* exhibited the lowest MIC (2.5–5.0 mg/mL) and MBC (5.0–10 mg/mL) against all tested pathogens, thus representing the highest antibacterial activity against the tested pathogens.

Various compounds can be mixed either by mixing essential oils (EO) and plant extracts and blending several types of plant extracts (Poimenidou et al., 2016; Mau et al., 2001). This approach has received significant attention because of its potential use as a natural additive in various foods. The combination of plant extracts and the main components of EO (-pinene, camphene, myrcene, -terpinene, p-cymene) can improve antibacterial and antifungal activity compared to individual hydrocarbons (Hossain et al., 2016; Nikkhah et al., 2017). Moreover, p-cymene, found in, both, plant extracts and EO, exhibits a high affinity for cell membranes, making it a membrane-exchanging impurity that assists carvacrol in penetrating cells, thereby increasing antibacterial efficacy (Poimenidou et al., 2016). Mixtures of *Calendula officinalis* extracts demonstrate excellent antibacterial effects against various bacteria owing to the complementary antibacterial activity of each extract fraction when used in combination (Mau et al., 2001). Therefore, blending various plant extracts is a natural and powerful method of enhancing their antibacterial properties when used as food additives.

306

307 **Optimization of active formulations**

308 In this study, a D-optimal mixture design optimization tool was used to predict the optimal
309 mixing ratio of natural extracts to achieve high antibacterial activity. The formulation 1 (F1)
310 mixing ratio was *Ecklonia cava* 58.40%, *Psidium guajava* 39.68%, and *Paeonia japonica*
311 (Makino) *Miyabe & Takeda* 1.92%. Table 3 shows the antibacterial test results of the composite
312 extracts prepared under the aforementioned conditions for the three pathogens. F1 exhibited
313 antibacterial activity against EC at 2.5 mg/mL for both MIC and MBC and against SAL at 1.25
314 and 2.5 mg/mL for MIC and MBC, respectively. In the case of LM, the MIC concentration was
315 2.5 mg/mL, and the MBC concentration was 5.0 mg/mL, indicating antibacterial activity.
316 Typically, a desirability value of 0.8–1.0 is considered favorable for product quality, and F1
317 scored 0.97.

318 Table 4 illustrates antibacterial activity by analyzing growth curves of the pathogens at
319 different concentrations of F1 (0.2%–M0.2, 0.5%–M0.5, and 1.0%–M1.0). This method was
320 used to determine the optimum concentrations for subsequent sausage production. The initial
321 concentrations of the three tested bacteria were 3–4 log CFU/mL. All concentrations of the
322 mixed extract gradually reduced the growth of all the three types of bacteria over 2 d. In
323 particular, F1 concentrations of 0.5 and 1% completely inhibited EC and LM after 2 d. Thus,
324 different concentrations of the natural extract mixture presumably controlled bacterial growth.
325 Additionally, growth of all the pathogens in the grapefruit seed group was inhibited after 1 d.
326 In contrast, the control and sorbic acid-treated groups showed increased bacterial growth after
327 2 d.

328 Developing natural extract mixtures using a central mixture design approach is an important
329 technique for creating natural preservatives to replace chemical preservatives in the food
330 industry. This helps minimize damage to the sensory properties of foods while effectively

331 controlling pathogens through the potential synergy between extracts. Combinations of
332 antimicrobial compounds can reduce undesirable organoleptic properties of foods by using
333 lower effective concentrations in foods (Tiwari et al., 2009).
334

Part 2. Experiment of sausages during storages

pH and color

pH and color are important quality-indicating properties of meat products, such as sausages, (Lee et al., 2021). The changes in pH and color of sausages in this study are presented in Table 6. The highest pH value at week 0 of treatment was observed for sorbic acid treatment (T1), followed by that of the control ($p<0.05$). The grape seed and natural extract treatments had the lowest pH values ($p<0.05$), and significant differences between these treatments were not observed ($p>0.05$). These findings could be attributed to the presence of phenolic compounds and organic acids in the natural extracts (Lee et al., 2021; Woo et al., 2023). The typical pH values of natural compounds vary the pH of meat products (Choi et al., 2022; Kim et al., 2019; Lee et al., 2021; Woo et al., 2023). Changes in pH during storage are important in the manufacture of meat products. In emulsified sausages, a stable pH during storage is important because oxidation and microbial growth in meat products are affected by pH (Song et al., 2017). Although microbial growth was majorly inhibited by heating (Shin et al., 2017), microbial changes were observed in the control group at weeks 3 and 4 (Table 5). In addition, pH of the sausages declined during storage due to the oxidation of lipids and proteins. Decay metabolites (volatile basic nitrogen) produced by microbes (gram-negative bacteria) during storage also affect the pH of sausages (Hwang et al., 2017); the TBARS value of the control was the highest ($p<0.05$). Therefore, these results may have affected the pH value of the control, and sausages containing natural extracts may have more stable quality characteristics than those of the control.

The typical color of the added natural extract has a critical impact on the color value of meat products (Kim et al., 2019). The color values of the emulsified sausages are listed in Table 6. Treatments with natural extracts had lower CIE L* and CIE b* values and higher CIE a* values than those of T1 and T2 ($p<0.05$). After 1 week, the CIE L* value increased, but decreased after

2 weeks. This result may be due to the exudate from the sausages at week 1. The light-scattering effects of moisture on the surface increased the CIE L* value of the sausages. During the storage period, sausages lose excessive moisture, causing them to dry and reduce their lightness (Shin et al., 2017; Song et al., 2017). They also tend to show a decrease in lightness, redness, and yellowness as the color pigments are oxidized and denatured over time (Lee et al., 2021; Shin et al., 2017; Woo et al., 2023).

TBARS

The antioxidant effects of the natural extracts on pork sausage were evaluated by analyzing TBARS during the 4 weeks of storage (Fig. 2). On oxidation, lipids form aldehydes as secondary oxidants, one of which, malondialdehyde (MDA) reacts with thiobarbituric acid. Thus, the TBARS value was used as a parameter of lipid oxidation. During the manufacturing and thermal treatment of the sausages, the TBARS values of the control increased owing to thermal stress. Compared with the control, sausages with synthetic or natural preservatives showed significantly lower TBARS values at 0 week of storage ($p<0.05$). Natural extracts were more effective in preventing lipid oxidation than those using sorbic acid and grapefruit seed extracts, regardless of the amount of ascorbic acid added. During the storage period, the control, T1, and T2 groups showed an increase in TBARS values until 3 weeks, followed by a decrease at 4 weeks. Elevated TBARS values due to lipid oxidation are associated with the rancid flavor of meat (Lai et al., 1995). The decrease in TBARS values after 4 weeks can be attributed to the degradation of MDA by further oxidation, which generates other alcohols and acid products (Azizkhani and Tooryan, 2015). The addition of natural extracts to pork sausages prevented an increase in TBARS values to less than 0.4 mg/kg during 4 weeks. The natural extract mixture composed of *Ecklonia cava*, *Psidium guajava*, and *Paeonia japonica (Makino) Miyabe & Takeda* showed excellent antioxidant activity with high polyphenol and flavonoid content

(Senevirathne et al., 2006; Camarena-Tello et al., 2018; Kim et al., 2016). In *Ecklonia cava*, numerous phlorotannins were contained, and especially 6,6′-bieckol, one of the phlorotannins, had prominent antioxidant activity with high yield (Li et al., 2009). In addition, according to a previous research, ethanol extract of *Psidium guajava* leaf showed remarkable antioxidant effect equivalent to 4.91 mM/mg of trolox (Tachakittirungrod et al., 2007). Also in another study, ethanol extract of *Paeonia japonica* showed high polyphenol content (125.1 mg/g) and flavonoid content (136.1 mg/g) (Kim et al., 2016). Plant phenolic compounds are known food antioxidants; however, their optimum concentration is important for effective antioxidant activity (Balasundram et al., 2006). Therefore, compared to sorbic acid and grapeseed extracts, the optimized natural extract and its concentration in our study were appropriate for use as an antioxidant for lipids in emulsion-type pork sausages.

Microbiological analysis

The total plate counts of sausages containing preservatives during 28 days of storage was shown in Table 5. Total microbes were not detected during the storage period in the treatments with preservatives added, including sausage in natural preservative extract. The control was not detected until the 14 days of storage. It was increased to 2.91 log CFU/g after 28 days. This result is in line with those reported by Qiu and Chin (2022), who found that the addition of lotus rhizome root powder with antibacterial activity reduces the total bacterial counts in sausages compared with those of the control during storage. Also, Coliform and *E. coli,* were not detected during the storage period in all treatments. The particular species of bacteria that contaminate the meat determine will determine the spoilage profile of muscle foods stored under environmental conditions (Tajik et al., 2014). Mesophilic bacteria are the most important spoilage microorganisms that deteriorate meat and meat products during storage (Alirezalu et al., 2019). The natural preservatives used in this study were mixed extracts of *Ecklonia cava*,

410 *Psidium guajava*, and *Paeonia japonica (Makino) Miyabe & Takeda*. It has been reported that
411 guava leaf extract shows high antibacterial activity against gram-positive bacteria and that the
412 flavonoid compounds with antibacterial activity (Arima and Danno, 2002; Jo et al. 2009).
413 *Ecklonia cava* and *Paeoniae radix* extracts were also reported for antibacterial activity (Ahn
414 1998; Chang et al., 1996; Lim et al., 2008; Park and Cho, 2010). Fu et al. (2007) reported a
415 combination of different plant-originated compounds could have additive, synergistic or
416 antagonistic effects depending on the type of microorganism, and the results were in the same
417 vein as our study. Therefore, these results suggest that the natural extracts could have
418 applications as a natural preservative for sausage.
419
420 **Sensory evaluations**
421 Table S1 shows the average sensory scores and standard deviations of the sausage samples.
422 Fig. 3 summarizes the results of the sensory evaluation. The changes in overall acceptability
423 are shown in Fig. 3A. After a storage of 4 weeks, the mean score for overall acceptability
424 significantly decreased ($p<0.05$) in the control group (no preservatives). Thus, the preservatives
425 in sausages not only controlled the growth of bacteria but also affected flavor. Compared to the
426 other groups, the control group showed higher TBARS values, which represent the degree of
427 lipid oxidation which is highly associated with the quality of sausages. Initially, the overall
428 acceptability value was the highest in T3 and remained the highest among the samples after 4
429 weeks. Natural extracts (T3) imparted an acceptable appearance and flavor to sausages. In
430 contrast, T2 had the lowest overall acceptability value among the samples. This may be because
431 natural preservatives, including natural extracts and grapefruit seeds, affect the flavor of
432 sausages. It was reported that chicken breast with grapefruit extract had a higher score of overall
433 acceptability than the untreated samples (Kang et al., 2017). However, in this study, the addition
434 of grapefruit seed extract to pork sausage had negatively affected overall acceptability. On the

other hand, a previous study (Velasco-Arango et al., 2021) showed that a higher mass fraction of guava extract, which is an ingredient of the natural extract in T3, led to a decrease in the acceptance of the sausage. It might be explained that the sensory results may vary depending on the amount of natural extract added and the target to which they are added.

The principal component analysis (PCA) biplot visualizes the sensory attributes and shows the separate groups of sausages with different storage times and types of preservatives (Fig. 3B). The biplot explains 73% of the total variation, including PC1 (44% of the variance) and PC2 (29%). R^2 was used to evaluate the goodness-of-fit of the PCA model. A higher R^2 value indicates higher explanatory power of the regression model (Yang, Lu, Huang, Huang, Ogata, & Lin, 2018). In this study, the PCA model performed well ($R^2X=0.73$) in discriminating between samples. In the PCA score plot, T2 and T3 showed different flavor profiles compared to those of the control and T1. Some sensory attributes, such as brownness, umami, and smoky odor, were strongly associated with T3, whereas T2 was associated with bitter taste. After storage for 4 weeks, the sensory characteristics of the samples weakened, and other sensory features were up- or downregulated. For example, some sensory properties, such as meaty odor, saltiness, and meaty taste, decreased in all samples after 4 weeks. Sourness affected the flavor of T1 after 4 weeks of storage. The sourness score decreased with storage in T1 but increased in the other samples. Pork odor and bitterness, which are located in the dimension opposite to overall acceptability, could negatively affect overall acceptability. In this study, the sensory properties of T2, which were located on the opposite dimension of overall acceptability, may have a negative effect on the quality of pork sausage, while those of T3 were related to a positive effect on the quality of pork sausage. Additionally, studies have shown that adding ascorbic acid to natural extracts increases the antibacterial effect (Gedikoğlu et al., 2022). However, no significant effect of ascorbic acid was observed in our study. These results suggest that the natural extract used in this study can served as a promising alternative preservative for sausages.

Conclusion

In this study, the optimal mixing ratio of three carefully selected natural extracts (*Ecklonia cava, Psidium guajava, Paeonia japonica (Makino) Miyabe & Takeda*) was established to obtain an extraction mixture with antibacterial and antifungal activity against various microorganisms. Storage tests were conducted by applying mixed extracts of various concentrations to sausages, and ascorbic acid was added to confirm the synergistic effect on the storage stability of sausages. Naturally derived *Ecklonia cava*, *Psidium guajava*, and *Paeonia japonica (Makino) Miyabe & Takeda* mixtures showed preservative effects similar to those using grapefruit seed extract. For the same amount of preservative, the natural extract group had a higher overall acceptability than that of the grapefruit seed extract treatment group in the sensory evaluation. Therefore, the mixture of *Ecklonia cava*, *Psidium guajava*, and *Paeonia japonica (Makino) Miyabe & Takeda* prepared in this study can be used as a potential natural preservative in meat products.

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674

675 **Table 1. Experimental design for plant extracts using the D-optimal method**

No.	Independent variable (%)		
	A : *Ecklonia cava*	B : *Psidium guajava*	C : *Paeonia japonica* (Makino) Miyabe & Takeda
1	49	48	3
2	4	25	71
3	1	72	27
4	94	1	5
5	49	48	3
6	3	94	3
7	94	1	5
8	26	71	3
9	47	4	49
10	1	50	49
11	4	2	94
12	47	4	49
13	32	35	33
14	64	17	19

676 Where A+B+C=100%

677

678 **Table 2. Antimicrobial and antioxidant activities of the natural extracts**

No.	MIC (mg/mL)			MBC (mg/mL)		
	EC	SAL	LM	EC	SAL	LM
1	2.50	2.50	5.00	2.50	2.50	10.00
2	10.00	10.00	20.00	10.00	10.00	20.00
3	10.00	5.00	5.00	10.00	10.00	20.00
4	2.50	2.50	5.00	2.50	5.00	10.00
5	2.50	2.50	5.00	2.50	2.50	10.00
6	5.00	5.00	5.00	2.50	5.00	10.00
7	2.50	2.50	2.50	5.00	2.50	10.00
8	5.00	2.50	5.00	2.50	2.50	10.00
9	2.50	2.50	5.00	5.00	5.00	10.00
10	10.00	10.00	10.00	10.00	10.00	20.00
11	10.00	5.00	20.00	10.00	20.00	40.00
12	2.50	2.50	5.00	5.00	5.00	10.00
13	5.00	2.50	5.00	5.00	10.00	10.00
14	2.50	2.50	5.00	2.50	5.00	10.00
Sorbic acid	2.50	5.00	5.00	40.00	40.00	160.00
Grape fruit seed	0.16	0.16	0.16	0.31	0.16	0.31

679 MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; EC,
680 *Escherichia coli*; SAL, *Salmonella* spp.; LM, *Listeria monocytogenes*. All mean values are
681 presented by the mean of three replicates.

682

683 **Table 3. The optimized formulation based on the constraints applied to the significant**
684 **variables and the corresponding antibacterial results are actual experimental values**

		Formulation 1
A: *Ecklonia cava*		58.40
B: *Psidium guajava*		39.68
C: *Paeonia japonica* (Makino) *Miyabe & Takeda*		1.92
Total (A+B+C)		100.00
Desirability		0.97
*MIC (mg/mL)	EC	2.50
	SAL	1.25
	LM	2.50
*MBC (mg/mL)	EC	2.50
	SAL	2.50
	LM	5.00

685 * Average value measured through actual three repeated experiments. MIC, minimum
686 inhibitory concentration; MBC, minimum bactericidal concentration; EC, *Escherichia coli*;
687 SAL, *Salmonella* spp.; LM, *Listeria monocytogenes*.
688

689 **Table 4. Foodborne pathogen counts (log CFU/mL) during storage in the broth with added**
690 **natural extracts**

691 (Unit: log CFU/mL)

Foodborne pathogen	EC					
Day	CON[1)]	SOR	GFS	F0.2	F0.5	F1.0
0	3.83±1.22^{Ca}	4.01±0.98^{Ba}	3.85±0.78^{Aa}	3.91±0.95^{Aa}	4.00±0.90^{Aa}	3.88±0.81^{Aa}
1	7.72±0.00^{Ba}	5.85±0.00^{Bb}	0.00±0.00^{Bd}	4.27±0.65^{Ac}	3.20±1.20^{Ac}	3.25±0.70^{Ac}
2	11.14±0.00^{Aa}	11.00±1.81^{Aa}	0.00±0.00^{Bb}	0.78±0.00^{Bb}	0.00±0.00^{Bb}	0.00±0.00^{Bb}
Foodborne pathogen	SAL					
Day	CON	SOR	GFS	F0.2	F0.5	F1.0
0	3.88±1.38^{Ca}	3.97±0.81^{Ba}	3.32±0.93^{Aa}	3.92±1.02^{Aa}	3.76±1.28^{Aa}	3.79±1.22^{Aa}
1	7.79±0.00^{Ba}	5.85±0.00^{Bb}	0.00±0.00^{Bd}	2.85±0.00^{Ac}	3.13±0.00^{Ac}	3.02±0.78^{Ac}
2	11.66±2.22^{Aa}	11.02±2.65^{Aa}	0.00±0.00^{Bb}	1.18±0.48^{Bb}	0.78±0.78^{Bb}	0.78±0.48^{Bb}
Foodborne pathogen	LM					
Day	CON	SOR	GFS	F0.2	F0.5	F1.0
0	4.13±1.13^{Ca}	4.10±0.48^{Ba}	4.06±1.13^{Aa}	4.10±0.00^{Aa}	4.06±1.26^{Aa}	3.85±0.65^{Aa}
1	6.78±0.00^{Ba}	5.74±0.00^{Bb}	0.00±0.00^{Bd}	3.26±0.00^{ABc}	2.87±0.78^{Ac}	2.69±0.30^{Bc}
2	11.30±1.35^{Aa}	10.87±1.70^{Aa}	0.00±0.00^{Bc}	2.21±1.41^{Bb}	0.00±0.00^{Bc}	0.00±0.00^{Cc}

692 [1)]CON, TSB broth; SOR, 0.2% sorbic acid; GFS, 0.2% grapefruit seeds; F0.2, 0.2% natural
693 extract; F0.5, 0.5% natural extract; F1.0, 1.0% natural extract; CFU, colony forming units

694 [A-C] means within a column in different letters are significantly different ($p < 0.05$).

695 [a-d] means within a row in different letters are significantly different ($p < 0.05$).

696

Table 5. Microbial counts of sausages added with natural extract during storage periods

	Storage period (weeks)	Control	T1	T2	T3	T4	T5	T6
Total plate counts	0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	3	2.31±0.03	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	4	2.91±0.11	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Coliform/ E Coli.	0	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

(Unit: log CFU/g)

T1, No additives; T2, 0.2% grapefruit seed; T3, 0.2% natural extracts; T4, 0.2% natural extracts and ascrobic acid; T5, 0.5% natural extracts; T6, 0.5% natural extracts and ascrobic acid; N.D., Not detected.

Table 6. pH and color of sausages added with natural extract during storage periods

Traits	Storage Period (weeks)	Treatments[1)]						
		Control	T1	T2	T3	T4	T5	T6
pH	0	6.05±0.01^{Cb}	6.08±0.02^{Aa}	5.99±0.01^{Ac}	5.98±0.02^{ABc}	5.97±0.01^{Bc}	5.97±0.04^{Bc}	5.99±0.01^{Ac}
	1	6.16±0.01^{Aa}	5.91±0.02^{Ce}	5.98±0.04^{Ab}	5.93±0.01^{Bde}	5.91±0.01^{Ce}	5.97±0.01^{Bbc}	5.95±0.02^{Bcd}
	2	5.96±0.01^{Db}	6.01±0.01^{Ba}	5.90±0.02^{Bc}	5.98±0.03^{ABab}	5.96±0.04^{Bb}	5.95±0.02^{Bb}	5.91±0.03^{BCc}
	3	6.04±0.02^{Cab}	6.07±0.01^{Aa}	6.00±0.01^{Ac}	6.04±0.01^{Ab}	6.05±0.02^{Aab}	6.06±0.01^{Aab}	5.88±0.02^{Cd}
	4	6.11±0.02^{Ba}	6.00±0.05^{Bb}	6.01±0.02^{Ab}	5.96±0.08^{Bb}	5.85±0.01^{Dc}	5.96±0.01^{Bb}	5.81±0.05^{Dc}
CIE L^*	0	74.19±0.54^{Bb}	75.28±0.94^{Ca}	73.69±0.99^{Db}	69.03±0.61^{Cc}	69.35±0.66^{Cc}	64.61±1.26^{De}	65.78±0.50^{Cd}
	1	76.13±0.85^{Ac}	85.85±1.37^{Aa}	85.47±0.92^{Abc}	79.89±0.45^{Abc}	80.52±0.52^{Ab}	75.80±1.04^{Abc}	76.03±0.59^{Ab}
	2	75.47±0.94^{Ab}	76.40±0.44^{Ba}	76.25±0.54^{Ba}	71.06±0.52^{Bc}	70.49±0.70^{Bc}	66.92±0.80^{Bd}	67.54±0.85^{Bd}
	3	75.64±0.52^{Aab}	76.68±0.41^{Ba}	75.11±0.56^{Cb}	68.95±2.55^{Cd}	70.40±0.49^{Bc}	65.91±0.49^{Ce}	66.04±1.00^{Ce}
	4	71.80±0.68^{Ca}	71.52±0.72^{Da}	69.81±0.99^{Ec}	64.05±0.94^{Dd}	64.17±0.75^{Dd}	58.73±0.85^{Ef}	60.35±0.39^{De}
CIE a^*	0	4.08±0.20^{Ac}	3.68±0.13^{Ad}	3.37±0.17^{Ae}	4.79±0.10^{Bb}	4.01±0.15^{Bc}	4.97±0.10^{Ba}	4.73±0.09^{Bb}
	1	3.62±0.47^{Bc}	3.05±0.35^{Bd}	3.53±0.12^{Ac}	5.04±0.25^{Aa}	4.48±0.16^{Ab}	5.13±0.21^{Aa}	5.17±0.09^{Aa}
	2	2.99±0.44^{Cc}	2.58±0.18^{Cd}	3.32±0.09^{Ab}	4.69±0.19^{Ba}	4.61±0.17^{Aa}	4.49±0.11^{Ca}	4.66±0.18^{Ba}
	3	2.31±0.35^{Dc}	1.95±0.09^{Dd}	3.17±0.21^{Ab}	4.62±0.13^{Ba}	4.50±0.19^{Aa}	4.51±0.10^{Ca}	4.69±0.12^{Ba}

	4	1.64±0.09^{Ed}	1.77±0.25^{Dd}	2.68±0.70^{Bc}	4.25±0.10^{Ca}	3.81±0.16^{Cb}	3.81±0.19^{Dd}	4.16±0.08^{Ca}
	0	10.32±0.28^{Be}	10.62±0.22^{Bd}	10.20±0.10^{Be}	11.02±0.19^{Bc}	11.24±0.25^{ABc}	12.24±0.39^{Bb}	12.58±0.23^{Ba}
	1	10.71±0.18^{Ad}	10.67±0.25^{ABd}	10.42±0.20^{ABd}	11.16±0.26^{ABc}	11.6±0.25^{Ab}	12.85±0.35^{Aa}	12.99±0.40^{Aa}
CIE b^*	2	10.85±0.43^{Ae}	10.88±0.11^{Ade}	10.55±0.15^{Af}	11.14±0.21^{ABcd}	11.37±0.29^{ABc}	12.47±0.15^{Bb}	13.06±0.40^{Aa}
	3	10.70±0.26^{Ad}	10.83±0.19^{ABcd}	10.64±0.23^{Ad}	11.45±0.40^{Ab}	11.15±0.55^{Bbc}	12.83±0.41^{Aa}	13.05±0.37^{Aa}
	4	9.58±0.20^{Cbc}	9.49±0.22^{Cbc}	9.35±0.57^{Cbc}	9.24±0.41^{Cc}	9.73±0.38^{Cb}	10.27±0.29^{Ca}	10.25±0.30^{Ca}

[1)]Control: no additives; T1: 0.2% sorbic acid; T2: 0.2% grapefruit seed; T3: 0.2% natural extract; T4: 0.2% natural extract and ascorbic acid; T5: 0.5% natural extract; T6: 0.5% natural extract and ascorbic acid. [A-E] means within a column with different letters are significantly different ($p < 0.05$). [a-e] Means within a row with different letters are significantly different ($p < 0.05$). Values are presented as mean ± SD with three replicates.

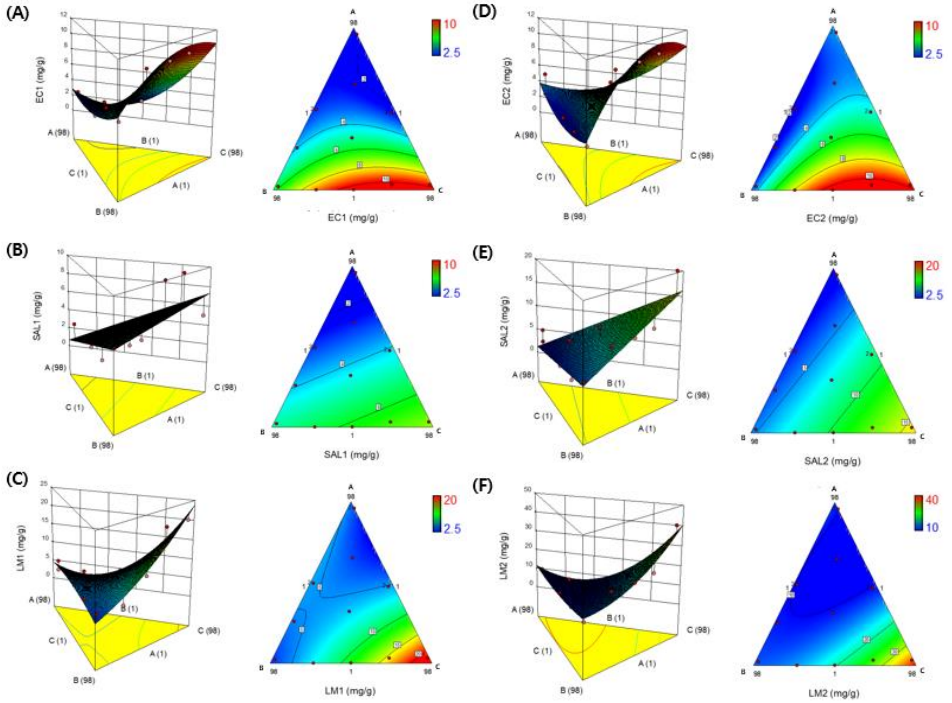
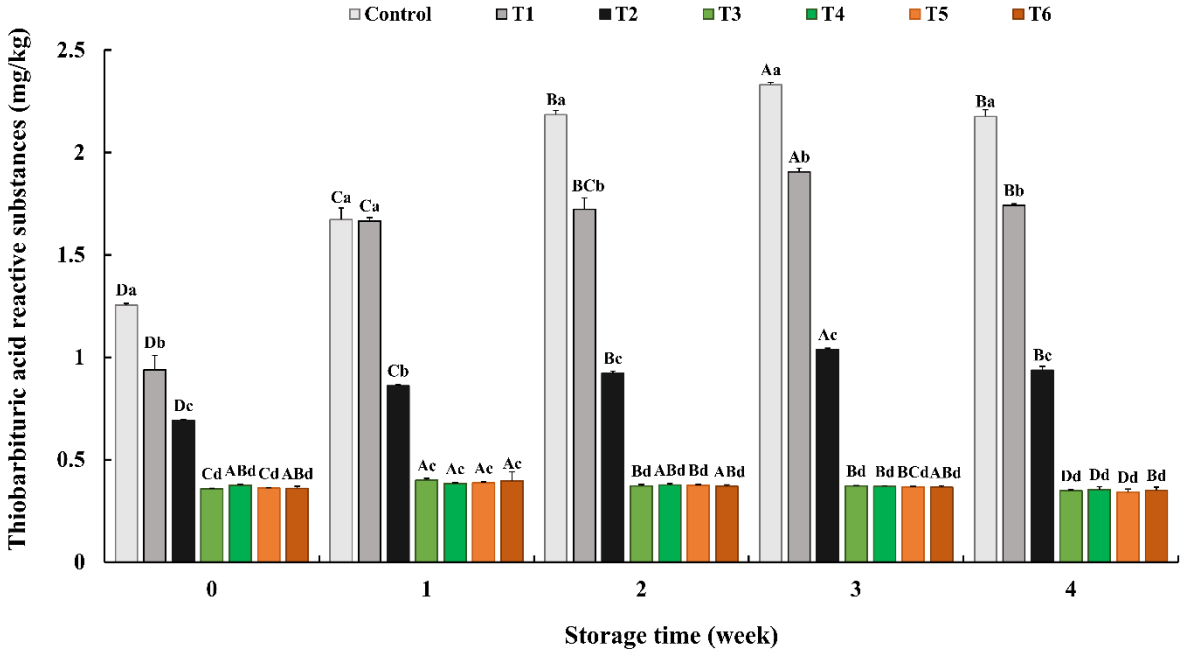


Figure 1. Contour and response surface plot (MIC, MBC) for the effects of *Ecklonia cava*, *Psidium guajava*, *Paeonia japonica (Makino) Miyabe & Takeda* on the antibacterial activity. (A): MIC results for EC, (B): MBC results for EC, (C): MIC results for SAL, (D): MBC results for SAL, (E): MIC results for LM, (F): MBC results for LM. Dark blue areas represent low MIC and MBC values (high antibacterial efficacy), while green to red areas represent medium to high MIC and MBC values (low antibacterial efficacy).



715

716 **Figure 2.** Thiobarbituric acid reactive substances (TBARS) of sausages added with natural
717 extract during storage periods**.** Control, No additives; T1, 0.2% sorbic acid; T2, 0.2% grapefruit
718 seed; T3, 0.2% natural extracts; T4, 0.2% natural extracts and ascrobic acid; T5, 0.5% natural
719 extracts; T6, 0.5% natural extracts and ascrobic acid. $^{A\text{-}D}$ means within a column in different
720 letters are significantly different ($p < 0.05$). $^{a\text{-}d}$ means within a row in different letters are
721 significantly different ($p < 0.05$). Values are presented as mean ± SD with three replicates.

722

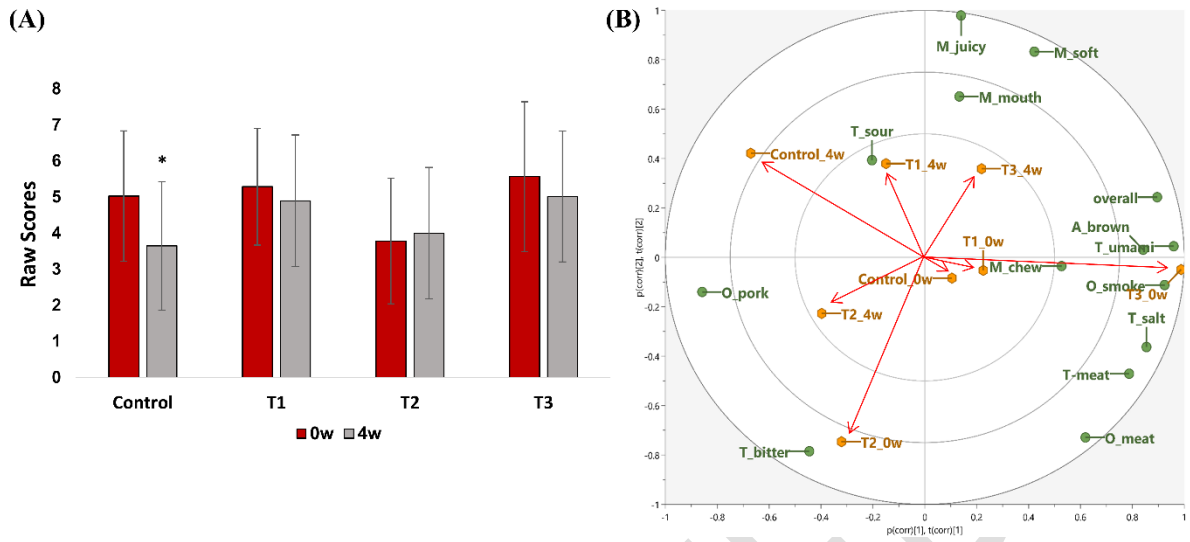


Figure 3. Summary of sensory evaluation. (A) overall acceptability and (B) principle component analysis biplot model based on rate-all-that-apply (RATA) intensities. The samples were kept at 4℃ for 0 week (0w) and 4 weeks (4w). Refer to Table S1 for sensory code. Control, no additives; T1, 0.2% sorbic acid; T2, 0.2% grapefruit seed; T3, 0.2% natural extracts; A, Appearance; O, odor; T, taste; M, mouthfeel.

Table S1. Average scores and SD of the RATA terms

Sample	Overall acceptability	Appear-ance (A)	Odor (O)			Taste (T)				Mouthfeel (M)			
		Brown-ness	Meaty	Pork	Smoky	Salti ness	Sour ness	Bitter-ness	Savory/ Umami	Meaty	Juici ness	Chewin ess	Tender-ness	Mouth coating
Control_0w	5.03 ±1.81^{b}	0.81 ±0.58^{a}	1.55±0.92^{bcd}	1.80 ±1.04^{b}	0.87 ±0.87^{ab}	1.87 ±0.84^{cd}	0.71 ±0.73^{abc}	0.55±0.63^{a}	1.41 ±1.02^{bc}	1.74 ±0.95^{b}	1.81 ±0.94^{b}	1.64±0.95^{ab}	1.67±0.97^{b}	1.2± 1.05^{a}
T1_0w	5.29 ±1.62^{b}	0.86 ±0.55^{a}	1.62±0.96^{cd}	1.75 ±1.09^{b}	0.94 ±0.87^{ab}	1.88 ±0.87^{cd}	0.59 ±0.69^{ab}	0.65±0.80^{a}	1.62 ±1.00^{cd}	1.81 ±0.94^{b}	1.93 ±0.94^{b}	1.26±0.87^{ab}	1.75±0.98^{b}	1.29 ±0.93^{a}
T2_0w	3.78 ±1.74^{a}	0.93 ±0.65^{a}	1.65±1.00^{cd}	1.74 ±1.07^{b}	0.93 ±0.94^{ab}	1.58 ±0.99^{bc}	0.65 ±0.78^{abc}	1.36±1.10^{b}	1.17 ±0.97^{ab}	1.65 ±1.07^{b}	1.13 ±0.89^{a}	1.51±0.93^{ab}	1.04±0.85^{a}	1.13 ±0.98^{a}
T3_0w	5.57 ±2.08^{b}	1.96 ±0.93^{c}	1.78±0.91^{d}	1.03 ±0.84^{a}	1.64 ±1.10^{c}	2.07 ±0.81^{d}	0.64 ±0.71^{ab}	0.61±0.65^{a}	1.80 ±1.09^{d}	1.86 ±0.99^{b}	2.06 ±0.97^{ab}	2.00±0.99^{b}	1.8 ±1.04^{b}	1.29 ±1.00^{a}
Control_4w	3.65 ±1.78^{a}	0.72 ±0.57^{a}	1.06±0.82^{a}	2.00 ±0.99^{b}	0.61 ±0.65^{a}	1.22 ±0.76^{a}	0.86 ±0.84^{bc}	0.65±0.74^{a}	0.99 ±0.87^{a}	1.22 ±0.94^{a}	2.32 ±0.80^{b}	1.61±1.05^{ab}	1.74±1.05^{b}	1.25 ±1.03^{a}
T1_4w	4.90 ±1.82^{b}	0.84 ±0.53^{a}	1.23±0.89^{ab}	1.70 ±0.94^{b}	0.74 ±0.76^{a}	1.29 ±0.75^{ab}	0.55 ±0.65^{a}	0.61±0.69^{a}	1.36 ±0.97^{bc}	1.57 ±0.93^{ab}	2.25 ±0.83^{b}	1.38±0.91^{ab}	1.84±0.96^{b}	1.41 ±1.12^{a}
T2_4w	4.00 ±1.82^{a}	0.87 ±0.57^{a}	1.62±0.89^{cd}	2.04 ±1.01^{b}	0.74 ±0.82^{a}	1.46 ±0.83^{ab}	0.68 ±0.68^{abc}	1.19±1.05^{b}	1.25 ±0.98^{ab}	1.55 ±1.01^{ab}	1.78 ±1.04^{b}	1.28±0.91^{ab}	1.30±0.97^{a}	1.36 ±1.01^{a}
T3_4w	5.01 ±1.81^{b}	1.42 ±0.85^{b}	1.42±0.96^{bc}	1.20 ±0.87^{a}	1.19 ±1.06^{b}	1.58 ±0.85^{bc}	0.93 ±0.90^{c}	0.62±0.77^{a}	1.54 ±1.05^{bcd}	1.54 ±0.88^{ab}	2.28 ±0.73^{b}	1.38±1.03^{ab}	1.68±0.98^{b}	1.38 ±0.99^{a}

Control: no additives; T1: 0.2% sorbic acid; T2: 0.2% grapefruit seeds; T3: 0.2% natural extract.
$^{a-d}$ means within a column with different letters are significantly different (p <0.05) according to Duncan's test.