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Inhibitory effect of natural extract mixtures on microbial growth and lipid oxidation of sausages during storage

Abstract

Large amounts of additives are used during the processing of meat products to maintain their quality and shelf life. With the growing interest in healthy eating, natural plant-based additives are being used as alternatives to synthetic additives. In this study, six types of natural extracts with excellent antibacterial activities were selected, and their antibacterial and antioxidant activities against four types of pathogens were evaluated in various combinations. In addition, the pH, color, amount of thiobarbituric acid reactive substances (TBARS), and growth of pathogenic microorganisms during the storage of sausages treated with various combinations of the extracts were analyzed. The natural extract mixtures exhibited different antibacterial activities, depending on the combination. Compared to grapefruit seed extract, a mixture of natural extracts extracted with ethanol (M4) reduced the *Escherichia coli* content by more than 99.9% after 8 days of storage and slowed the growth of *L. monocytogenes* and *Salmonella* spp. by more than 80% at 14 days. Compared to untreated (NC) and grapefruit extract (PC)-treated sausages, sausages treated with the natural extract mixtures showed a significant decrease in the CIE L* and an increase in the CIE a* and CIE b* ($p < 0.05$). The pH value was significantly lower in sausages with natural extract mixtures than in NC and PC sausages ($p < 0.05$). The natural plant extract mixtures significantly prevented lipid oxidation ($p < 0.05$). In sum, different types of natural extract mixtures have a synergistic effect when used together, suggesting that natural preservatives can generally inhibit the growth of microorganisms and oxidation of processed meats.

Keywords: Natural preservative, sausages, antibacterial activity, microorganisms, oxidation

Introduction

The control of microorganisms is one of the most improtant issues in the food industry. Many pathogenic microorganisms have been reported to be responsible for foodborne illness and food spoilage [1]. Foodborne pathogenic bacteria have been isolated from meat products, frozen fruits, and freshly cut vegetables [2]. In general, synthetic chemical preservatives are used to prevent the growth of pathogenic and spoilage-causing microorganisms in the food industry, and the use of synthetic chemical preservatives is regulated by each country [3, 4]. Nevertheless, there are concerns regarding the residual toxicity of synthetic chemical preservatives and microbial resistance to conventional synthetic preservatives [5]. Some chemical preservatives have carcinogenic and teratogenic properties [6]; therefore, it is necessary to develop natural preservatives using natural materials that can replace chemical preservatives, reduce the proliferation of foodborne pathogens, and improve food safety.

As consumer awareness of the benefits of natural additives increases, the preference for products with natural additives and clean labels has increased [7, 8]. In addition, the food industry has continuously shown interest in the development and use of plant-derived natural preservatives [7, 9]. Natural antibacterial agents, including plant extracts, essential oils, enzymes, bacteriocins, bacteriophages, and fermentation ingredients, have been reported to be promising alternatives to chemical antibacterial agents [10]. Among edible plant extracts, those belonging to Fabaceae and *Ocimum* and *Hibiscus sabdariffa* have shown antibacterial activities against *L. monocytogenes*, *Salmonella* spp., and *Escherichia coli*, respectively [11-13]. In addition, a study reported that a mixture of *L. tridentata*, *F. cernua*, and *O. ficus-indica* extracts was more effective in inhibiting the growth of *E. aerogenes* and *S. typhi* than their separate ethanol extracts [1]. Edible plant resources have not only antibacterial effects but also physiological effects; therefore, they have health benefits [9]. Plant extracts contain many physiological compounds that interact synergistically [14]; therefore, a greater effect can be expected when they are used in combination than when each extract is used individually.

Grapefruit seed extract (GFSE) is a natural substance extracted from the seeds and pulp of grapefruit that contains many flavonoids and other polyphenols [15]. The antibacterial activity of GFSE was investigated by Reagor, Gusman [16] on gram-negative bacteria (*Salmonella*) and gram-positive bacteria (*Staphylococcus aureus*); it showed excellent antibacterial effects against various pathogenic bacteria in other studies as well [15, 16]. However, when GFSE is applied to sausage manufacturing, its antimicrobial activity is generally limited to specific strains, such as *Listeria monocytogenes* [17].

It is important to maintain the quality of the product, as sausages are prone to spoilage and growth of pathogenic microorganisms, and their high fat content is prone to lipid oxidation [18]. Preservatives in sausages are responsible for improving quality, shelf life, and safety [19]. Recently, the increasing interest in clean-label meat products has led to increased efforts towards replacing synthetic preservatives with natural ones [20]. Plant-derived antimicrobials can extend the shelf life of sausages and, in some cases, can also improve the quality and color stability [21, 22]. The use of plant extracts instead of synthetic preservatives is expected to contribute to the production of healthy processed meat products and clean-label foods.

The purpose of this study was to evaluate the broad-spectrum antimicrobial activity of various plant extracts against pathogenic microorganisms and to confirm their potential as natural preservatives in sausages.

Materials and Methods

Preparation of natural extracts

Forty-nine natural extracts obtained from previous studies [23] and forty-seven plants were freeze dried and ground into a powder using a grinder (Cgolenwall, China) for extraction. A mixture was prepared by selecting a 6 natural product with excellent antibacterial activity from ninety-six natural extracts (Supplementary Table 1). A total of 3 g of natural plant powder was mixed with 40 mL of 50% (v/v) ethanol solution. The mixture was then stirred at 120 rpm for 24 h. The extracts were centrifuged at 3,500 rpm for 5 min, and the supernatants were collected and filtered using filter paper to remove any impurities. Residual ethanol was evaporated using a rotary evaporator (Eyela N-3000; Shanghai Eyela Co. Ltd., China). The extracts were lyophilized and stored in a deep freezer at -70℃.

Bacterial inoculum preparation

For this study, we used four foodborne pathogenic bacteria related to foodborne illnesses from meat products, namely *L. monocytogenes* (gram-positive), *C. perfringens* (gram-positive), *Salmonella* spp. (gram-negative), and *E. coli* (gram-negative). *L. monocytogenes* NCCP 10920, *L. monocytogenes* NCCP 10943, *L. monocytogenes* ATCC 13932, *L. monocytogenes* ATCC 51774, and *L. monocytogenes* ATCC BAA 839 were activated in 10 mL tryptic soy broth (TSB; Becton, Dickinson, and Company, Sparks, MD, USA) with 0.6% yeast extract (TSBYE) at 30℃ for 24 h. *C. perfringens* NCCP 10846, *C. perfringens* NCCP 10920, *C. perfringens* NCCP 10970, *C. perfringens* NCCP 15911, and *C. perfringens* NCCP 10976 were activated in 10 mL pre-reduced TSB and

incubated under anaerobic conditions at 37℃ for 24 h. *Salmonella* spp. (*Enteritidis* NCCP 14645, *Typhimurium* NCCP 12219, *Typhimurium* NCCP 16207, *Montevideo* NCCP 10140 and *Kentucky* NCCP 11686) and *E. coli* strains (NCCP 13717, NCCP 13718, NCCP 13719, NCCP 13720, NCCP 13721) were activated in 10 mL TSB at 37℃ for 24 h. Subsequently, 0.1 mL aliquots of the bacterial cultures were subcultured in the same medium under the same conditions. The cultures were then centrifuged and washed twice with 0.85% sterile saline (Cleancle, JW Pharmaceutical, Dangjin, Republic of Korea). The same bacterial strains were mixed because they undergo strain variation when they grow, and the bacterial mixtures were then used as inoculum for the experiment.

Agar diffusion assay

Agar spot assays were used to detect the antimicrobial activity of the natural plant extract mixtures against various foodborne pathogenic bacteria. Bacterial mixtures diluted with 0.85% sterilized saline, adjusted to 6–7 log CFU/mL, were uniformly spread on Muller–Hinton agar (MHA; Becton, Dickinson, and Company) using cotton swabs and then air dried for 15 min at room temperature. Aliquots (10 μL) of the natural plant extract mixture (50 mg/mL) were spotted onto MHA plates. The plates were incubated at 30℃ (*L. monocytogenes*) or 37℃ (*C. perfringens*, *Salmonella* spp., and *E. coli*) for 24 h, depending on the growth of the strains, and the appearance of inhibitory zones was observed.

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The MICs of the natural plant extract mixtures against foodborne pathogenic bacteria (*L. monocytogenes*, *C. perfringens*, *Salmonella* spp., *and E. coli*) were determined using the two-fold dilution method. The natural plant extract mixtures were dissolved in sterile TSB broth containing 10% DMSO. Then, they were was transferred to serial dilutions of TSB broth to obtain final concentrations of 15%, 7.5%, 3.75%, 1.875%, 0.9375%, 0.4688%, 0.2344%, 0.1172%, 0.0586%, 0.0293%, 0.0146%, and 0.0732%. A bacterial suspension (10 μL) was added to each sample to a final concentration of 6–7 log CFU/mL. A 96-well microtiter plate was incubated for 24 h with the bacterial strains under cultivation conditions. During incubation (4, 8, and 24 h), microbial growth was determined by estimating the turbidity of each well, measured at 600 nm using a spectrophotometer microplate reader. The lowest concentration of each extract that showed no visible bacterial growth was defined as the MIC. Therefore, the MBCs of the bacteria from the complete broth microdilution assay were placed onto TBA plates

and incubated for 24 h. After incubation, the colonies on the TBA plates were examined. The lowest concentration at which no visible growth was observed on the agar plates was defined as the MBC.

Preparation of natural extract mixtures

To prepare the natural preservative mixture of sorbic acid, four candidate substances showing antibacterial effects were selected from 48 natural extract candidate substances whose antibacterial activity was measured in this study. In addition, for the development of universal natural preservatives, two types of natural extracts (*Nelumbo nucifera* and *Ecklonia cava*) that have shown antibacterial effects in previous studies were used [23], and a total of six natural extracts were combined to demonstrate their potential as universal natural preservatives in various combinations. The profile of the natural extract mixtures is presented in Table 2. M1 was a mixture of natural extracts prepared at the minimum concentration to control *L. monocytogenes*, *C. perfringens*, *Salmonella* spp., and *E. coli*. M2 was a mixture of natural extracts prepared at the minimum concentration to control *L. monocytogenes*, *C. perfringens*, *Salmonella* spp., and *E. coli*, with economic feasibility. M3 was a combination of all six natural extract candidates. M4 was a combination of five candidate substances. M5 was prepared at 1/2 the concentration of M4. M6 has the same composition as M1 along with the addition of natural extracts effective against gram-negative and gram-positive bacteria and one more natural extract against the four types of bacteria.

Antimicrobial effect of natural extract mixtures

The natural plant extract mixtures were dissolved in sterile TSB broth containing 10% DMSO, and the diluted bacteria (*L. monocytogenes*, *C. perfringens*, *Salmonella* spp., *and E. coli*) were inoculated at 3 log CFU/mL. The mixtures were incubated at 30°C (*L. monocytogenes*) or 37°C (*C. perfringens*, *Salmonella* spp., and *E. coli*) depending on the growth of the strains and were spread on the agar plates at 0 h, 3 h, 6 h, 12 h, 24 h, 36 h, and 48 h. After incubating the plates for 24 h at the optimum incubation temperature, the colonies were counted.

Antioxidant activity of natural extracts

The total polyphenol and flavonoid content and DPPH and ABTS radical cation scavenging activities were measured to determine the antioxidant activity of the natural extract mixture. Total polyphenol content was determined according to the method described by Folin and Denis [24]. Briefly, sample extracts (20 μL) were combined with 40 μL of Folin–Ciocalteu reagent, to which 160 μL of sodium carbonate solution (Na2CO3, 75

g/L, w/v) was added. The mixture was vortexed and incubated in a dark room for 1 h at room temperature. Absorbance was measured at 725 nm using a spectrophotometer (Optizen 2120 UV Plus; Mecasys, Daejeon, Republic of Korea). The total polyphenol content was calculated using a standard curve prepared with gallic acid. Total flavonoid content was determined using the $AlCl_3$ colorimetric method [25]. The diluted sample extract (200 μL) was mixed with 100 μL of 5% $NaNO_2$, and 100 μL of 10% $AlCl_3$ was added after 5 min, followed by 0.6 mL of 1N NaOH. After allowing the mixture to react in the dark for 10 min, the absorbance was read at 415 nm. A standard curve was prepared using catechin, and the results were expressed as milligrams of catechin equivalents per gram (mg CE/g) of the sample.

The electron-donating ability was measured according to the DPPH free radical scavenging method described by Blois [26]. Extracts of the mixture sample (10 μL) were mixed with 190 μL of 0.4 mM DPPH solution. The mixtures were left in the dark for 10 min at room temperature, and the absorbance values were measured at 517 nm using a spectrophotometer (Optizen 2120 UV Plus; Mecasys). The half-maximal inhibitory concentration (IC_{50}) was expressed as the concentration of the sample that decreased the absorbance of DPPH by 50%. DPPH free radical-scavenging activity. The ABTS assay was based on the method described by Re [27]. The ABTS stock solution was prepared by mixing an equivalent amount of 7 mmol/L ABTS with 2.45 mmol/L potassium persulfate solution and kept in the dark for 12–16 h at room temperature. The ABTS stock solution was diluted to obtain a working solution with an absorbance value of approximately 1.4–1.5 at 734 nm. The ABTS working solution (1 mL) was mixed thoroughly with an appropriately diluted sample (50 μL). After allowing the mixture to react for 30 min in the dark, the absorbance was read at 734 nm. A standard curve was prepared using ascorbic acid, and the ABTS radical cation scavenging activity was expressed as the IC_{50} value.

Application of natural extract mixtures in sausage preservation

Preparation of emulsion sausage

Lean meat (fresh pork ham) and pork back fat were chopped using a 3-mm plate. The sausages were prepared using chopped lean meat (50%), pork back fat (25%), and ice water (25%). The sausages were prepared according to the method described by Lee et al. [23]. Lean meat was homogenized, ground for 20 s in a silent cutter, and then mixed with ice water. Salt (1.5%) and phosphate (0.15%) were added to the mixture for 1 min, and pork back fat was added after 4 min. Natural extract mixtures were added after 3 min and combined using a silent cutter. The meat batter was stuffed into a collagen casing and then cooked at 85℃ for 30 min in a smoke chamber

(MAXi3501 chamber; Kerres, Postfach, Germany). The sausage was cooled until the core temperature reached 21℃. Each portion of the sausage was placed in a polyethylene bag and stored until further use.

Antimicrobial effect of natural extract mixture on foodborne pathogen in sausages

Sausage samples were cut into pieces of approximately 10 g and inoculated with 3 log CFU/mL of pathogenic microorganisms (*L. monocytogenes, C. perfringens, Salmonella* spp., and *E. coli*). Each inoculated specimen was vacuum-packed in a sterile plastic bag and stored at 20℃ for up to 14 d. Cell counts of pathogenic microorganisms in sausages were analyzed on days 0, 4, 8 and 14. Then, 30 mL of 0.85% sterile saline was added to the sample bag and the sample was vigorously mixed for 30 s. The solution was serially diluted with 0.85% sterile saline. Diluents were plated on selective media, and the plates were incubated (at 30℃° or 37℃°) for 24 h. Only typical pathogenic microorganism colonies were counted.

Changes of pH, color and thiobarbituric acid reactive substances

For the measurement of pH, sausage and distilled water were homogenized at a ratio of 1:10. The pH of the homogenates was measured during refrigerated storage using a pH meter (Mettler Toledo, Schwerzenbach, Switzerland). The color of the sausages was measured using a colorimeter (CR-410, Minolta Ltd., Tokyo, Japan) and standardized using a white plate (L* = +97.83, a* = −0.43, b* = +1.98). The colors were expressed as CIE L* (lightness), CIE a* (redness), and CIE b* (yellowness). Thiobarbituric acid reactive substances (TBARSs), which represent the degree of lipid oxidation in meat products, were measured as described in a previous study [28]. Briefly, each sample (10 g) was homogenized with distilled water (50 mL) and 0.2% BHT (0.2 mL) at 10,000 rpm for 2 min using a homogenizer (AM-7; Nihonseiki Co. Ltd., Osaka, Japan). The homogenates were mixed with distilled water (47.5 mL), 4 N HCl (2.5 mL), and an antifoaming agent. The mixtures were boiled, and the distillate was collected. The distillate was reacted with 0.02-M thiobarbituric acid dissolved in 90% acetic solution at 95℃ for 35 min at a ratio of 1:1. After the reaction, the absorbance of the reactant was measured at 532 nm using a spectrophotometer (Optizen 2120UV plus; Mecasys, Daejeon, Republic of Korea). The amounts of TBARSs were calculated as previously described [29].

Statistical analysis

All experimental data were analyzed using the SPSS statistical software (SPSS Ver. 20.0, IBM Inc., IL, USA). One-way analysis of variance was performed using the general linear model procedure to investigate the effects of the natural extract mixtures. Two-way analysis of variance was performed to investigate the addition of natural extract and the storage period were considered fixed terms for evaluation of sausage. The significance of the differences among the mean values was determined by Duncan's multiple range test with a confidence level of $p < 0.05$. The data were expressed as mean values and standard deviations.

Results and Discussion

Determination of MIC and MBC of natural extracts against pathogens

In this study, the antimicrobial effects of 48 natural product candidates prepared by ethanol extraction were measured by spot assay (data not shown). Four natural extracts *(Paeonia japonica* (*Makino*) *Miyabe* & *Takeda*, *Rhus chinensis Mill*, *Paeomia suffruticosa*, and *Psidium guajava*) showed antibacterial activity against four pathogens (*L. monocytogenes, C. perfringens, Salmonella* spp., and *E. coli*). In addition, based on the results of previous studies that *Nelumbo nucifera* and *Ecklonia cava* extracts showed excellent antibacterial activity against pathogens, they were selected as materials for further research [23]. Serial dilution analyses were performed to obtain the MICs and MBCs for the six selected natural extracts and grapefruit seed extracts (positive control). The MICs and MBCs of most of the natural plant extracts were higher than those of the grapefruit seed extract (Table 1).

Paeonia japonica (Makino) Miyabe & Takeda extract showed more pronounced antibacterial activity against gram-negative bacteria considering that the MIC (37.5 mg/mL, 4.69 mg/mL) and MBC (37.5 mg/mL, 37.5 mg/mL) values of *Salmonella* spp. and *E. coli* were lower than those of the gram-positive bacteria. In a previous study, the difference in the antibacterial effect according to the extraction solvent of *Paeonia japonica (Makino) Miyabe & Takeda* was studied, and the antibacterial effect against the gram-negative *Staphylococcus aureus* and *Salmonella* spp. was demonstrated [30]. This medicinal plant extract contains cetyl alcohol as an antibacterial substance and acts as a natural antibiotic [31]. *Rhus chinensis Mill.* extract showed antibacterial activity against four pathogens, and for each pathogen, the MIC was 0.15–150 mg/mL and the MBC was 4.69–75 mg/mL. It was confirmed that the extract exhibited high antibacterial activity against non-spore-forming bacteria. These results are in agreement with previous studies showing that it is effective in inhibiting the proliferation of various bacteria,

such as food-poisoning bacteria and pathogenic bacteria in fish [31, 32]. In addition, the component showing antibacterial activity of the extract is known to be stable even at 80℃, so it has high industrial use [32]. *Paeomia suffruticosa* extract had the same or lower MIC (18.75 mg/mL, 4.69 mg/mL) and MBC (9.38 mg/mL, 37.5 mg/mL) values against the gram-positive bacteria, *L. monocytogenes* and *C. perfringens*, than against the gram-negative ones. Hwang also reported that the same concentration of extracts inhibited the growth of *L. monocytogenes* and *Bacillus* spp., which are gram-positive bacteria, by 100% among food microorganisms [33]. *P. guajava* leaf extract showed overall low MIC (1.17–4.69 mg/mL) and MBC (4.69-37.5 mg/mL) values against the four pathogens. *P. guajava* leaves are rich in bioactive phenolic compounds, such as flavonoids and tannins, and are non-toxic, allowing them to be used medicinally [34]. *Nelumbo nucifera* (seed pod) extracts showed antibacterial activity against the four pathogens; the MIC was 0.29–75.0 mg/mL and MBC was 4.69–37.5 mg/mL. Lee found that the *N. nucifera* (seed pod) extract showed the most extensive microbial inhibition zone (*B. subtilis*, *S. aureus*, and *P. aeruginosa*) among all *N. nucifera* parts, showing similar results to those described in the current study [31]. *Ecklonia cava* extract showed low MIC and MBC values for all bacteria except *C. perfringens*. Seaweeds, such as *E. cava*, are known to contain specific metabolites that exhibit various biological activities, such as antibacterial, antioxidant, and anti-inflammatory activities, and this extract has been reported to show strong antibacterial activity against marine bacterial pathogens [35]. Grapefruit seed extract had MIC values of 1.17–2.34 mg/mL and MBC values of 2.34–4.69 mg/mL and showed antibacterial activity against all the four pathogens. Previous studies by Heggers also reported that grapefruit seed extract has excellent antibacterial effects against a variety of pathogenic bacteria (*Staphylococcus aureus and Salmonella* spp.).

Recent research has shown growing interest in edible plant extracts as a way to control the proliferation of pathogenic microorganisms [18]. The results of this study confirmed that the antibacterial effect of the extracts differed depending on the type of strain. Therefore, to manufacture an antibacterial agent that has a universal antibacterial effect against various microorganisms, it is necessary to confirm the antibacterial activity of various natural extracts and to combine them in different ways. The results of this study can be used as indicators of the complexities involved in the manufacturing process of natural extracts.

Antioxidant compound contents and antioxidant activity

The total polyphenol and total flavonoid contents of the mixtures are listed in Table 3. The polyphenol contents of extracts were 116.02–386.70 mg GAE/g, with M2 having the highest content. While the total polyphenol content of the grapefruit seed extract (29.70 mg/GAE/g) was the lowest. . The flavonoids comprise a large group

of polyphenolic compounds that occur in plants and vegetables [36]. The flavonoid content expressed in catechin equivalents (CE) ranged from 69.72 to 212.49 mg CE/g. The total flavonoid content also showed a trend similar to that of the total polyphenol content.

The DPPH and ABTS radical scavenging activities of the mixtures are shown in Table 3. In the DPPH assay, the IC_{50} values of the treatments were 0.07–0.38 mg/g, which were higher that the IC_{50} of vitamin C (0.04 mg/g), while the IC_{50} of the grapefruit seed extract was the highest at 0.99 mg/g. A low IC_{50} value indicates a high DPPH radical scavenging activity. The DPPH radical scavenging capacity of these treatments decreased in the following order: Vit C>M2,M4>M1,M6,M5>M3> grapefruit seed extracts. The ABTS assay showed a similar tendency to that of the DPPH assay. These results demonstrated that there was a positive correlation between the total polyphenol content and antioxidant activity. Previous studies reported that phenolic compounds had a good association with antioxidant activities [37]. Maisuthisakul et al. [38] reported that phenolic compound exhibit antioxidant activity by inactivating lipid free radicals or preventing decomposition of hydroperoxides into free radicals.

Inhibition of bacterial growth by different combinations of natural extracts

Four bacteria were inoculated into various combinations of natural extracts and cultured for 48 h under optimal culture conditions. The bacterial growth curves are shown in Fig. 1. The initial concentration of the four tested bacteria was 2–5 log CFU/m. In normal medium without extracts (NC), all four bacteria grew steadily for 48 h, resulting in a final concentration of 12–13 log CFU/mL. On the other hand, in the medium containing 0.2% grapefruit seed extract (PC), all the bacteria generally died after 3 h of incubation, indicating the presence of strong antibacterial activity. The M1, M4, and M6 media also inhibited the growth of all the bacteria. In the M1, M4, and M6 media, the growth of *C. perfringens* and *Salmonella* spp. was inhibited after 6 h of incubation. In addition, *E. coli* growth was inhibited in the culture from 12 h onward, and *L. monocytogenes* growth was inhibited in the culture from 24 h onward. In the other group, the overall bacterial growth was hindered. In the M2 media, the bacterial concentrations of *C. perfringens* and *Salmonella* spp. decreased only after 6 h, and in the M3 media, only the proliferation of *Salmonella* spp. decreased. The concentration of *Paeomia suffruticosa* in the M2 media was higher than that in the M3 media (Table 2). Table 1 shows that *P. suffruticosa* exhibited relatively high antibacterial activity against *C. perfringens*, which may have affected the growth inhibition of the bacteria. In contrast, the M3 group showed a high concentration of *Paeonia japonica (Makino) Miyabe & Takeda*, indicating its effectiveness against gram-negative bacteria. It was confirmed that the M5 group only inhibited the growth of

the gram-negative *Salmonella* spp. and *E. coli*. The M5 group had the same concentration of *Paeomia suffruticosa* as the M3 group, indicating that it may not be effective in inhibiting the growth of gram-positive bacteria. Consequently, a combination of plant extracts can inhibit bacterial growth, and each combination has a different effect. Therefore, we further examined the application of M1, M4, and M6 groups, which showed universal effects on various microorganisms.

Antimicrobial effect of natural extract mixture on food-borne pathogens in sausages

The NC, PC-treated, and sausages treated with the plant extract combinations (M1, M4, and M6) were inoculated with four bacteria, and growth was evaluated for 14 days (Table 4). The initial concentration of *L. monocytogenes* was approximately 2.3 log CFU/mL, and the initial concentration of other bacteria was 3–4 log CFU/mL. The concentrations of gram-positive bacteria, *L. monocytogenes* and *Salmonella* spp., showed a tendency to increase growth regardless of the type of natural preservative used. The final concentrations of *L. monocytogenes* and *Salmonella* spp. in the NC group were 7.23 log CFU/mL and 7.59 log CFU/mL, respectively, and the final concentrations in the PC group were 6.94 log CFU/mL and 6.36 log CFU/mL, respectively. In addition, the M4 and M6 groups had final concentrations ranging from 5.30 to 6.78 log CFU/mL. Therefore, the M4 and M6 groups showed approximately 23% lower growth rates than the PC group. The *E. coli* cell counts in the NC and PC groups increased to 8.18 CFU/mL and 8.10 log CFU/mL, respectively. On the other hand, the M4 and M6 groups showed an increase the number of bacteria up to the 4 days, but a decrease by more than 99.9% was observed on day 8. In the growth of *C. perfringens*, the group of natural preservatives slowed the growth rate of bacteria compared to the group that did not. However, the growth of bacteria was inhibited more in the PC group than in the M1, M4, and M6 groups.

Based on these results, it was confirmed that a mixture of natural plant extracts inhibited the proliferation of various bacteria. Grapefruit seed extract was the most effective in inhibiting the growth of *C. perfringens*, and the natural plant extract mixtures M4 and M6 were more effective in inhibiting the growth of *L. monocytogenes*, *Salmonella* spp., and *E. coli* than grapefruit seed extract. In particular, M4 and M6 can induce the death of *E. coli*. Rivera et al. [1] measured the antimicrobial activity of a mixture of ethanol extracts of a semi-desert plant and paddle cactus. There observed a difference in antibacterial activity according to the mixing ratios of the extract, and appropriate mixtures of the plant extracts were effective in suppressing the growth of food-borne pathogens. The natural extracts contain bioactive compounds such as tannin, alkaloids and quinones [39](Vaou et al., 2021). These compounds affect the cytoplasmic membrane structure and permeability, making it impossible to function

properly [39] (Vaou et al., 2021). It is also known to inhibit the quorum sensing of pathogens and efflux pump related with antimicrobial resistance [39-41](Radulovic et al., 2013; Savoia, 2012; Vaou et al., 2021). Therefore, plant extract mixtures suitable for sausage manufacture may exhibit better antibacterial activity against a wider range of bacteria than grapefruit seed extracts.

Color of sausages prepared with natural extracts mixtures

The color values of the emulsion sausages according to the storage period are shown in Table 5. The color of the sausages was affected by the natural plant extract mixtures and storage period. On day 0, M2-treated samples showed the lowest CIE L* value and NC-treated samples showed the highest value ($p < 0.05$). NC-treated samples showed the lowest CIE a* value and M5-treated samples, the highest ($p < 0.05$). M2- and M7-treated samples showed the highest CIE b* value and NC-treated samples showed the lowest CIE b* value ($p < 0.05$). Compared to NC- and PC-treated samples, samples treated with natural plant extracts showed a decrease in CIE L* and a* significant increase in CIE a* and b* values ($p < 0.05$). The addition of natural plant extracts to meat products may result in chromaticity changes [23]. According to Kim et al. [42], the chromaticity may change depending on the concentration of the natural plant extract being added. On comparing the CIE L* values on days 0 and 15, we observed a significant increase in the values in all treatment groups ($p < 0.05$); the NC-treated samples showed no significant difference in the CIE a* values ($p > 0.05$), while the PC-treated samples showed a decrease ($p < 0.05$). However, treatment with the plant extracts increased significantly ($p < 0.05$). Redness can be reduced by the oxidation of myoglobin to metmyoglobin in the meat during storage [43, 44]. The results observed for M2, M5, and M7 may be related to antioxidant activity. Antioxidation by the addition of phenolic compounds improves the color stability [45]. In addition, there might be an effect of color development due to phenolic compounds present in the plant extract [46].

pH in sausages prepared with natural extracts mixtures

The pH of sausage is an important factor that can affect the quality and risk of microbial growth in the products [47, 48]. The pH values of the samples and the variations during storage are shown in Fig. 2. The pH value of PC-treated sausages did not significantly differ from that of NC sausages. Meanwhile, the pH value of sausages treated with natural plant extract mixtures was lower than that of NC sausages ($p < 0.05$). These results were influenced by the abundance of organic acids in the mixtures, such as citric, malic, and tartaric acids [49]. The grapefruit

seed extract also has an acidic pH [50]; however, the concentration of grapefruit seed extract, which was determined to be an effective dose for antimicrobial activity, was lower than that of other extracts in the mixtures. During refrigerated storage, the pH values decreased slightly in all the treated sausages. The pH decline during storage can occur because of the production of additional organic compounds by aerobic microorganisms [51].

TBARSs in sausages prepared with natural extracts mixtures

Lipid oxidation in meat can occur due to exposure to thermal and oxidative stress, which decreases the nutritional value, safety, and sensory properties of food products [52]. The TBARS values of sausages treated with grapefruit seed extract and natural plant extract mixtures are presented in Fig. 3. The addition of grapefruit seed extract to the sausage significantly increased lipid oxidation during the manufacturing process and storage of the products, and the amount of TBARSs was significantly higher in the PC-treated sausages than in NC sausages ($p < 0.05$). Each plant extract mixture showed a remarkable effect in preventing lipid oxidation in the sausages, and they also prohibited lipid oxidation until 15 d of refrigeration. These results can be attributed to the considerable amounts of polyphenols and flavonoids in the plant extract mixtures, as shown in Table 3. *P. guajava* extract, which was present in M1, M4, and M6, contains an abundant amount of polyphenols, especially gallic acid [53]. The grapefruit seed extract also contained these antioxidants, but at a much lower amount than the other mixtures, and it also contained ascorbic acid and tocopherol [54]. However, it acts as a pro-oxidant in sausages. The antioxidant effects of natural plant extracts can be hindered by the extraction method and storage conditions because of their high sensitivity to external factors [55]. Ascorbic acid, tocopherol, and some phenolic compounds can also promote oxidation when combined with iron and copper in foods or when the concentration of specific antioxidants is too high [56]. Therefore, all natural plant extract mixtures were found to be suitable for the prevention and inhibition of lipid oxidation in sausages.

Conclusion

We evaluated the antimicrobial activities of 96 natural extract candidates against *L. monocytogenes*, *C. perfringens*, *Salmonella* spp., and *E. coli*, and selected six natural materials with excellent antibacterial activities. The antioxidant an antimicrobial effects of the six selected natural extracts on sausages were investigated. Lipid oxidation and growth levels of *C. perfringens* were also analyzed. Overall, our findings confirmed the antimicrobial activities and lipid oxidation effects of the six selected natural extracts during storage, suggesting

that these natural products may be good substitutes for grapefruit seed extract. Accordingly, the extracts prepared in this study show the potential for application as natural preservatives for meat products.

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Table legend

Table 1. Antimicrobial activity of natural extracts expressed as minimum inhibitory concentraions (MICs) and minimum bactericidal concentration (MBC) in mg/mL

Table 2. Contents of natural plant extract mixtures

Table 3. Antioxidant compound contents and antioxidant activities of nature extract mixtures

Table 4. Cell counts of pathogenic microorganisms in sausages during storage

Table 5. Color of sausages prepared with natural extract mixtures during the refrergiated stroage

Table 1. Antimicrobial activity of natural extracts expressed as minimum inhibitory concentraions (MICs) and minimum bactericidal concentration (MBC) in mg/mL

No. of natural extracts	Natural plant extracts	LM[1)]		CP[2)]		SAL[3)]		EC[4)]	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
1	***Paeonia japonica* (*Makino*) *Miyabe & Takeda***	75.00	75.00	150.00	150.00	37.50	37.50	4.69	37.50
2	***Rhus chinensis Mill.***	0.15	37.50	150.00	75.00	4.69	4.69	2.34	9.38
3	***Paeomia suffruticosa.***	18.75	9.38	4.69	37.50	18.75	18.75	18.75	37.50
4	***Psidium guajava***	1.17	4.69	2.34	37.50	4.69	4.69	4.69	4.69
5	***Nelumbo nucifera***	0.59	9.38	75.00	37.50	4.69	4.69	0.29	9.38
6	***Ecklonia cava***	4.69	18.75	150.00	150.00	2.34	2.34	2.34	2.34
7	**Grapefruit seed sxtract**	2.34	2.34	1.17	4.69	2.34	2.34	1.17	2.34

All values were presented by mean of three replicates.
[1)]*Listeria monocytogenes*; [2)]*Clostridium perfringens*; [3)]*Salmonella* spp.; [4)]*Escherichia coli*

Table 2. Contents of natural plant extract mixtures

Unit : mg/mL

No. of natural extracts	Natural plant extracts	M1	M2	M3	M4	M5	M6
1	***Paeonia japonica (Makino) Miyabe & Takeda***	-	-	37.50	18.75	9.38	18.75
2	***Rhus chinensis Mill.***	-	37.50	4.69	18.75	9.38	-
3	***Paeomia suffruticosa.***	-	37.50	9.38	18.75	9.38	18.75
4	***Psidium guajava***	37.50	37.50	4.69	18.75	9.38	37.50
5	***Nelumbo nucifera***	-	-	4.69	18.75	9.38	9.38
6	***Ecklonia cava***	18.75	-	2.34	-	-	18.75

[1)]Not used

M1: mixture 1; M2: mixture 2; M3: mixture 3; M4: mixture 4; M5: mixture 5; M6: mixture 6

Table 3. Antioxidant compound contents and antioxidant activities of nature extract mixtures

Treatments	Antioxidant compound contents		Antioxidant activities	
	Total polyphenol (mg GAE[1)]/g)	Total flavonoid (mg CE[2)]/g)	DPPH radical scavenging activity(IC_{50} mg/g)	ABTS radical scavenging activity(IC_{50} mg/g)
Vit C	-	-	0.04±0.00[a]	0.10±0.00[a]
M1	254.45±4.85[d]	159.52±2.61[e]	0.17±0.00[c]	0.15±0.01[ab]
M2	386.70±1.57[f]	212.49±10.80[g]	0.07±0.00[b]	0.11±0.00[a]
M3	123.47±0.57[b]	89.63±0.2[c]	0.38±0.00[d]	0.25±0.00[c]
M4	334.14±7.20e	196.0±4.58[f]	0.08±0.00[b]	0.14±0.00[ab]
M5	116.02±6.02[b]	69.72±1.61[b]	0.18±0.01[c]	0.32±0.00[d]
M6	181.82±7.89[c]	115.96±1.93[d]	0.17±0.01[c]	0.23±0.00[c]
Grapefruit seed extracts	29.70±2.25[a]	7.90±0.09[a]	0.99±0.00[e]	2.95±0.04[e]

[1)]GAE: gallic acid equivalent [2)]CE: catechin equivalent

Table 4. Cell counts of pathogenic microorganisms in sausages during storage

Unit : log CFU/g

Time (day)	LM[1)]					Time (day)	CP[2)]				
	NC	PC	M1	M4	M6		NC	PC	M1	M4	M6
0	2.30±1.00[a]	2.30±1.00[a]	2.30±1.00[b]	2.11±1.00[b]	2.48±1.00[c]	0	3.51±1.00[c]	3.23±1.70[b]	4.28±3.22[b]	3.13±1.40[c]	3.30±1.00[c]
4	7.11±4.81[a]	7.14±5.60[a]	7.07±5.76[a]	1.00±1.40[b]	4.11±2.51[bc]	4	4.57±1.00[bc]	4.55±1.00[a]	4.40±1.00[b]	4.32±1.00[bc]	4.21±1.00[bc]
8	7.00±5.85[a]	8.15±6.65[a]	8.33±4.78[a]	4.46±1.40[a]	5.70±4.62[ab]	8	6.97±4.63[ab]	5.56±2.70[a]	7.10±5.00[a]	6.56±5.01[a]	5.79±4.56[ab]
14	7.23±5.95[a]	6.94±4.65[a]	7.44±6.31[a]	5.42±3.26[a]	6.78±5.59[a]	14	7.85±6.83[a]	4.82±2.65[a]	6.03±4.48[ab]	6.42±5.13[ab]	6.39±4.61[a]
Time (day)	**SAL[3)]**					**Time (day)**	**EC[4)]**				
	NC	PC	M1	M4	M6		NC	PC	M1	M4	M6
0	3.63±2.53[b]	3.24±1.40[b]	3.15±1.48[b]	3.10±1.54[b]	3.06±1.65[b]	0	3.64±2.20[b]	3.38±2.00[b]	3.45±1.30[b]	3.40±1.95[b]	3.42±1.40[b]
4	7.57±6.10[a]	5.79±4.31[ab]	6.95±5.30[a]	4.90±3.00[ab]	4.30±3.00[ab]	4	9.71±7.60[a]	7.89±5.98[a]	7.00±5.30[a]	4.70±3.70[a]	5.00±1.00[a]
8	7.35±4.70[a]	5.87±4.38[ab]	6.18±4.18[a]	5.13±3.98[a]	4.81±3.40[a]	8	8.15±6.75[a]	7.86±5.40[a]	6.40±5.18[a]	0.00±0.00[c]	0.00±0.00[c]
14	7.59±6.24[a]	6.36±6.54[a]	6.81±4.48[a]	5.31±4.22[a]	5.30±0.00[a]	14	8.18±6.30[a]	8.10±6.18[a]	6.48±5.00[a]	0.00±0.00[c]	0.00±0.00[c]

All values were presented by mean of three replicates.

[a-c] Means within a row with different letters are significantly different.

[1)]*Listeria monocytogenes*; [2)]*Clostridium perfringens*; [3)]*Salmonella* spp.; [4)]*Escherichia coli*

NC: negative control (sausage with no preservative); PC: positive control (sausage with 0.2% grapefruit seed extract); M1: sausage with mixture 1; M4: sausage with mixture 4; M6: sausage with mixture 6

Table 5. Color of sausages prepared with natural extract mixtures during the refrergiated stroage

Trait	Treatments	Time (day)						
		0	1	3	5	8	12	15
CIE L*	NC[1)]	74.56±0.82^{Ad}	74.50±0.79^{Ad}	74.87±0.64^{Ad}	77.93±1.12^{Ac}	79.55±0.32^{Aab}	78.58±0.95^{Abc}	80.03±0.69^{Aa}
	PC	71.02±0.60^{Bc}	73.02±0.97^{Bb}	73.36±0.53^{Bb}	77.23±1.30^{Aa}	77.57±0.83^{Aa}	78.26±1.44^{Aa}	77.74±1.24^{Ba}
	M1	51.12±0.68^{Cb}	49.25±0.89^{Cb}	51.02±1.30^{Cb}	56.10±1.96^{Ba}	55.93±1.92^{Ba}	55.95±3.05^{Ba}	56.10±1.78^{Ca}
	M4	41.04±0.58^{Ec}	41.03±0.92^{Dc}	42.45±0.82^{Dbc}	45.81±3.70^{Cab}	44.38±3.93^{Dbc}	48.87±0.76^{Ca}	48.49±1.82^{Da}
	M6	42.99±0.58^{Db}	41.50±1.01^{Db}	42.37±1.08^{Db}	47.20±2.86^{Ca}	48.34±1.92^{Ca}	48.45±1.41^{Ca}	48.70±1.44^{Da}
CIE a*	NC	3.00±0.12^{Eab}	2.94±0.17^{Eb}	3.07±0.25^{Dab}	3.32±0.27^{Da}	3.15±0.09^{Dab}	3.01±0.18^{Dab}	2.97±0.35^{Db}
	PC	3.73±0.12^{Da}	3.22±0.15^{Dab}	3.21±0.17^{Dabc}	3.10±0.67^{Dabc}	2.91±0.48^{Dabc}	2.39±0.91^{Dbc}	2.70±0.68^{Dc}
	M1	4.94±0.16^{Cc}	5.04±0.11^{Cc}	5.06±0.29^{Cc}	5.93±0.16^{Ca}	5.61±0.29^{Cab}	5.34±0.50^{Cbc}	5.79±0.26^{Ca}
	M4	9.76±0.15^{Ab}	9.21±0.25^{Ab}	9.01±0.26^{Ab}	10.88±0.88^{Aa}	10.78±0.67^{Aa}	10.88±0.31^{Aa}	11.10±0.62^{Aa}
	M6	7.72±0.15^{Bcd}	7.09±0.23^{Bde}	6.83±0.16^{Be}	8.63±0.64^{Ba}	8.45±0.40^{Bab}	7.79±0.38^{Bbc}	8.35±0.77^{Babc}
CIE b*	NC	9.68±0.56^{Db}	9.33±0.44^{Db}	9.26±0.22^{Eb}	10.52±0.47^{Ca}	10.45±0.35^{Ba}	10.53±0.38^{Da}	10.51±0.39^{Ca}
	PC	10.93±0.67^{Cabc}	10.20±0.35^{Cbc}	9.89±0.20^{Dc}	10.82±0.71^{Cabc}	11.38±1.28^{Ba}	10.89±0.51^{Dabc}	11.20±0.49^{Cab}
	M1	13.07±0.27^{Ab}	12.26±0.42^{Ab}	12.72±0.53^{Ab}	15.81±0.96^{Ba}	16.24±0.98^{Aa}	15.41±0.62^{Ca}	15.64±0.76^{Ba}
	M4	12.17±0.19^{Bb}	11.63±0.27^{Bc}	11.26±0.19^{Cc}	16.67±1.86^{ABa}	16.28±1.28^{Aa}	16.58±0.31^{Ba}	17.10±0.98^{Aa}
	M6	12.96±0.23^{Ab}	11.91±0.13^{ABc}	11.76±0.41^{Bc}	18.07±1.11^{Aa}	17.28±0.71^{Aa}	17.42±0.43^{Aa}	17.67±0.96^{Aa}

All values were presented by mean of three replicates.

$^{a-e}$ Means within a row with different letters are significantly different.

$^{A-E}$Means column with different letters are significantly different.

$^{1)}$NC: negative control (sausage with no preservative); PC: positive control (sausage with 0.2% grapefruit seed extract); M1: sausage with mixture 1; M4: sausage with mixture 4; M6: sausage with mixture 6

Figure legends

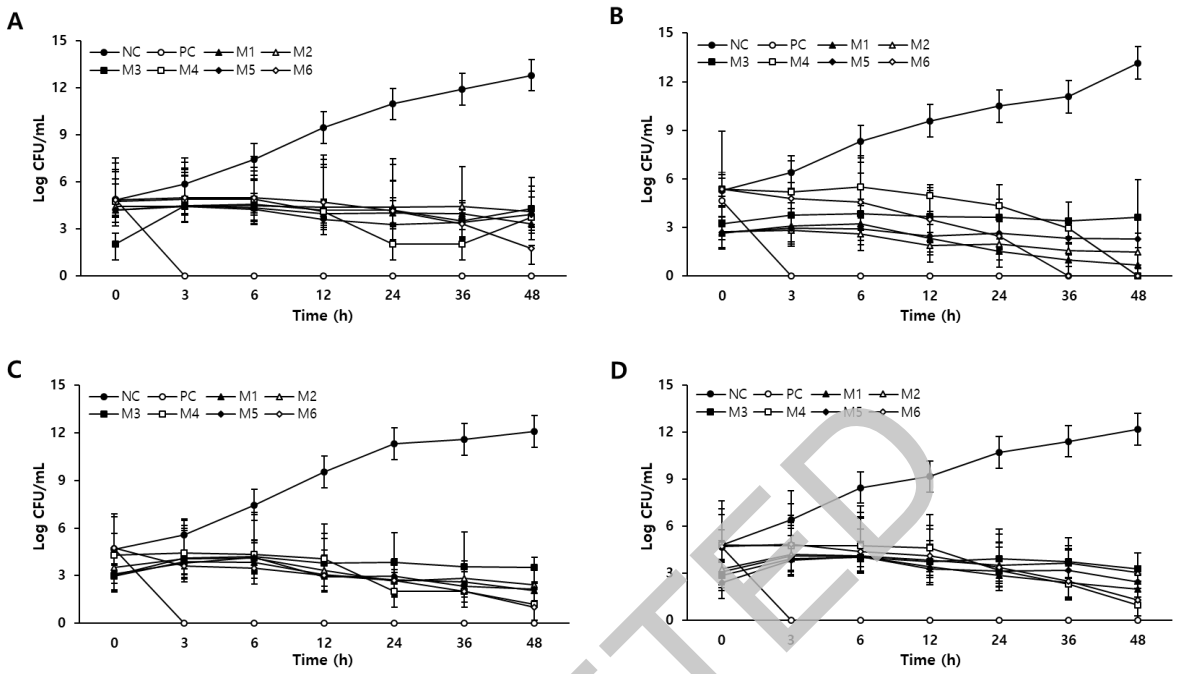
Fig 1. Growth curves of pathogenic bacteria in the natural plant extract media

A, *Listeria monocytogenes*; B, *Clostridium perfringens*; C, *Salmonella* spp.; D, *Escherichia coli*

NC (●), negative control (TSB broth); PC (○), positive control (0.2% grapefruit seed extract); M1 (▲), mixture 1; M2 (△), mixture 2; M3 (■), mixture 3; M4 (□), mixture 4; M5(◇), mixture 5; M6 (◆), mixture 6

Fig 2. pH in sausages prepared with natural extract mixtures during the refrergiated stroage. NC, negative control; PC, positive control (0.2% grapefruit seed extract); M1, mixture 1; M4, mixture 4; M6, mixture 6. $^{a-d}$ Different letters within the same storage daymeant significantly different. $^{A-D}$ Different letters within the same treatments meant significantly different.

Fig 3. Thiobarbituric acid reactive substances in sausages prepared with natural extract mixtures during the refrergiated stroage. NC, negative control; PC, positive control (0.2% grapefruit seed extract); M1, mixture 1; M4, mixture 4; M6, mixture 6. $^{t-z}$ Different letters within the same storage daymeant significantly different. $^{W-Z}$ Different letters within the same treatments meant significantly different.



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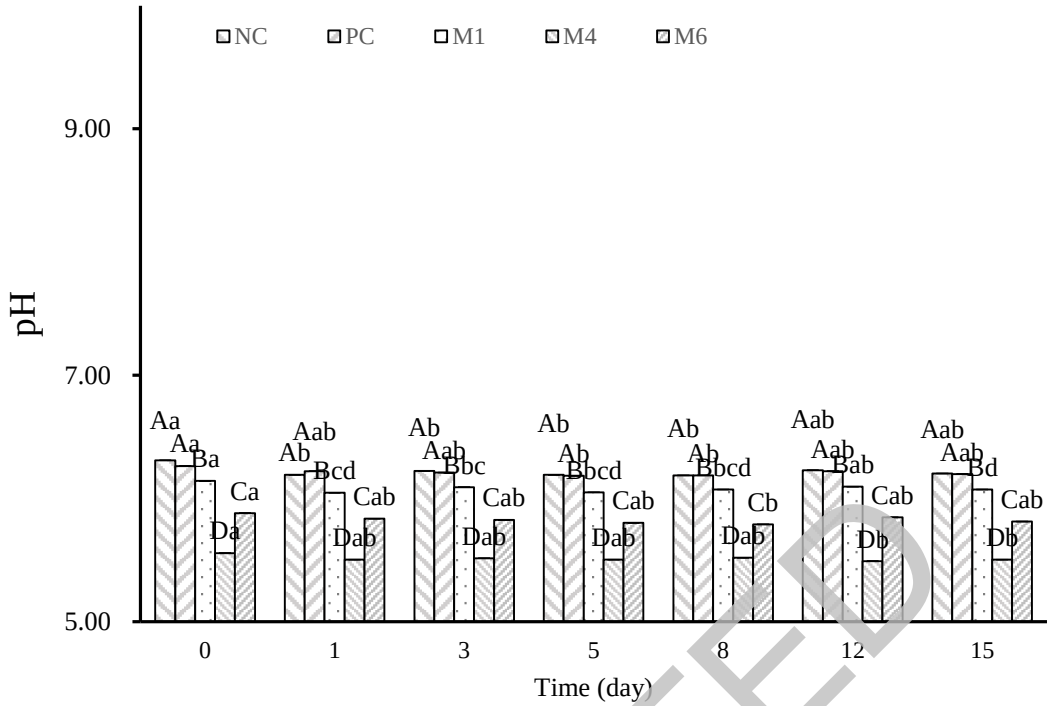

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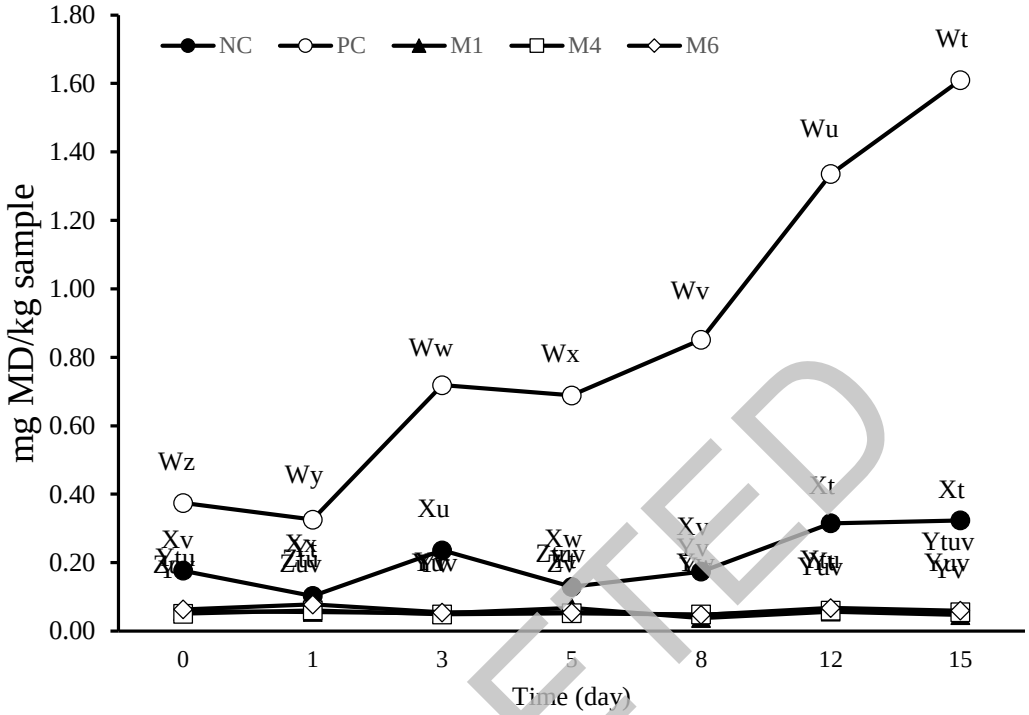

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