Nitrite formation from vegetable sources and its usage

as a preservative in cooked sausage

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Abstract

BACKGROUND: Due to the potential health risk associated with nitriles, nitrite alternatives from natural sources in meat products have been investigated. We compared the nitrate contents of young radish, lettuce and commercial vegetable powder (cabbage and Chinese cabbage), as well as investigated the effect of incubation time and salt addition on vegetable nitrite formation from vegetable sources. The antioxidant and antimicrobial effects of vegetable nitrite in cooked sausage were also compared with sodium nitrite.

RESULTS: Young radish produced the greatest amount of nitrite after 24 h of incubation at 38 °C. On average, an approximately 32% reduction of nitrite was observed in sausage during 4 weeks of storage. Lipid oxidation in sausage was significantly prevented by vegetable nitrite produced from vegetable powder or young radish. The color of the sausage prepared with young radish was most similar to that of the sausage with sodium nitrite. The addition of young radish to sausage significantly prevented the growth of L. monocytogenes at 4 °C and S. aureus at 8 °C.
CONCLUSION: Young radish was more effective as a natural antioxidant and antimicrobial agent as compared to commercial vegetable powder, which is currently used to make natural meat products, indicating that young radish has a high potential as a natural preservative.

Keywords: natural nitrite source; young radish; cooked sausage; natural antimicrobial; pathogen

INTRODUCTION

Nitrite is responsible for many important and distinctive characteristics of cured meat products such as sausage and ham. For one thing, it contributes to the notable reddish-pink color\(^1\) and the flavor of the meat product\(^2\), on the other hand, it also serves as an antioxidant that effectively controls the lipid oxidation that leads to rancid flavors in meat products.\(^3\), \(^4\) Nitrite also plays an important role in controlling the growth of pathogens, specifically \textit{Clostridium botulinum}.\(^5\), \(^6\) However, residual nitrite in meat products may react with certain amines in food to produce N-nitroso compounds, such as nitrosamines, which are known as carcinogens.\(^7\), \(^8\) Although controlled use of nitrite in processed meats generally poses no risks of toxicity, the issue of carcinogenic nitrosamines formed from nitrite in meat products remains a concern for consumers. Many consumers have a negative perception of nitrite as a food ingredient in processed meat products. Thus, the concentration of sodium nitrite in meat products is permitted up to 70 mg kg\(^{-1}\) in Korea,\(^9\) whereas the U.S. limits the concentration of sodium nitrite to 156 mg kg\(^{-1}\) for sausage and 120 mg kg\(^{-1}\) for bacon.\(^10\)

The use of natural and organic ingredients in processed meat products at retail stores have significantly increased due to consumer preference for organic and natural foods.\(^11\) Natural
and organic foods are often called “preservative free products” or “no nitrite added products” in the meat market. Thus, natural meat products have been made of natural ingredients, such as vegetable powder, with high nitrate content. It is reported that commercially available vegetable powders made with celery and cabbage contain 1,678 and 2,120 mg kg\(^{-1}\) nitrate, respectively. Furthermore, the use of nitrite alternatives from natural sources, including cherry powder and tomato pulp powder, in meat products has been researched owing to the potential health risk associated with nitrites from the formation of nitrosamines. At present, vegetable nitrite sources used to make natural and organic meat products are limited to celery powder, owing to the subtle flavor of celery and the minimal color effect on the product. Thus, there is a need to identify other nitrite alternatives from vegetable sources to replace conventional sodium nitrite in processed meat products.

Since nitrate is not a reactive curing compound, nitrate in vegetable sources must be first reduced to nitrite for curing reactions. Nitrate-to-nitrite reduction can be accomplished with a bacterial starter culture, such as \textit{Staphylococcus xylosus} or \textit{Staphylococcus carnosus}, which has a specific nitrate-reducing ability. These organisms can achieve nitrate reduction at 15-20 °C. In their investigation of the most favorable brine incubation conditions for nitrite formation from nitrate by the nitrate-reducing culture, Krause \textit{et al.} reported that nitrate reduction occurred most quickly in the solution without NaCl at 38 °C.

In the present study, we compared the nitrate content of young radish, lettuce, and commercial vegetable powder (cabbage and Chinese cabbage), which are capable of producing a high level of nitrite. We also investigated the effect of incubation time and salt addition on vegetable nitrite formation from vegetable sources in the curing brine mixture. In addition, we compared the effect of vegetable nitrite formed from young radish and commercial vegetable powder on color, lipid oxidation, and control of the pathogen growth in cooked sausage with sodium nitrite.
MATERIALS AND METHODS

Sample preparation

Freeze-dried vegetable powder (cabbage, *Brassica oleracea* var. *capitata* and Chinese cabbage, *Brassica rapa* L. spp. *pekinensis*) was purchased from Saeddeum-won (Yeonggwang-gun, Jeollanamdo, Korea). In order to test the vegetables that contain high levels of naturally occurring nitrates, white radish, spinach, young radish, and lettuce were purchased from local retail markets in Seoul, Korea. The vegetables were rinsed and frozen at -18 °C for 24 h in a household freezer. Before freeze-drying, the frozen samples were cut into pieces and freeze-dried for 3 days using a freeze dryer (FD8508, IlShin, Korea).

Analysis of the nitrate content

The freeze-dried samples were finely ground into powder for 2 min using a blender (HICHEN, HM-220, Korea). Their nitrate contents were then analyzed using the modified method initially proposed by Cataldo *et al.* Specificallly, 0.5 g of each powder was transferred to a conical tube and 10 mL of hot distilled water (90-95 °C) was added. After a thorough mixing, the conical tube was heated in a water bath (80 °C) for 30 min and then cooled. The sample solution in the conical tube was filtered using ashless filter paper and the filtered extract was used for the nitrate content analysis. 0.8 mL of salicylic acid reagent (5 g of salicylic acid in 100 mL 96% sulphuric acid) was added to 0.2 mL of the extract and then mixed. After leaving the sample at room temperature for 20 min, 19 mL of 2 N NaOH was added and stored for 48 h. The optical density (OD) of the samples at 410 nm was read using an ELISA reader (Bio-Tek Instrument., Richmond, VA, USA). The nitrate content values were calculated using a nitrate standard curve. All measurements were performed in duplicate.
Incubation conditions of brine solution for nitrite production from vegetable sources

The brine solutions for the production of nitrite from nitrate by a nitrate-reducing culture were first prepared by adding 15 g kg\(^{-1}\) vegetable powder (cabbage and Chinese cabbage), 80 g kg\(^{-1}\) dextrose, NaCl (0, 30, and 60 g kg\(^{-1}\)), and 0.1 g kg\(^{-1}\) starter culture consisting of *Staphylococcus carnosus* and *Staphylococcus xylosus* (Aurapa Wuerzungen, Bietigheim-Bissingen, Germany) to the water while mixing. The brine solutions were then immediately placed in a 38 °C incubator for 0, 12, and 24 h.

After incubation, the amount of nitrite was determined using the AOAC method with modifications to accommodate the measurement in the brine solutions. Five mL of the sample solution was transferred to a 500 mL volumetric flask, which was then filled up to 500 ml of the final volume with distilled water. Approximately 30 mL of the sample was then transferred to a 50 mL volumetric flask. Under a fume hood, 2.5 mL of sulfanilamide reagent (0.5 g sulfanilamide in 150 mL 150 g kg\(^{-1}\) acetic acid) was added. After 5 min, 2.5 mL of the NED reagent (0.2 g N-(1-naphthyl) ethylenediamine dihydrochloride in 150 mL 150 g kg\(^{-1}\) acetic acid) was added and filled up to 50 ml of final volume with the sample. The solution was stabilized for 15 min at room temperature for color development and then transferred into 96 well microplates and the absorbance was measured at 540 nm using an ELISA reader. The nitrite content was calculated using the equation of the standard curve.

Preparation of sausage

In order to investigate the effect of vegetable nitrite in processed meat products as an alternative to sodium nitrite, sausages with vegetable powder (VN), young radish (YN), or sodium nitrite (SN) were prepared, the formulations of sausage with SN, VP, or YP are shown in Table 2. Foreleg of pork was purchased from a local supermarket in Seoul, Korea and was ground with a blender. Pre-made vegetable nitrite solution without NaCl was prepared with
0.01% starter culture and vegetable powder or young radish. Then, 125g of sausage was prepared by adding pre-made vegetable nitrite solution to the ground pork at a volume of 250 g kg\(^{-1}\) of the total meat weight. After mixing all the ingredients including meat, NaCl, dextrose, and water, the mixed meat was then cured for 24 h at 4 °C. Then 50g kg\(^{-1}\) starch was then added into the cured meat mixture. 70 mg kg\(^{-1}\) sodium nitrite was also added to the ground pork for comparison. Each cured meat mixture with SN, VN, or YN was transferred to a sausage maker (Time Square, Nonsan, Korea) and stuffed into collagen casings of 26mm in diameter (Esfood, Gunpo, Korea). All treatments were steamed with an electric steamer (Fine Art, Incheon, Korea) for 35 min until the internal temperature of the sausages reached 75 °C. The samples were cooled at room temperature. Considering the packing method of hand-made sausage at retail markets, all samples were packed aerobically. A control group was also prepared without any preservatives (nitrite-free).

**Residual nitrite analysis**

In order to analyze the change in the amount of residual nitrite in the sausage containing sodium nitrite (SN) and vegetable nitrite formed from commercial vegetable powder (VN) or young radish powder (YN) at 4°C during storage periods, residual nitrite was determined by the diazotization method described in the Korean Food Standards Codex.\(^9\) Ten g of ground sausage sample was transferred into Erlenmeyer flasks and approximately 80 °C of hot distilled water was added. After mixing well, 10 mL of 0.5 N NaOH and 10 mL of 120 g kg\(^{-1}\) zinc sulfate reagent were added to the sample in the Erlenmeyer flasks, which was then heated at 80 °C for 20 min in a water bath and cooled. After cooling, 20 mL of ammonium acetate reagent was added and stabilized for 10 min. The sample solution in the Erlenmeyer flask was filtered using ashless filter paper and the filtrate was used for the nitrite content analysis. 1 mL of sulfanilamide reagent (0.5 g sulfanilamide in 100 mL HCl) and 1 mL of
NED reagent (0.12 g N-(1-naphthyl) ethylenediamine dihydrochloride in 100 mL distilled water) were added to 20 mL of the filtrate and 3 mL of distilled water was added for a total volume of 25 ml, which was then stabilized for 20 min. The optical density (OD) of each sample at 540 nm was measured using an ELISA reader. The residual nitrite content was calculated using the standard curve for a nitrite solution. Residual nitrite was measured subsequently at weeks 0, 1, 2, 3, and 4.

**Analysis of sausage color**

In order to analyze the change of color at a retail market, the sausages were stored for 20 days at 4 °C. The color of sausage was measured using a Hunter Colorimeter (Color Techno System Co., Japan). The white calibration plate with ‘L*’ value (lightness), ‘a*’ value (redness) and ‘b*’ value (yellowness) was used for calibration. Three measurements (CIE L*, a*, b*) were taken at random location on 5 g of slice of sausage containing SN, VN, or YN. The color of sausage was measured subsequently on days 0, 5, 10, 15, and 20. All measurements were performed in duplicate.

**Thiobarbituric acid reactive substances (TBARS):**

The TBARS analysis was performed with a modified method to measure the degree of lipid oxidation in the sausages. Each sausage was made by adding SN, VN, or YN and stored at 4 °C. The TBARS values were measured on days 0, 5, 10, 15, and 20. Five grams of ground samples were weighed into a conical tube (45 mL) with 15 mL of distilled water. It was homogenized with 50 μL of 72g kg⁻¹ butylated hydroxytoluene (BHT) in ethanol for 30 sec to stop the oxidation-reaction and then placed in the dark room for 15 min. The homogenate was centrifuged at 3,500 rpm for 15 min using a Multi-tube Carrier Refrigerated Centrifuge (VS-550; Vision Scientific, Yuseong, Daejeon, Korea) and filtered using No.1
filter paper (ADVANTEC, Bunkyo, Tokyo, Japan). One mL of the filtered solution was transferred into a conical tube (15 mL) and 2 mL of the TBA/TCA solution was added. The mixture was vortexed, heated at 100 °C for 15 min and then refrigerated for 10 min. It was then centrifuged at 2,000 rpm for 15 min using a Multi-tube Carrier Refrigerated Centrifuge. The absorbance of the supernatant was measured using an ELISA reader at 532 nm. The TBARS value (mg malondialdehyde per kg of meat) was calculated.25

**Preparation of bacterial strains**

In order to investigate the antimicrobial effect of natural nitrite on foodborne pathogens, a strain of *L. monocytogenes* (ATCC 15313) was obtained from the Korean Research Institute of Bioscience & Biotechnology (KRIIBB) and strains of enterohemorrhagic *E. coli* (EHEC: NCCP 13720 and 13721) were obtained from the Ministry of Food and Drug Safety (MFDS). Enterotoxin A producing *S. aureus* (ATCC 13565) was purchased from the Korean Culture Center of Microorganisms (KCCM). *L. monocytogenes* was maintained in tryptic soy broth (TSB, Difo, Sparks, MD, USA) with 6 g kg⁻¹ yeast extract (Oxoid Ltd, Hants, UK). Both EHEC and *S. aureus* were maintained in TSB. Stock cultures in TSB containing 200 g kg⁻¹ glycerol (Sigma-Aldrich, St. Louis, USA) were stored at -80 °C. The stock culture of each pathogen was thawed at room temperature and 0.01 mL of thawed stock culture of the pathogen was inoculated into a 25 mL Erlenmeyer flask containing 10 mL of TSB with 6 g kg⁻¹ yeast extract or TSB. Thereafter, the flask was sealed with a silicone cap and incubated aerobically at 36 °C for 24 h at 2.33 Hz on a rotary shaker (VS-8480SR, Vision, Korea). Viable cell counts of all the pathogens ranged from 9 to 10 log CFU/mL at the end of the incubation period. One mL of the stationary phase of overnight culture was transferred into 9 mL of 1 g kg⁻¹ sterilized peptone water (BD, Sparks, MD, USA), which was serially diluted before inoculation into the sausage sample.
Inoculation and enumeration of pathogens

In order to compare the antimicrobial effect, 5 g of cooked sausage was weighed and transferred into a sterile petri dish and inoculated with 50 μl of the dilution of foodborne pathogen (L. monocytogenes, EHEC or S. aureus) using a sterile pipette to give a target population of approximately 2.0-3.0 log CFU/g. Considering the packing method of hand-made sausage at retail markets, all samples were again packed aerobically. The samples inoculated with L. monocytogenes, EHEC, or S. aureus were then stored at 4, 8, and 10 °C. At selected times post inoculation, depending on the incubation temperature, each sample was homogenized (Stomacher, Interscience, Paris, France) for 2 min in 10 mL of 1 g kg\(^{-1}\) sterilized peptone water. One hundred microliters of dilution was spiral plated (Whitley automatic spiral plater, Don Whitley Scientific, West Yorkshire, UK) onto selective media, PALCAM, eosin methylene blue agar (EMB: Difo, Sparks, Md, USA) and Baird-Parker agar (BP: Oxiod Ltd, Hants, UK), respectively, in duplicate and incubated aerobically at 36 °C for 24-48 h. The colonies on the duplicated plates of each sample were counted with an automated colony counter (Scan 1200, Interscience, Saint Nom, France).

Moisture, pH and AW measurement

Moisture content was determined by the direct oven drying method. The loss in weight after oven-drying 1 g each of the sample at 105 °C for 24 h to constant weight was expressed as g kg\(^{-1}\) moisture content. Five grams of samples were homogenized with 45 mL of distilled water for 30 sec in a blender. The pH of each sample was measured with a pH meter (IQ Scientific Instruments, CA, USA) at room temperature. To determine the water activity (Aw), 5 g of the samples were collected and measured at 25 °C using an Aqualab Lite (Decagon...
Devices, Inc. Pullman, WA, USA). The pH and Aw values were obtained by the average of three measurements for each sample. All experiments were performed in duplicate.

Statistical analysis

Each experiment was replicated at least twice at different times. For each replication, three to five measurements of each parameter were performed. The data were analyzed with the SAS software, version 9.3 (SAS Institute, Cary, N.C.). The significance of differences among treatments (vegetable powder, young radish, and sodium nitrite) was determined by one-way ANOVA followed by Duncan’s test for multiple comparisons. A probability level of $p<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Analysis of the nitrate content in vegetable powder (cabbage and Chinese cabbage), young radish, and lettuce

Nitrite can be formed by reducing nitrate in vegetables with a nitrate-reducing starter culture. Thus, the higher the nitrate content in vegetable, the larger the amount of nitrite produced.\textsuperscript{20, 26} It is also well known that increasing incubation time and temperature can also increase the level of nitrate to nitrite conversion.\textsuperscript{16, 20} In the present study, we first identified natural sources containing a high amount of nitrate to produce a large amount of nitrite. The nitrate content was first screened to identify vegetables that contain a high level of nitrate and, therefore, could produce the most nitrite. In the preliminary work, the amount of nitrate in several vegetables, including white radish, spinach, young radish, and lettuce, was first screened. Based on the results, young radish and lettuce were selected as natural nitrate sources for further study.

In this study, the nitrate content of freeze-dried young radish, lettuce, and commercial vegetable powder (cabbage and Chinese cabbage) were compared. The mean nitrate contents
in commercial vegetable powder, young radish, and lettuce were 740 mg kg\(^{-1}\), 3,931 mg kg\(^{-1}\), and 291 mg kg\(^{-1}\), respectively (Table 1). The nitrate content in each sample was significantly different \((p<0.05)\). Among the tested samples, the freeze-dried young radish had the highest amount of nitrate that was even higher than that of vegetable powder, which was commercially used to make natural meat products. Depending on the plant species and environmental factors, including the conditions of cultivation and storage, nitrate content can vary from 1 to 10,000 mg kg\(^{-1}\).\(^{27,28}\) Previous studies conducted in Korea\(^{29}\) and Turkey\(^{30}\) reported that the nitrate content in cabbage was approximately 725 mg kg\(^{-1}\) and 510 mg kg\(^{-1}\) (ranging from 124 to 819 mg kg\(^{-1}\)), respectively. Another study from China\(^{13}\) reported that the mean nitrate content in the Chinese cabbage was 2,120 mg kg\(^{-1}\) (ranged from 337 to 3,600 mg kg\(^{-1}\) ), which was higher than the amount measured in the present study. Several studies analyzed the nitrate content in young radish in comparison to other vegetables. The nitrate content in young radish was analyzed and the mean value was 3,565 mg kg\(^{-1}\) (ranging from 20 to 6,888 mg kg\(^{-1}\)),\(^{31}\) which was similar to the present study. In 2003, Chung \textit{et al.}\(^{29}\) also reported seasonal variation of nitrate content in lettuce, ranging from 247 to 3,283 mg kg\(^{-1}\) in winter (November-March) and 884 to 4,488 mg kg\(^{-1}\) in summer (April–October). Compared to the 2003 results, the lettuce nitrate content in the present study was evaluated in summer and a lower level of nitrate was measured. The nitrate level of vegetables can be affected by various environment factors such as soil, fertilizer, and ground water, especially large amounts of nitrogen, including residual soil mineral nitrogen and the nitrogen present in the vegetables remains in the soil after harvest.\(^{32}\) The present results confirm that more nitrite can be generated from the nitrate in young radish powder and, similarly to commercial vegetable powder, young radish powder can be used as another vegetable nitrate source.
Effect of incubation time and NaCl concentration on nitrite formation from vegetable sources

The effects of incubation conditions for time and NaCl concentration on producing the maximum amount of nitrite from nitrate in vegetable sources were investigated and the data are also presented in Table 2. The vegetable nitrite solution with commercial vegetable powder was prepared and used to assess the effect of incubation conditions for nitrite formation in the present work. Since the incubation temperature for commercial nitrate reducing cultures is 38-42 °C, 38 °C was used in this study to minimize the time necessary for nitrite formation in the brine solution, combined with 0, 12, and 24 h incubation times and 0, 30, and 60 g kg⁻¹ NaCl concentration.

Overall, the amount of nitrite produced from the vegetable powder solution increased as the incubation time increased from 12 to 24 hr. However, the production of nitrite was inhibited as the NaCl concentration increased (Table 2). Although no significant difference between 12 and 24 h incubation time without NaCl was observed, the maximum amount of nitrite (70 mg kg⁻¹) was produced without NaCl after 24 h incubation at 38 °C in the present study. In a previous study, 208.5 mg kg⁻¹ nitrite was produced from nitrate using vegetable juice powder solution without NaCl after 12 h at 38 °C. However, the nitrite was not measured after 24 h and decreased to 187.2 mg kg⁻¹ after 48 h. The results of previous studies as well as the present study indicate that the maximum nitrite forms after 24 h incubation and that nitrite production decreases when the incubation time is over 24 h. Since nitrite is produced by the reduction of nitrate using starter cultures such as Staphylococcus xylosus and Staphylococcus carnosus, the incubation process is necessary for the nitrate to nitrite conversion. 33 Another study 22 reported that nitrite was not formed without the incubation process. The greatest amount of nitrite from vegetable powder solution was also produced without NaCl after 24 h incubation at 38 °C in the present study. S. carnosus and S. xylosus

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are considered NaCl tolerant and survive well in the presence of NaCl,\textsuperscript{20} however, the conversion of nitrate to nitrite is faster and more complete when salt is excluded from the brine mixture. Thus, NaCl addition to the ground meat during manufacturing of processed meat can be recommended.

The amounts of nitrite produced from freeze-dried commercial vegetable powder, young radish, and lettuce powder were also compared before and after 24 h incubation without NaCl at 38 °C (Table 3). The nitrite level was below 1 mg kg\textsuperscript{-1} in all samples before incubation. After incubation, the amount of nitrite from commercial vegetable powder, young radish, and lettuce powder increased to 54, 68, and 40 mg kg\textsuperscript{-1}, respectively. Although the nitrate content in each sample (commercial vegetable powder: 740 mg kg\textsuperscript{-1}, young radish: 3,931 mg kg\textsuperscript{-1}, and lettuce: 291 mg kg\textsuperscript{-1}) was significantly different ($p<0.05$), similar amounts of nitrite were generated after incubation. However, the greatest amount of nitrite was produced in young radish after incubation, which was not significantly different as compared to commercial vegetable powder. Another study\textsuperscript{20} also reported similar results.

**Comparison of residual nitrite in sausage during refrigerated storage**

For the comparison of the change of residual nitrite in sausage during storage, sodium nitrite (SN), commercial vegetable powder (VN), or young radish (YN) was added to the ground pork to make sausage (Table 4). Pre-made vegetable nitrite solution was added to the ground pork at a volume of 250 g kg\textsuperscript{-1} of the total meat weight. The initial concentrations of nitrite in the sausages containing SN, VN and YN were 70, 54 and 68 mg kg\textsuperscript{-1}, respectively. Table 5 shows the concentrations of residual nitrite in sausages containing vegetable nitrite (VN and YN) and SN during storage at 4 °C. The residual nitrite concentration decreased over time in all samples. After 2 weeks, no significant further reduction in residual nitrate was observed in the sausage containing VN or YN, while residual nitrite in the sausage with
SN decreased throughout the 4 weeks of storage. Although a greater nitrite reduction was observed in SN (37% reduction) than in VN (30% reduction) and YN (30.4% reduction) during 4 weeks of storage, the sausage with SN had significantly more residual nitrite than the sausages with VN or YN after 4 weeks storage. In addition, there were significant differences in the amount of nitrite measured among the sample ($p<0.05$) and the highest residual nitrite level was observed in the sausage with SN throughout 4 weeks of storage.

The trend of decreasing residual nitrite levels over storage time was also observed in previous studies. The decrease in the nitrite content during processing was reported to be due to the conversion of nitrite into nitric oxide and nitrous oxide in the mixing step, as well as nitrite oxidation to nitrate over time. Nitrous oxide is known to be responsible for the cured meat color, a nitrosohemochromogen. Nitrite level was also reported to be affected by storage time and temperature. In the present study, storage time might be the principal factor to decrease residual nitrite level, because constant storage temperature (4°C) was maintained.

**Effects of vegetable nitrite from vegetable sources on the color of sausage during refrigerated storage**

In order to compare the effect of vegetable nitrite from commercial vegetable powder(VN), and young radish (YN) with sodium nitrite (SN) on the color of sausage, the values of $L^*$, $a^*$ and $b^*$ of sausage were measured (Table 6 for the results of color measurement). Significant differences in the values of $L^*$, $a^*$, and $b^*$ were observed between the control (nitrite-free) and the other treatments (SN, VN and YN) during refrigerated storage ($p<0.05$). The color of the sausage with SN was the lightest among the samples on day 0, while the color of the control, which had no sodium nitrite or vegetable nitrite, was significantly lighter than that of the other samples after 20 days of storage ($p<0.05$). The
color of sausage with VN or YN was significantly ($p<0.05$) darker than the control and the sausage with SN during storage. These results indicate that the inherent greenness of cabbage, Chinese cabbage, and young radish may have influenced the color of sausage.

In addition, the color of sausage with SN was of the most red color as compared to the control and the other treatments (VN and YN), which was confirmed by a* values during storage ($p<0.05$) (Table 6 and Fig. 1). The color of the sausage with YN was less red than that of the sausage with SN, but it was significantly more red than the control and the sausage with VN ($p<0.05$). Overall, a* values in all samples decreased during storage, however a* values of the sausages with SN and YN were significantly higher as compared to the control and the sausage with VN on the final day of storage. These results imply that cabbage and Chinese cabbage powder, which is currently used in meat products, have little effect on meat color expression during 20 days of storage. On the other hand, young radish powder can be used more effectively as an alternative for reddish-pink color expression in meat products. For b* value, significant differences were observed on day 0 among the samples ($p<0.05$), but no significant differences were observed on the final day of storage ($p>0.05$).

**Effects of vegetable nitrite from vegetable sources on lipid oxidation in sausage during refrigerated storage**

Addition of natural nitrite produced from commercial vegetable powder (VN) and young radish (YN) significantly prevented lipid oxidation like sodium nitrite (SN) as measured by TBARS ($p<0.05$) (Fig. 2). As expected, the progress of lipid oxidation was observed in the control during 20 days of refrigerated storage (4 °C) and the TBARS value of the control (nitrite-free) increased sharply during refrigerated storage ($p<0.05$). However, the TBARS values of the other treatments increased slowly as compared to the control and the sausage with SN had the lowest TBARS level. On the final day of storage, no significant difference in
TBARS value was observed between the sausage with SN and the sausage with YN. Nitrite is an effective antioxidant in meat products and is known to possess antioxidant activity at concentrations as low as 40-50 mg kg⁻¹ nitrite. Therefore, the initial amount of nitrite from the sausages with SN (70 mg kg⁻¹), VN (54 mg kg⁻¹), or YN (68 mg kg⁻¹) in the present study would be sufficient to prevent or reduce lipid oxidation. Several studies also reported that the typical quality characteristics of cured meat, such as color and flavor, are achieved when at least 50 mg kg⁻¹ nitrite is used. In addition, a starter culture such as S. carnosus used for the reduction of nitrate to nitrite can generate catalase more significantly in the presence of nitrate. Since catalase is an important antioxidant enzyme, which degrades hydrogen peroxide, the use of the starter culture could contribute to additional antioxidant protection. Consequently, the antioxidant effect by the starter culture used in natural meat products may be greater than in conventional meat products without the starter culture. Addition of natural antioxidants to processed meat products is one of the important strategies in the development of healthier and novel meat products. It is well known that green vegetables are rich in many bioactive components, including polyphenols, which are the major active components responsible for the antioxidant activity of vegetables. In this present study, we found that young radish has a high potential as a natural antioxidant.

Antimicrobial effect of vegetable nitrite from vegetable sources on pathogen in sausage at various refrigerated temperatures

The antimicrobial effects of vegetable nitrite from commercial vegetable powder (VN), young radish (YN), and sodium nitrite (SN) on the growth of L. monocytogenes (Fig 3), EHEC (Fig. 4) and S. aureus (Fig. 5) in sausage were compared at 4, 8, and 10 °C. At 4 °C, significant differences in L. monocytogenes growth were observed between the control (nitrite-free) and the other treatments (SN, VN and YN) from day 6 to day 15 (p<0.05) (Fig. 3).
The final populations of *L. monocytogenes* in the sausage were affected by the presence of vegetable nitrite or sodium nitrite. The growth of *L. monocytogenes* was the most delayed in the sausage with YN at 4°C during 15 days of storage. In comparison with *L. monocytogenes*, the growth of EHEC (Fig. 4A) and *S. aureus* (Fig. 5A) was not observed at 4 °C, regardless of the presence of nitrite. On the other hand, the growth of *L. monocytogenes* was not effectively inhibited at 8 °C (Fig. 3B) and 10 °C (Fig. 3C), regardless of the presence of sodium nitrite or vegetable nitrite. However, SN was slightly better to control the growth of *L. monocytogenes* at 10 °C on day 15. Nitrite has an inhibiting effect on the growth of *L. monocytogenes*. However, the addition of nitrite at a low concentration did not totally control the growth of *L. monocytogenes* on the meat products. In addition to nitrite, pH and temperature affect the growth of foodborne pathogens. The antimicrobial effect of nitrite is enhanced at lower pH (pH 5.0 to 6.0) in the presence of reducing agents, such as ascorbic acid. The impact of pH and nitrite from celery juice concentrate on the growth of *L. monocytogenes* in broth and on ham slices has been studied. The results demonstrate that the pH of celery juice affects antimicrobial action of nitrite, therefore, a larger pH reduction may be needed at 100 mg kg⁻¹ in order to reduce *L. monocytogenes* growth or a greater concentration of nitrite is needed. In the present study, the pH of sausage was 6.06 with the control (nitrite free), 6.13 with SN, 5.71 with VN, and 5.69 with YN, indicating that addition of VN and YN reduced the pH of the sausage (Table 4) and may affect the antimicrobial activity. However, the pH of sausage decreased gradually over storage time. However, no significant differences in pH values were observed among the treatment on the final day of storage (data not shown).

In comparison to *L. monocytogenes*, the growth of EHEC was inhibited in the sausage with nitrite at 8 and 10 °C. After 15 days of storage, the population of EHEC in the sausage with YN, VN, and SN was significantly lower than that in the control (*p*<0.05). The lowest
population of EHEC in the sausage with SN was observed at 8 °C (Fig. 4B) during 15 days of storage. In the case of *S. aureus*, storage temperature significantly affected *S. aureus* growth in sausage (Fig. 5). At 8 °C, the growth of *S. aureus* in the control was significantly faster compared to other samples (*p*<0.05) from 3 to 15 days of storage; furthermore, addition of nitrite to sausage inhibited the growth of *S. aureus*. The lowest population of *S. aureus* in the sausage with YN was observed on day 15 (Fig. 5B). At 8 °C, the growth of *S. aureus* was more effectively controlled in the sausage with nitrite as compared to that of *L. monocytogenes* (Fig. 3B) and EHEC (Fig. 5B). However, the growth of *S. aureus* was not controlled by SN, VN, or YN at 10 °C (Fig. 5C). The nitrite concentration in natural meat products manufactured with vegetable sources, such as celery, cabbage, and the Chinese cabbage, ranges from 35 to 70 mg kg⁻¹. According to the regulations of Korea Food Additives Standard Division, the nitrite concentration in conventional meat products is limited to 70 mg kg⁻¹. Previous studies reported that the addition of celery powder as the natural curing resulted in 48 mg kg⁻¹ nitrite, which was shown to be insufficient for inhibition of *L. monocytogenes* growth. In this study, the amount of nitrite from VN and YN was 54 mg kg⁻¹ and 68 mg kg⁻¹, respectively, which is lower than the recommendation level of 70 mg kg⁻¹. Although the difference in the amount of nitrite between YN and SN is negligible, the nitrite formed from YN prevented more effectively the growth of *L. monocytogenes* than SN at 4 °C during 15 days of storage. This result indicates that the vegetable nitrite formed from YN can be used as an alternative of SN during the manufacturing of processed meat. On the final storage day, the growth of *S. aureus* at 8 °C and EHEC at 10 °C was inhibited about 1~2 log CFU/g in sausage with nitrite; however, the growth of most foodborne pathogens in this study was not significantly inhibited, regardless the presence of nitrite at 10 °C. This result indicates that there is high risk due to the growth of foodborne pathogens in meat products, regardless of the presence of nitrite at 10°C, which is the recommended refrigerated storage temperature.
temperature at retail markets in Korea.

CONCLUSIONS

The highest nitrate amount was measured in young radish and was higher than that of commercial vegetable powder that is currently used in natural meat products. The greatest amount of nitrite due to nitrate reduction was produced by adding vegetable source containing nitrate and a starter culture without NaCl after 24 h incubation at 38°C. Although the residual nitrite level in sausage gradually decreased over 28 days of storage, antioxidant effect of nitrite was maintained, regardless of nitrite source. The color of the sausage prepared with young radish was most similar to that of the sausage with sodium nitrite. The TBARS values of the sausage with sodium nitrite and young radish were the lowest among the samples, indicating the prevention of lipid oxidation due to sodium nitrite and vegetable nitrite. Addition of vegetable nitrite produced from young radish to sausage significantly reduced the growth of \textit{L. monocytogenes} at 4 °C, \textit{S. aureus} at 8 °C, and EHEC at 8 and 10 °C. Therefore, when used as a preservative for processed meat products, young radish is superior to commercial vegetable powder that is currently used to make natural meat products. Thus, young radish powder can be recommended as a vegetable nitrite source for making processed meat products. However, further research is needed to determine the impact of young radish powder on various sensory characteristics of processed meat products.
REFERENCES


Table 1. The nitrate content in vegetable powder, young radish and lettuce

<table>
<thead>
<tr>
<th>Vegetable source</th>
<th>Moisture content (g kg⁻¹)</th>
<th>Nitrate content (mg kg⁻¹) Mean ± SD</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetable powder*</td>
<td>68.5±8.3</td>
<td>740±40ᵇ</td>
<td>639</td>
<td>826</td>
</tr>
<tr>
<td>Young radish</td>
<td>53.2±5.9</td>
<td>3931±200ᵃ</td>
<td>3352</td>
<td>4244</td>
</tr>
<tr>
<td>Lettuce</td>
<td>63.1±5.4</td>
<td>291±18ᶜ</td>
<td>251</td>
<td>339</td>
</tr>
</tbody>
</table>

*cabbage and Chinese cabbage.

ᵃ~ᶜ Means(n=3) ± SD in column with different superscripts are significantly different by Duncan’s multiple range test at p < 0.05.

Table 2. Effects of NaCl concentration and incubation time on nitrite formation for curing solution
containing vegetable powder* and a starter culture†

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>Incubation time (h)</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl concentration (g kg⁻¹)</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>Water</td>
<td>904.9</td>
<td>874.9</td>
<td>844.9</td>
</tr>
<tr>
<td>Dextrose</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Vegetable powder</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Starter culture</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>NaCl</td>
<td>0</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Total</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

Nitrite content (mg kg⁻¹) ††

A⁵⁴±11  C⁴±1  C³±1  A⁷⁰±⁸  B¹⁹±⁴  B¹¹±²

*starter culture: Staphylococcus carnosus and Staphylococcus xylosus.

†† Means (n=3) ± SD, which were measured in sausage.

A–C Means in the same row with different superscripts are significantly different by Duncan’s multiple range test at p < 0.05.

Table 3. Effect of incubation* on nitrite content of vegetable powder, young radish and lettuce
<table>
<thead>
<tr>
<th>Vegetable source</th>
<th>Nitrite content (mg kg(^{-1}))</th>
<th>Pre-incubation</th>
<th>Post-incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetable powder†</td>
<td>&lt; LOD</td>
<td></td>
<td>54±8(^{ab})</td>
</tr>
<tr>
<td>Young radish</td>
<td>&lt; LOD</td>
<td></td>
<td>68±16(^{a})</td>
</tr>
<tr>
<td>Lettuce</td>
<td>&lt; LOD</td>
<td></td>
<td>40±11.4(^{b})</td>
</tr>
</tbody>
</table>

*incubation for 24 h at 38 °C
†cabbage and Chinese cabbage
LOD: limit of detection
\(^{a-b}\) Means(n=3) ± SD in column with different superscripts are significantly different by Duncan’s multiple range test at \(p < 0.05\).
Table 4. Formulations for sausage containing sodium nitrite (SN) and natural nitrite solution prepared with vegetable powder (VN) and young radish (YN)

<table>
<thead>
<tr>
<th>Ingredient (g kg⁻¹)</th>
<th>Control</th>
<th>SN</th>
<th>VN</th>
<th>YN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pork forelegs</td>
<td>935</td>
<td>935</td>
<td>935</td>
<td>935</td>
</tr>
<tr>
<td>Sodium nitrite*</td>
<td>-</td>
<td>0.087</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Natural nitrite</td>
<td>-</td>
<td>-</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>solution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Starch</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Water</td>
<td>230</td>
<td>230</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dextrose</td>
<td>20</td>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>pH</td>
<td>6.06</td>
<td>6.13</td>
<td>5.71</td>
<td>5.69</td>
</tr>
<tr>
<td>Aw†</td>
<td>0.987</td>
<td>0.979</td>
<td>0.985</td>
<td>0.989</td>
</tr>
</tbody>
</table>

* based on 70 mg kg⁻¹ recommendation level.²²
† was measured in sausage containing SN, VN, or YN.
Table 5. Analysis of residual nitrite* in cooked sausages during storage at 4 °C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Storage (week)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN</td>
<td></td>
<td>A27±1a</td>
<td>B22±1a</td>
<td>C21±1a</td>
<td>D18±1.7a</td>
<td>E17±1a</td>
</tr>
<tr>
<td>VN</td>
<td></td>
<td>A10±1c</td>
<td>B9±1c</td>
<td>C8±1.7c</td>
<td>C7±1.7c</td>
<td>C7±2.6c</td>
</tr>
<tr>
<td>YN</td>
<td></td>
<td>A23±1.1b</td>
<td>B20±0.5b</td>
<td>C17±0.5b</td>
<td>C16±1b</td>
<td>C16±1b</td>
</tr>
</tbody>
</table>

* Residual nitrite determination reported in mg kg⁻¹ of sample

Treatment: SN, sodium nitrite added; VN, vegetable powder added; YN, young radish added.

a-c Means (n=3) ± SD in the same column with different superscripts are significantly different by Duncan’s multiple range test at p < 0.05.

A-E Means (n=3) ± SD in the same row with different superscripts are significantly different by Duncan’s multiple range test at p < 0.05.
Table 6. Effect of nitrite addition on color (L*, a*, b*) in cooked sausages during storage at 4 °C

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Days of storage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>L*</td>
<td>Control</td>
<td>83.7±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SN</td>
<td>86.6±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>VN</td>
<td>82.5±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>YN</td>
<td>74.6±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>a*</td>
<td>Control</td>
<td>2.3±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SN</td>
<td>11.6±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>VN</td>
<td>4.6±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>YN</td>
<td>7.5±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>b*</td>
<td>Control</td>
<td>26.7±2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>SN</td>
<td>16.7±1.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>VN</td>
<td>26.5±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>YN</td>
<td>21.6±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Treatment: Control, nitrite-free; SN, sodium nitrite added; VN, vegetable powder added; YN, young radish added.

<sup>a</sup>-<sup>d</sup> Means (n=3) ± SD in the same column within the same parameter with different superscripts are significantly different by Duncan’s multiple range test at p < 0.05.
Figure captions

Figure 1. Photographs of sausages made with sodium nitrite and vegetable nitrite from vegetable sources. A: nitrite-free, B: sodium nitrite added, C: vegetable powder added, D: young radish added.

Figure 2. The graph shows the TBARS values over time for different treatments. The legend indicates Control, SN, VN, and YN.
Figure 2. Effect of nitrite addition on TBARS values of cooked sausages during storage at 4 °C.

a-d Means (n=3) of same day with different superscripts are significantly different by Duncan’s multiple range test at $p < 0.05$. Control: nitrite-free, SN: sodium nitrite added, VN: vegetable powder added, YN: young radish added.
Figure 3. *L. monocytogenes*

A (4°C)

B (8°C)

C (10°C)

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Figure 3. Effect of nitrite addition on the growth of *L. monocytogenes* in cooked sausages during storage at 4 °C (A), 8 °C (B) and 10°C (C).

a–c Means (n=3) with different superscripts are significantly different by Duncan’s multiple range test at *p* < 0.05. Control: nitrite-free, SN: sodium nitrite added, VN: vegetable powder added, YN: young radish added.
Figure 4. EHEC

A (4°C)

B (8°C)

C (10°C)

log CFU/g

Day

log CFU/g

Day

log CFU/g

Day

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Figure 4. Effect of nitrite addition on the growth of EHEC in cooked sausage during storage at 4 °C (A), 8 °C (B) and 10°C (C). \(^{a-d}\) Means (n=3) with different superscripts are significantly different by Duncan’s multiple range test at \(p < 0.05\). Control: nitrite-free, SN: sodium nitrite added, VN: vegetable powder added, YN: young radish added.
Figure 5. *S. aureus*

A (4°C) 

B (8°C) 

C (10°C) 

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**Figure 5.** Effect of nitrite addition on the growth of *S. aureus* in sausages during storage at 4 °C (A), 8 °C (B) and 10°C (C). a-c Means (n=3) with different superscripts are significantly different by Duncan’s multiple range test at $p < 0.05$. Control: nitrite-free, SN: sodium nitrite added, VN: vegetable powder added, YN: young radish added.