Arugula and pre-converted arugula extract as natural Nitrate/Nitrite sources for heat-treated sausages

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Abstract. The aim of this study was to investigate using arugula or pre-converted extracts as nitrite alternatives in heat-treated fermented sausages. Sausages with nitrite, 150 mg/kg of NaNO₂, 1.2% arugula extract, and 1.5% pre-converted arugula extract were formulated. Natural nitrate sources added resulted in significantly lower oxidation content compared to negative control groups at initial storage. The addition of natural nitrate sources influenced colour, pigments, and conversion rate of sausages. Pre-converted arugula extract showed little effect on the residual nitrite content. The result of colour, oxidation, and nitrite analysis suggest pre-converted arugula is a potential nitrite replacer, but arugula as a nitrate source is limited to provide the functions of nitrite.

1. Introduction
Curing is one of the meat preservation methods that uses as ingredients salt, nitrate, and nitrite [2]. Nitrate/nitrite are utilized to form cured meat pigment, enhance the flavour, and provide antimicrobial and antioxidant effects in meat products. It is well known that nitrite is also responsible for producing carcinogenic N-nitrosamines in certain meat products under some processing conditions [3]. This concern has led researchers to seek natural nitrite alternatives. In recent years, much attention has been paid to products formulated with natural additives [4,5]. An alternative curing method to avoid the direct addition of synthetic nitrite is by utilizing those vegetables that have high nitrate content and starter cultures that possess nitrate reductase activity to convert the nitrate to nitrite (pre-conversion).

Spinach, beets, radishes, celery, lettuce, and cabbage are vegetables with high nitrate content [6]. It is stated that serious health concerns about nitrate and nitrite are mostly associated with high intake doses. However, the use of nitrate and nitrite obtained from natural sources does not have harmful toxicological effects on human health [6,7]. Arugula species (Eruca spp. and Diplotaksis spp.) are commercially important salad crops grown around the world [8]. The European Food Safety Authority (EFSA) found that the nitrate content in arugula ranged from 4800 to 6400 ppm in various regions and seasons in Europe [8]. Despite the high nitrate content of arugula species, there is no study regarding the effects of using arugula as a nitrite alternative in meat products. Therefore, the objective of this study was to determine the effects of arugula extract or pre-converted arugula extract on some quality parameters, lipid and protein oxidation of heat-treated fermented sausages.
2. Materials and methods

Preparation of pre-converted nitrite sources from arugula extract (PAE)
Ten grams of dried arugula was ground into powder form, mixed with 100 mL of distilled water, then 0.025% of an active nitrate reductase culture containing *Staphylococcus carnosus* (S-B-61 BactofermTM, Chr. Hansen Inc., USA) was added, and the mixture was incubated with shaking at 30°C for 24 h. The mixture was filtered using Whatman No. 1 filter paper and evaporated in a rotary evaporator (Heidolph Hei Vap ValG1, Germany) at < 50°C. The extract was stored in amber flasks in the dark (pre-converted arugula extract: nitrate content = 514.21 ppm, nitrite content = 56.38 ppm, pH = 5.33, L*=14.24±0.47, a*=-0.46±0.03, b*=13.96±0.32).

Preparation of nitrate sources from arugula extract (AE)
Ten grams of dried arugula powder was mixed with 100 mL of distilled water, then the mixture was left 20 minutes at 80°C in a water bath. The solution was filtered from a 0.45 µm filter, and the mixture was evaporated in a rotary evaporator (Heidolph Hei Vap ValG1, Germany) at < 50°C. The extract was stored in amber flasks in the dark. (Arugula extract: nitrate content = 4,689.27 ppm, nitrite content = 23.82 ppm, pH = 5.34, L*=14.24±0.54, a*=-0.47±0.84, b*=13.96±0.24).

Preparation of heat-treated fermented sausages
Fresh boneless beef cuts and beef fat were obtained from a local manufacturer. Production of heat-treated fermented sausages was carried out according to Zungur et al. [9]. Beef was trimmed of visible fat and connective tissue. Four different formulations of heat-treated fermented sausage, 4 kg each, were prepared: C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-converted arugula extract), AR (1.2% arugula extract). Fat and meat were separately minced through a plate with 3 mm holes and mixed with spices, curing agents (salt, sodium ascorbate, saccharose, and nitrite or natural nitrite source). Arugula extracts (150 ppm nitrate equivalent and 150 ppm nitrite equivalent) were added to the formulation to replace sodium nitrite. Sausage formulations are given in Table 1.

**Table 1.** Formulation of heat-treated fermented sausages

| Groups | Beef (g) | Fat (g) | Salt (g) | Spice (g) | Sugar (g) | Ascorbate (g) | NaNO₂ (g) | AE (g) | PAE (g) |
|-------|---------|--------|---------|----------|----------|--------------|----------|-------|--------|
| C     | 4000    | 800    | 96      | 180      | 19.2     | 1.92         | 0        | 0     | 0      |
| CN    | 4000    | 800    | 96      | 180      | 19.2     | 1.92         | 0.76     | 0     | 0      |
| PA    | 4000    | 800    | 96      | 180      | 19.2     | 1.92         | 0        | 0     | 75.51  |
| AR    | 4000    | 800    | 96      | 180      | 19.2     | 1.92         | 0        | 60.64 | 0      |

C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-converted arugula extract), AR (1.2% arugula extract).

The nitrate/nitrite amounts in arugula and pre-converted arugula extracts were calculated to be equivalent to 150 ppm nitrate and 150 ppm nitrite, respectively.

Physicochemical parameters
Total moisture and ash analysis were performed according to AOAC [10], fat content determined according to Flynn and Bramblet [11], protein content was determined by using an automatic nitrogen analyser (FP 528 LE-CO, USA) based on the Dumas method. pH was measured three times by using a pH-meter (WTW pH 330i/SET, Germany) equipped with a penetration probe. The colour of the samples was measured in triplicate using a portable colorimeter (CR-200, Konica Minolta, Japan) with D65 illuminant setting and 10° standard observer and expressed as CIE L* (lightness), a* (redness) and b* ( yellowness).
Lipid and protein oxidation analyses
Oxidative stability of sausages was analysed by determining peroxide value (PV) [12] and 2-Thiobarbituric acid reactive substances (TBARS) [12]. Protein carbonyls were measured by estimation of total carbonyl groups according to Levine et al. [13] with some modifications as described by Srinivasan et al. [14]. Sulphhydryl groups (thiol content) were determined according to Srinivasan et al. [14].

Residual Nitrite content
Residual nitrite content of heat-treated sausages determined by using AOAC method [10].

Nitrosomyoglobin and total pigments contents
The content of nitrosomyoglobin and total pigments were identified with the method described by Horsey [15]. Conversion ratio was calculated from the nitrosomyoglobin concentration divided by the total pigment concentration.

Statistical analyses
The data grouped by treatment were analysed by one-way ANOVA using the SPSS software version 11 (SPSS, 2001). A significance level of p < 0.05 was used for all evaluations.

3. Results and discussion
Chemical composition and pH values of sausages grouped by treatment are given in Table 2. The moisture content of all treatments was lower than 50% which is a limit set by the Turkish Food Codex [16]. The highest moisture content was found in PA and AR treatments due to the addition of extracts in liquid form. Similarly, Ozaki et al. [17] reported an increase in moisture content when radish powder and chitosan were added to the formulation as nitrite alternatives. Fat contents of the sausages were between 18.52%-24.45%. The highest fat content was found in PA treatment (P<0.05). The highest protein content was recorded in CN and AR treatments (P<0.05). Addition of chard powder [18] and beetroot flours [6] to fermented sausage formulation as nitrite alternatives did not change the chemical composition of the sausages. In our study, the pH was between 5.56-5.58 and significant differences were recorded among the pH of the sausages. pH 5.6 is the maximum pH value allowed for heat-treated fermented sausages in Turkey [16]. Similarly, cooked pork loins treated with nitrite, spinach juice, and fermented spinach juice had similar pH values [4]. Even though some researchers reported addition of acidic or basic additives to product formulation for nitrite replacement [18,19], in our study, arugula extract did not affect pH.

Table 2. Physicochemical parameters of heat-treated fermented sausages

| Physicochemical parameters | Samples   | C         | CN        | PA        | AR        |
|----------------------------|-----------|-----------|-----------|-----------|-----------|
| Moisture (%)               | 47.71±0.92| 47.43±0.54| 49.53±0.36| 49.44±0.26|
| Fat (%)                    | 21.09±1.75| 21.95±1.77| 24.45±1.25| 18.52±1.01|
| Protein (%)                | 23.76±1.22| 26.30±0.69| 22.44±0.09| 27.42±0.45|
| Ash (%)                    | 4.11±0.15 | 3.71±0.33 | 3.66±0.14 | 3.76±0.21 |
| pH                         | 5.58±0.01 | 5.56±0.01 | 5.56±0.01 | 5.56±0.01 |

All values are mean ± standard deviation of three replicates (n = 3).
* Means within a row with different letters are significantly different (P<0.05).
C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-concentrated arugula extract), AR (1.2% arugula extract).

The L* and a* values of internal surfaces were not affected by the treatments (Table 3), and regarding b*, it was found that C sausage, with no added nitrite or extract, was yellower than other sausages (P<0.05). The use of fermented celery and beet extracts did not affect L* values; however, the additives resulted in increasing effect on b* values [20]. In our study, the lowest external L* value was found in
CN treatment (P<0.05). External L*, a*, and b* values recorded were between 33.10-37.79, 10.27-13.74 and 10.81-14.95 respectively. The results showed that significant differences were obtained in colour features depending on formulations (P<0.05). The positive control formulated with nitrite had a more intense red colour as expected (P<0.05). The most visible change was seen in L* values, where PA and AR treatments were lighter compared to C and CN treatments. L* values did not change with the addition of 2% celery juice powder whilst a* values were higher in Bologna sausages formulated with celery juice [19]. The reason for lower a* values in our PA and AR treatments is probably due to the natural behaviour of extracts in giving a darker colour when mixed with water.

Nitrosomyoglobin, total pigment concentrations, and pigmentation conversion rates were lower in treatments without nitrite (CN; negative control) than other treatments, as expected. The pre-conversion process had a significant effect on nitrosomyoglobin formation, and similar results have been reported by other researchers [18]. Total pigment concentrations were between 109.63-155.93 ppm. The bioconversion process was found to be effective with regard to total pigment content, (P <0.05), as PA treatment had a greater amount of total pigment compared to other treatments (P <0.05). The conversion rate of fermented sausages was between 22.64-67.14%. CN had the highest conversion rate, followed by PA (P<0.05). The highest total pigment amount was found in the PA sausages. This result supports the idea that natural nitrate sources as nitrite alternatives would be more effective in curing if the nitrate is converted to nitrite. Sausages without sodium nitrite showed the lowest conversion rate (P<0.05). The curing efficiency of pork patties ranged between 9.16-82.66% and the highest efficiency was found in patties formulated with 60 ppm nitrite + 1 % pre-converted nitrite from Swiss chard powder, and addition of pre-converted nitrite from Swiss chard powder increased the total pigment concentration in pork patties [21].

Residual nitrite content of our heat-treated fermented sausages was between 8.45-22.59 ppm, and sausages formulated with 150 ppm sodium nitrite had the highest residual nitrite, as expected (p<0.05). Residual nitrite contents of the sausages treated with extracts were the same as the negative control. This result indicates that residual nitrite content was likely influenced by the presence of pre-converted nitrite. Kim et al. [22] reported that residual nitrite levels of pork sausage was affected by the presence of pre-converted nitrite in spinach extract. Similar to our results incorporation of celery, parsley, red beet, and spinach powders into cooked sausages lowered the residual nitrite levels [18], and Sindelar et al. [23] showed that the residual nitrite content of meat products treated with vegetable juice powder was higher when a greater amount of pre-converted nitrite was added to meat products.

**Table 3.** Colour parameters (L*, a*, b*), nitrosomyoglobin, total pigment, residual nitrite concentrations and conversion rate

| Color parameters | Samples | C | CN | PA | AR |
|------------------|---------|---|----|----|----|
| **L** | | | | | |
| Internal surface | L’ | 49.23±0.63 | 49.65±1.19 | 48.37±1.15 | 49.21±0.95 |
| | a* | 10.40±0.35 | 10.98±0.66 | 11.34±0.83 | 11.38±0.66 |
| | b* | 10.88±0.36 | 12.23±0.75 | 12.36±0.39 | 12.81±0.75 |
| External surface | L’ | 34.52±0.90 | 33.10±0.63 | 37.79±1.15 | 37.30±1.10 |
| | a* | 11.76±0.77 | 13.74±0.54 | 10.27±0.48 | 10.48±0.66 |
| | b* | 14.95±1.14 | 10.81±0.47 | 13.25±0.61 | 13.37±0.72 |
| Nitrosomyoglobin (ppm) | | | | | |
| | 24.77±1.30 | 102.64±2.48 | 96.77±1.34 | 42.76±1.86 |
| Total pigment (ppm) | | | | | |
| | 109.36±0.98 | 152.89±0.15 | 155.93±0.77 | 124.14±1.14 |
| Conversion rate (%) | | | | | |
| | 22.65±1.35 | 67.14±1.68 | 62.06±1.15 | 34.44±1.51 |
| Residual nitrite (ppm) | | | | | |
| | 8.45±0.13 | 22.59±1.09 | 8.78±0.54 | 9.50±1.15 |

All values are mean ± standard deviation of three replicates (n = 3).

**Table 3.** Colour parameters (L*, a*, b*), nitrosomyoglobin, total pigment, residual nitrite concentrations and conversion rate

PVs and TBARS values are shown in Table 4. The PV of treatments ranged between 5.63 and 20.64 meqO₂/kg. Initially, C and AR sausages had the highest PVs (P<0.05), whilst CN and PA had similar
PVs. PVs decreased in the second month then remained stable. CN and PA had similar PVs at the end of the storage. It could be said that primary oxidation was more pronounced in C treatment, which was formulated without nitrite.

The incorporation of nitrite effectively retarded TBARS values. Lipid oxidation in AR and C sausages was more intense compared to other treatments. Nevertheless, CN had the lowest TBARS value followed by PA (P<0.05). This result indicates the potential of PAE to replace nitrite up to some point. A study carried out by Ozaki et al. [17] reported that the use of beet and radish powders as natural nitrite sources did not affect the TBARS values on days 45 and 60 of storage. On the other hand, Kim et al. [4] stated that replacing nitrite with pre-converted spinach juices lowered lipid oxidation.

The total amount of carbonyl and sulfhydryl is accepted as one of the indicators of protein oxidation [24]. Carbonyl and sulfhydryl contents are shown in Table 4. Protein oxidation of PA and AR, which contained added with natural nitrite sources, did not differ from each other (P>0.05). The treatment with pre-converted nitrite source had higher sulfhydryl content compared to C (P<0.05). During the storage, pre-converted arugula extract was more effective in inhibiting protein oxidation than arugula extract. Sulfhydryl content of all treatments decreased throughout the storage; however, the addition of arugula extract or pre-converted arugula extract was effective against sulfhydryl loss. Similar to our result, Martínez-Zamora et al. [24] indicated that natural nitrate sources such as chard-beet with rosemary or citrus extract protected the chorizo against thiol loss. Initial carbonyl contents of our sausages were similar. At the end of the storage, the carbonyl contents of the sausages ranged between 5.07-5.67 nmol/mg. The addition of natural nitrite sources was found to be effective in maintaining carbonyl contents. It has been observed that nitrite and nitrite alternatives added to the formulation have a significant effect on lipid oxidation, resulting in a lower carbonyl content amount, thus providing a slower progression of protein oxidation in these examples.

### Table 4. Oxidation parameters of heat-treated fermented sausages

| Samples | C | CN | PA | AR |
|---------|---|----|----|----|
| 0th day | 18.62 ±0.41 | 13.71 ±1.17 | 14.78 ±1.03 | 17.53 ±0.98 |
| 1st month | 24.53 ±1.15 | 19.86 ±1.03 | 16.72 ±0.47 | 20.64 ±1.65 |
| 2nd month | 8.05 ±0.49 | 3.72 ±1.06 | 6.63 ±1.29 | 7.24 ±0.09 |
| 3rd month | 8.83 ±0.61 | 3.40 ±1.85 | 5.63 ±1.15 | 7.77 ±0.37 |
| 0th day | 1.01 ±0.01 | 0.54 ±0.10 | 0.75 ±0.04 | 1.70 ±0.02 |
| 1st month | 1.35 ±0.02 | 1.07 ±0.06 | 1.13 ±0.01 | 1.67 ±0.02 |
| 2nd month | 1.38 ±0.01 | 1.20 ±0.10 | 0.81 ±0.01 | 1.36 ±0.04 |
| 3rd month | 1.23 ±0.03 | 0.67 ±0.01 | 1.10 ±0.10 | 1.37 ±0.06 |
| 0th day | 36.70 ±0.70 | 43.86 ±1.08 | 38.79 ±0.91 | 38.67 ±0.68 |
| 1st month | 35.13 ±0.23 | 41.60 ±1.38 | 39.05 ±0.59 | 35.02 ±0.52 |
| 2nd month | 32.18 ±0.95 | 40.02 ±1.10 | 38.32 ±0.93 | 35.24 ±0.86 |
| 3rd month | 30.53 ±0.28 | 34.80 ±0.61 | 37.16 ±0.70 | 35.72 ±0.28 |
| 0th day | 5.18 ±0.22 | 4.31 ±0.29 | 4.74 ±0.25 | 4.87 ±0.64 |
| 1st month | 5.44 ±0.02 | 4.51 ±0.08 | 5.04 ±0.06 | 5.27 ±0.02 |
| 2nd month | 5.52 ±0.02 | 4.57 ±0.02 | 5.24 ±0.04 | 5.37 ±0.03 |
| 3rd month | 5.62 ±0.02 | 5.07 ±0.05 | 5.42 ±0.02 | 5.54 ±0.02 |

All values are mean ± standard deviation of three replicates (n = 3).

"abcd" Means within a row with different letters are significantly different (P<0.05).

"def" Means within a column with different letters are significantly different (P<0.05).

C (negative control, nitrite free), CN (positive control, 150 ppm sodium nitrite), PA (1.5% pre-converted arugula extract), AR (1.2% arugula extract).

### 4. Conclusion

In this study, it was shown that replacing nitrite with arugula extract or pre-converted arugula extract resulted in lower redness and nitrosomyoglobin and residual nitrite levels. At the same time, adding pre-converted arugula extract resulted in sausages that had similar quality parameters and oxidation levels.
to sausages treated with nitrite. Therefore, arugula extract has promising potential for use in heat-treated sausages as a nitrite source.

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