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Use of lysozyme from chicken egg white as a nitrite replacer in an Italian-type chicken sausage

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ABSTRACT

Background: Sodium or potassium nitrite is widely used as a curing agent in sausages and other cured meat products. Nitrite has strong antimicrobial and antioxidant effects and generates cured meat color. Nitrite, however, can react with secondary or tertiary amines in meat to form carcinogenic, teratogenic and mutagenic N-nitroso compounds. Several findings have been suggested that high consumption of processed meat may increase the risk of cancer, and emphasized that dietary nitrosamines are positively associated with cancer. Lysozyme is one of the major egg proteins that have antimicrobial and antioxidant characteristics. Therefore, lysozyme can be used in meat processing to prevent microbial growth and oxidative degradation in meat products during storage. This study was focused on evaluating the antimicrobial and antioxidant effects of lysozyme extracted from egg white as a replacer of nitrite in a cooked Italian-type chicken sausage.

Methods: Four curing treatments including 100% nitrite (control), 100% lysozyme (treatment 1), 25% nitrite + 75% lysozyme (treatment 2) and 50% nitrite + 50% lysozyme (treatment 3) were used to prepare Italian-type chicken sausage samples. Recipe was developed with 64% meat, 17% binder (bread crumble), 12% ice, 4% vegetable oil, 2% salt, spices 1% (chili, black pepper, cardamom). Prepared samples were cooked in an 80 °C smoke house to a core temperature of 65 °C and cooled in cold water to 20-25 °C subsequently packed in polyethylene and stored in a freezer (-18 °C). The antimicrobial effect lysozyme was tested using *Escherichia coli* and *Salmonella.* The growth of these pathogens at 0, 3 and 5 days of storage of spore inoculation was determined. Antioxidant activity of lysozyme was determined using the TBARS value during the 25 d storage period. The redness (a*), lightness (L*), and yellowness (b*) of sausages were analyzed using a Minolta color meter (CR 410, Konica Minolta Inc., Japan). The proximate

composition (AOAC, 2002) of frozen (-18 °C) sausage samples and sensory properties of cooked samples were determined.

Results: 50% nitrite + 50% lysozyme (treatment 3) was as effective as control (100% nitrite) in suppressing the growth of *Escherichia coli*, *Salmonella* and limiting lipid oxidation in the Italian-type chicken sausage. Treatment 3 was not significantly different from the control for the lightness (L*), redness (a*) and yellowness (b*) values ($P > 0.05$) but showed the best sensory characteristics among the treatments ($p=0.002$). Moisture content of control sample was significantly higher ($p=0.000$) than other treatments while crude protein, crude fat, crude fiber and ash content were not differ significantly. In term of the cost, both treatment 3 and control have shown approximately equal values.

Conclusion: This study demonstrated that lysozyme can be used as an effective nitrite replacer in the Italian-type chicken sausage. Replacing 50% of nitrate salt with 50% lysozyme did not show any negative effects in controlling microbial growth, preventing lipid oxidation, and color changes but improved the sensory characteristics.

Keywords: Italian-type chicken sausage, nitrite, lysozyme, antimicrobial, antioxidant

BACKGROUND

Meat is a highly nutritious food that can provide high-quality proteins, essential fatty acids, important minerals, and vitamins for human beings. However, the preference of meat has been affected since 1970's due to adverse incidences published by researchers and media, suggesting that eating meat is among the important causes of life-style diseases. Especially, cured and smoked meat products play major roles on the development of the diseases [1].

Sodium or potassium nitrite is widely used as a curing agent in Italian-type chicken sausages and other cured meat products [1]. Nitrite hampers spoilage and the development of food poisoning by anaerobic microorganisms, especially *Clostridium botulinum*, and delays the development of oxidative rancidity, develops the characteristic flavor of cured meats by reacting with myoglobin, and stabilizes the red color [2]. Nitrite, however, can react with secondary or tertiary amines in meat to form carcinogenic, teratogenic and mutagenic N-nitroso compounds (NNC) [2, 3].

Several findings have been suggested that high consumption of processed meat may increase the risk of cancer, and dietary nitrosamines have positive associations with cancer [4-8]. Wojciak et al. [7] reported that the preference of people towards the organic foods, which are absent chemical additives especially nitrate and nitrite salt, is high. Thus, many studies have been conducted to develop natural ingredients to substitute synthetic ingredients.

Lysozyme is one of the natural proteins present in egg white [4]. It is widely used as a food additive because it has antimicrobial and anti-oxidative effect against several pathogenic bacteria [8, 9, 10]. Lysozyme belongs to a class of enzymes that lyses the cell wall of certain Gram-positive bacteria by splitting the 1-4 linkages between N-acetylnuramic acid and N-acetylglucosamine of the peptidoglycan, which are important components of bacterial cell walls

[8, 9]. Radziejewska et al. [10] reported that lysozyme is capable of controlling food-borne pathogens such as *Listeria monocytogenes* and *Clostridium botulinum*. Lysozyme monomer effectively controls gram positive bacteria [11] and has antiviral and antitumor activity [8]. The objective of this study was to evaluate the use of lysozyme as a nitrite replacer in an Italian-type chicken sausage.

MARTIALS AND METHODS

Preparation of Italian-type chicken sausages: Chicken breast meats were purchased from a local market. Lysozyme powder was prepared according to the method described by Abeyrathne et al. [14]. Breast meats were first cut into small pieces using a knife and chilled at 4°C. The chicken meat pieces were divided into four equal portions.

Four treatments of Italian-type chicken sausage were prepared using ingredients commonly used in Italian-type chicken sausage preparations. Nitrite: lysozyme ratios in the four treatments were 100:0 (125 ppm nitrite), 0:100 (125 ppm lysozyme), 25:75 (21.25 ppm nitrite and 93.75 ppm lysozyme), and 50:50 (62.5 ppm nitrite and 62.5 ppm lysozyme). The sausage recipe was developed with 64% meat, 17% bread crumbs, 12% ice, 4% vegetable oil, 2% salt, 1% spices (chili, black pepper, and cardamom). All ingredients were mixed using a grinder (Jaipan Family Mate, IS 4250, India) and stuffed into cellulose casings (28 mm). Prepared samples were cooked at 80 °C in smoke house to a core temperature of 65 °C and cooled in cold water to 20-25 °C. Samples were subsequently packed in polyethylene bags and stored in a freezer (-18 °C) for further analyses.

Proximate analysis: Proximate compositions of the Italian-type chicken sausage samples were determined as outlined by AOAC [15-17]. Briefly, moisture content was measured by drying the samples (2 g) at 102 °C for 15 h. Crude fat and crude protein contents were measured by using the Soxhlet extraction system (TT 12/A, Gerhardt Ltd., Germany), and the Kjeldahl method (VAPO45, Gerhardt Ltd., Germany), respectively.

Color analysis: The color values of the Italian-type chicken sausage samples were analyzed using a color meter (CR 410, Konica Minolta Inc., Japan), which was calibrated against a white reference tile. The values of lightness (CIE L*), redness (CIE a*), and yellowness (CIE b*) were obtained using the average value of three repeated measurements taken from different locations on each sample.

Microbiological analysis

***Escherichia coli* (*E. coli*) test:** Microbiology test was conducted according to Sri Lanka Standards [18] method for *Escherichia coli* with slight modifications. Spore inoculation was done prior to microbial analysis using the following procedure: an *E. coli* strain obtained from a processed meat product was used for the inoculation. The stock culture of *E. coli* was grown in an Eosin Methylene Blue Agar (Oxoid, England, 37.5 g/L). Using a sterile micropipette tip, bacterial colony was gently scraped and the tip was rinsed out with 9 ml of peptone water (Himedia, India, 15 g/L), and mixed with the Italian-type chicken sausage. The paste was stuffed

into casings and the Italian-type chicken sausage samples were cooked to 65 ^{o}C, cooled, packed and stored at 4 ^{o}C.

For microbiological analysis, 10 g of meat from each sample were homogenized with 90 ml of sterile water using a sterile commercial blender (Jaipan Family Mate: IS 4250, India) for 1 min. The homogenates were diluted in series to prepare 10^{-2}, 10^{-3} and 10^{-4} diluted samples. One-ml portion of the diluted samples were spread in each pre-poured and dried standard Eosin Methylene Blue Agar media (Oxoid, England, 37.5 g/L) and then incubated at 37 ^{o}C for 24 hr. Typical *E. coli* colonies were counted as colony forming units (CFU) after the incubation period using a colony counter (Galaxy 230) and the results were expressed as log10 CFU/g of Italian-type chicken sausage sample. Three replicates per each treatment were prepared and the colonies were counted at 0, 3, and 5 days of storage at 4 C to analyze whether there is a suppression of growth for *E. coli*.

***Salmonella* identification:** *Salmonella* identification test was conducted according to Sri Lanka standards [19]. Spore inoculation procedure was the same as *E. coli* whereas *Salmonella* were grown in Xylose-Lysine-Deoxycholate Agar (Oxoid, UK, 3 g/L). *Salmonella*-inoculated meat samples were prepared for each treatment as in the *Escherichia coli* study. For the *Salmonella* test, 1 g of each sample was measured into a sterile vacutainer and 10 ml of peptone water (Himedia, India, 15 g/L) was poured in. Then, the sample was mixed well using a vortex mixer (Jeiotec: VM 96B, Korea) and kept in a 37 ^{o}C incubator (Gemmy 888, Taiwan) for 48 hr. After incubation, samples were spread on the pre-poured replicate plates prepared with dried standard Xylose-Lysine-Deoxycholate Agar (Oxoid, UK, 3 g/L). The inoculated plates were kept in the incubator at 37 °C for 24 hr to detect the presence of *Salmonella*. The test was replicated 3 times and the colonies were counted at 0, 3, 5, 7 and 21 days after incubation to see the growth of *Salmonella*.

Assessment of lipid oxidation: Samples for lipid oxidation assay were prepared by mixing 1 g of meat sample with 9 ml distilled water. The sample was thoroughly homogenized using a homogenizer (Vortex Genie, B model, VM 96 B) at 3000 rpm for 30 seconds. Ascorbic acid (0.5 ml, 99.7% w/w) was added to the sample and incubated at 37 ^{o}C for 16 hr. At the end of incubation, 1 ml of the sample was transferred to a 15 ml Falcon tube, added with 2 ml of thiobarbituric acid/trichloracetic acid solution (20 mM TBA/15% TCA) (w/v), 50 µl of butylated hydroxyanisole in 90% ethanol, and vortex-mixed. The mixture was incubated in a 90 °C water bath for 15 min to develop color. The sample was cooled in an ice bath for 10 minutes and centrifuged at 3000 x g for 15 min (Gemmy, Taiwan). The absorbance of the supernatant solution was measured using a UV-VIS Spectrophotometer (model galaxy, UV 1200) at 532 nm against a blank prepared with 1 ml of distilled water and 2 ml TBA/TCA solution. The amounts of TBARS were expressed as mg of malondialdehyde (MDA) per L of homogenate.

Sensory evaluation: Representative Italian-type chicken sausage samples were sliced into approximately equal size and placed in odorless, disposable paper plates. Each sample was coded separately with a three-digit number and served for the sensory evaluation. Clean water was

provided to clean the mouth between each sample. Thirty panelists (Age group 21-26 years /male & female) assessed the appearance, texture, color, mouth feel and overall acceptability scores of the samples at Day 0 using a 5-point hedonic scale.

STATISTICAL ANALYSIS

All measurements were carried out in triplicate (n=3), and results were subjected to a one-way analysis of variance (ANOVA) using Minitab 16 software (Minitab, Inc., USA). Differences between means were determined by the least significant difference test at $P < 0.05$. Non parametric data (only sensory data) were analyzed using Friedman test.

RESULTS

Oxidation assessments: At Day 10, the oxidation values of the control and treatment 3 (50% nitrite: 50% lysozyme) were significantly different (P=0.000) from those of the treatments 1 (100% lysozyme) and 2 (25% nitrite + 75% lysozyme) [Table 1].

Table 1. Malondialdehyde (MDA) content (mg/ kg) of Italian-type chicken sausage added with chicken egg white lysozyme

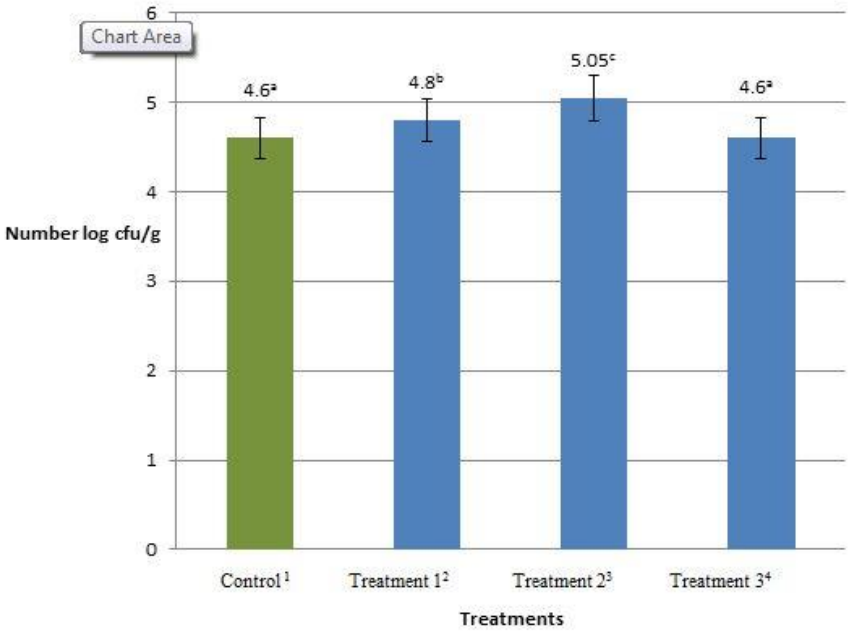
Treatment	Day 0	Day 05	Day 10	Day 15	Day 20	Day 25
Control[1]	$0.079^{a}\pm0.004$	$0.070^{a}\pm0.001$	$0.065^{a}\pm0.004$	$0.088^{a}\pm0.003$	$0.076^{a}\pm0.001$	$0.069^{a}\pm0.003$
Treatment 01[2]	$0.088^{a}\pm0.003$	$0.085^{a}\pm0.005$	$0.179^{b}\pm0.005$	$0.074^{a}\pm0.004$	$0.085^{a}\pm0.002$	$0.075^{a}\pm0.001$
Treatment 02[3]	$0.064^{a}\pm0.002$	$0.098^{a}\pm0.002$	$0.164^{b}\pm0.004$	$0.081^{a}\pm0.002$	$0.086^{a}\pm0.001$	$0.071^{a}\pm0.004$
Treatment 03[4]	$0.073^{a}\pm0.001$	$0.096^{a}\pm0.003$	$0.140^{c}\pm0.003$	$0.073^{a}\pm0.001$	$0.086^{a}\pm0.003$	$0.074^{a}\pm0.002$

[a-c]Different letters within a column are differ significantly(p<0.000) 100% nitrite[1], 100% lysozyme[2], 25% nitrite + 75% lysozyme[3] and 50% nitrite + 50% lysozyme[4]

According to the mean comparison, the lowest oxidation rate was observed in control samples followed by treatment 3. Except for Days 5 and 10, the values within a treatment were not differ significantly (P > 0.05) [Table 1].

Antimicrobial activity of lysozyme: The antimicrobial activity against *E. coli* was determined by spore inoculation and the suppression of microbial growth. Both control and treatment 3 (lysozyme 50%: curing salt 50%) have shown the lowest microbial growth among all treatments (4.6 cfu/g) (p =0.001). Although, 50% of nitrite was replaced with lysozyme in treatment 3 (50% nitrite: 50% lysozyme), the results (antimicrobial activity) remained the same as the control (Figure 1).

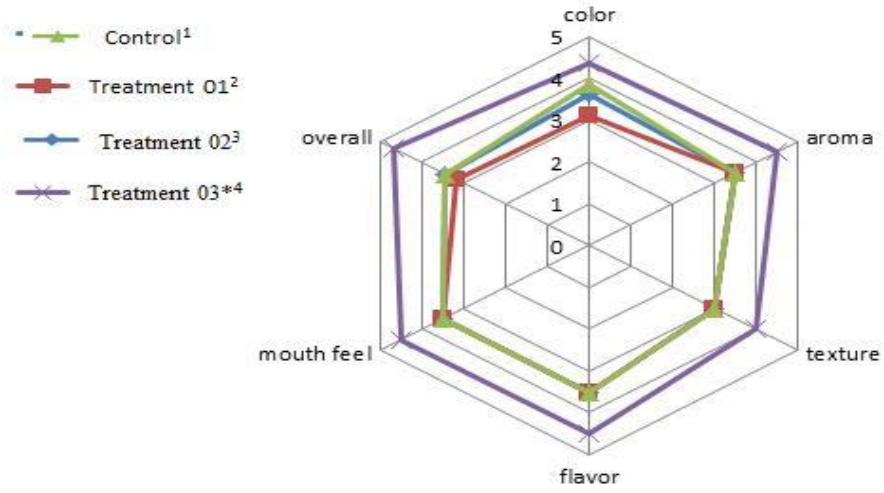
The presence or absence of *Salmonella* was an indicator of antimicrobial effects. After 5 days of storage, all the samples representing all treatments were negative in *Salmonella.* Lysozyme was more effective on *Salmonella* than *E.* coli. This may be due to the differences in cell-wall composition of the two microorganisms.



100% nitrite[1], 100%, lysozyme[2], 25% nitrite + 75% lysozyme[3] and 50% nitrite + 50% lysozyme[4]

Figure 1: Microbiology analysis of Lysozyme incorporated Italian-type chicken sausage against *Escherichia coli* day 5. [a-c]Different letters are differ significantly (p=0.001).

Sensory analysis: Sensory results indicated that treatment 3 (50% nitrite: 50% lysozyme) had significantly higher flavor, color, aroma, texture, odor and overall acceptability than other three treatments (p=0.002) (Figure 2). This indicated that replacing 50% nitrite with lysozyme significantly increased the sensory attributes of the Italian-type chicken sausages.



100% nitrite[1], 100% lysozyme[2], 25% nitrite + 75% lysozyme[3] and 50% nitrite + 50% lysozyme[4]

Figure 2: Sensory analysis of the lysozyme incorporated Italian-type chicken sausage. *Treatment 3 is significantly differ from other treatments for all evaluated sensory properties (Data not shown) (p=0.002).

Color analysis: From the oxidation, microbiology, and sensory evaluation point of view, treatment 3 (50% lysozyme: 50% nitrite) was the best among the four treatments. However, treatment 3 was not significantly different from the control for the lightness (L*), redness (a*) and yellowness (b*) values (P > 0.05) (Table 02).

Table 2: Color analysis for control and three treatments

Color parameter	Control[1]	Treatment 1[2]	Treatment 2[3]	Treatment 3[4]
L*(Lightness)	$32.93^{a} \pm 1.51$	$32.72^{a} \pm 0.98$	$31.96^{a} \pm 1.23$	$32.9^{a} \pm 1.01$
a*(Redness)	$16.57^{b} \pm 1.01$	$15.86^{b} \pm 1.32$	$16.07^{b} \pm 0.86$	$16.77^{b} \pm 0.66$
b*(Yellowness)	$7.53^{c} \pm 0.96$	$8.42^{c} \pm 1.20$	$8.66^{c} \pm 0.88$	$8.00^{c} \pm 0.45$

[a-c]Different letters within a raw are differ significantly (p=0.000). No significant difference in L*(p=0.575), a* (p=0.162) and b* (p=0.174) among treatments. 100% nitrite[1], 100% lysozyme[2], 25% nitrite + 75% lysozyme[3] and 50% nitrite + 50% lysozyme[4]

Proximate analysis: Moisture content of the control (100% nitrite) samples was significantly higher (p=0.000) than treatment 3 (50% lysozyme: 50% nitrite), whereas crude protein, crude fat, crude fiber and ash content were not significantly different (P >0.05) (Table 3).

Table 3: Proximate analysis for control and treatment 3

Character	Control[1]	Treatment 03[2]
Moisture	66.90 ± 1.92^{a}	62.80 ± 1.67^{b}
Crude protein	$18.14^{a} \pm 1.24$	$18.77^{a} \pm 1.04$
Crude fat	$12.54^{a} \pm 0.87$	$12.67^{a} \pm 0.97$
Crude fiber	$1.82^{a} \pm 0.44$	$1.92^{a} \pm 0.36$
Ash	$0.16^{a} \pm 0.02$	$0.22^{a} \pm 0.06$

[a-b]Different letters within a raw are differ significantly(p=0.000). No significant difference in crude protein (p=0.643), crude fat (p=0.090), crude fiber (p=0.245) and ash (p=0.058) among treatments. 100% nitrite[1] and 50% nitrite + 50% lysozyme[2]

Cost analysis: Cost analysis of control (100% nitrite) and treatment 3 (50% lysozyme: 50% nitrite) indicated that they were approximately the same. Therefore, it can be concluded that the use of lysozyme from chicken egg white as an antimicrobial and antioxidant supplement in large-scale Italian-type of chicken breast sausage production is cost effective and economically viable.

DISCUSSION

This study investigated the antimicrobial and antioxidant effects of lysozyme extracted from egg white as a replacer of nitrite in Italian-type of chicken breast sausages. Liu et al. [20] and Mine [13] reported that lysozyme has a strong antioxidant property as it scavenges free radicals and hydroxyl molecules, leading to decreased oxidative stresses. Results of this study also proved reduced oxidation in the presence of lysozyme in Italian-type of chicken breast sausages.

Heme-compounds of muscle contain iron ions that catalyze lipid oxidation reactions. Curing salt reacts with heme pigments to form cured pigments. However, iron retained in the heme, usually in the reduced (Fe^{2+}) form, makes it unavailable as a catalyst for lipid oxidation and formation of nitroso- and nitrosyl compounds that have antioxidant properties. Even though curing salts are a popular antioxidant agent, lysozyme could be able to replace it due to its antioxidant capacity. When curing salt was combined with lysozyme in equal propotion, it generated lower oxidation value (p=0.000). The results proved a positive potential for lysozyme as an antioxidant in Italian-type chicken breast sausages. However, the oxidation values increased dramatically on day 5 and decreased after that. The reason was not clear in meat products but similer resutls have been observed in fish proteins. Malonaldehyde is capable of cross-linking with amino acids to form amidine linkages and may also interact with other components of meat such as nucleosides, nucleic acids, amino acids of phospholipids and other aldehydes which are the end products of lipid oxidation. This may be the reason why the levels of TBARS decreased with time [21]. Also, Odote and Obiero [21] mentioned that the decrease in levels of TBARS after an increase is because the carbonyls are unstable and react easily with other compounds. It is also known that oxygen accessibility, degree of tissue disruption, and storage temperatures can affect shelf-life and are important for rancidity development in meat.

Durance [22] reported that enzyme activity can be enhanced by certain substances including EDTA, butylparaben, tripolyphosphate, as well as some naturally-occurring antimicrobial agents. This study showed that combining curing salt (nitrite) with lysozyme increased the antimicrobial ability of lysozyme. Antimicrobial activity of lysozyme is clearly displayed in gram positive bacteria as it hydrolyzes the cell walls of gram - positive bacteria [8, 23, 19]. Ibrahim [24] also reported that *E. coli* was less sensitive to lysozyme than *Salmonella*. Even though, heat denaturation of lysozyme resulted in the progressive loss of enzymatic activity, a greatly improved antimicrobial action against gram negative bacteria was reported by Ahn [25] and Radziejewska et al. [11]. After 5 days of storage, all the samples were negative for *Salmonella*. Ahn et al. [25] reported that the high-affinity binding of lysozyme to bacterial lipopolysaccharide resulted in reversible inactivation of its enzymatic activity. This study also indicated a possibility of using lysozyme as an antimicrobial agent for gram-negative bacteria, *i.e. Escherichia coli*. Ibrahim et al. [24, 26] reported that lysozyme can be lethal to gram-negative bacteria if the interactions with the bacterial membrane can be strengthened by modifying the enzyme surface hydrophobicity.

Sensory analysis results demonstrated that replacing 50% of nitrite with lysozyme significantly improved the sensory attributes of the Italian-type chicken breast sausages. The combination of lysozyme with curing salts has given a better result than other treatments for many sensory attributes. Hen egg lysozyme elicits a sweet taste sensation for human beings. Pearson [27] reported that curing salt is also considered as a color and flavoring agent. Rather than acting alone, when those characters are combined together it appeared to act synergistically. Therefore, treatment 3 has shown significant modifications in the sensory attributes of the final product.

The oxidation, microbiology and sensory results indicated that treatment 3 (50% lysozyme: 50% nitrite) was among the best. So, treatment 3 was subjected to color, proximate analysis and cost analysis to determine the further characteristics and economic viability of the product. Color

of the cured meat product is an important quality characteristic. Ahn [25] reported that lysozyme has an ability to bind with Fe^{2+} and directly affects color. Curing salt improves its own curing color and flavor on meat products. Therefore, no significant differences were observed between the two selected treatments - treatment 3 and control. Yellowness occurs as the result of Maillard browning reaction [28]. It seems that both curing salt and lysozyme induced the same rate of Maillard browning reaction in the meat product. Therefore, adding lysozyme did not significantly modify the L*, b* and a* values of treatment 3 and control. The proximate compositions of treatment 3 and control demonstrated that only moisture content was altered. It was also reported that lysozyme can bind with water molecules [29, 30, 31] and this may be the reason why treatment 3 had lower moisture content than other treatments.

CONCLUSION

This study has clearly shown that the control and treatment 3 (50% lysozyme: 50% nitrite) significantly suppressed the microbial activity of the meat product. Lysozyme reduced the oxidation but did not affect the color and other sensory properties, and maintained the quality of the final product. Therefore, partially replacing curing salt with lysozyme is possible and recommended. Results of this study also demonstrated that the production cost of treatment 3 was approximately the same as control. Therefore, it is proven that lysozyme can be effectively used as a partial nitrite replacer in Italian-type chicken breast sausages.

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