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Thyme and Clove Essential Oils as Antioxidants and Antimicrobial in Beef Sausage

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Abstract: Sausage is one of the well-liked foodstuffs in many areas. However, its quality is vulnerable to deteriorate owing to the lipid oxidation and microbial contamination. This research aimed to study the influence of thyme and clove essential oils (EOs) with or without nitrite on the chemical composition, microbial growth, and lipid oxidation rate in sausage during frozen storage-18°C for three months. The obtained results showed that the main component in clove EO and thyme EO is eugenol and thymol. Adding sodium nitrite to sausage resulted in declines in moisture and crude lipids and increases in protein and ash contents of sausage. Adding the tested EOs increased nitrite's effect on the chemical composition of sausage. Besides, these EOs lowered TBARS values, residual nitrite, and TBC in sausage. They also resulted in the rise of nitrite's efficacy in reducing TBARS values and TBC in sausage. *Proteus* was more sensitive than *Klebsiella*, whereas *E. coli* showed more resistance when adding nitrite or EO to sausage. The tested EOs increased the inhibitory influence of nitrite on *Proteus*, *Klebsiella*, *Aspergillus niger*, and *Candida albicans* in sausage. We concluded that the EOs of clove and thyme have antioxidant and antimicrobial efficacies in raw beef sausage during frozen storage. Also, they have a synergistic impact on nitrite. Therefore, it is suggested that these EOs, especially thyme, could be utilized to prolong shelf-life, prevent deterioration of sausage, and lessen the added nitrite's proportion to sausage for avoiding the formation of carcinogenic N-nitrosamines.

Keywords: Clove Essential Oil, Thyme Essential Oil, Antioxidant Activity, Antimicrobial Activity, Nitrite, Beef Sausage

1. Introduction

Sausages are one of the popular food items in many regions. Sausage quality is subject to deterioration due to lipid oxidation and microbial contamination. Hence, the additional factors which own antioxidant and antimicrobial effects are useful for maintaining the sausage quality and prolonging its shelf life. For achieving these goals, sausage manufacturers have used many artificial food additives with antioxidant and antimicrobial properties in the last few decades, such as nitrite [1]. Nitrite is used in making sausage in the form of sodium nitrite.

Nitrite owns antioxidant influence. It is an essential ingredient useful for producing the characteristic red color and flavor in sausage. Also, nitrite prevents unwanted microorganisms' growth, particularly *Clostridium botulinum*. Despite the beneficial nitrite role in manufacturing sausage, the nitrite usage in sausage became controversial. The reason for that is the formation of carcinogenic nitrosamines through the interaction of secondary amines of meat with nitrite [2]. Thus, there is a developing awareness to lower or exclude the nitrite added to sausage, which resulted in the pressure for using natural additives [3]. And as a result, the researchers of food industries search about natural food additives. Prerequisite in these food additives, they have to possess antioxidant and antibacterial activities against lipid oxidation and microbial inroads in sausage. At the same time, they have to own a synergistic impact on nitrite to lower the percentage of the added nitrite quantity. Consequently, there is nowadays a considerable interest in examining the ability of many natural additives such as essential oils from many plants to delay lipid oxidation and reduce microbial growth to preserve foods such as sausage [4].

Essential oils (EOs) are volatile liquids that are aromatic and oily. They are extracted from various plants' parts, such as buds, bark, flowers, leaves, peels, seeds, etc. Many EOs are known to be rich sources in antioxidants that inhibit oxidative damage [5]. As a result, more attention has been paid to testing the ability of several EOs to act as antioxidant and antimicrobial. This test goals to find EOs that act as a "safe" substitute for artificial antioxidants and synthetic antimicrobials. Hence, nowadays, research is directed to detect EOs that can prevent the growth of both harmful and pathogenic organisms and inhibit fat oxidation for prolonging the shelf life of the food products [4,6].

Clove (*Syzygium aromaticum* (L.)) EO displayed a clear antioxidant impact *in vitro* when using 2, 2'-diphenyl-1-picrylhydrazyl radical scavenging (DPPH) method [7-8]. Also, clove EO displayed antibacterial and antifungal influences *in vitro* [9]. Thyme (*Thymus vulgaris*) EO exhibited antioxidant impact *in vitro* [10-11]. This EO also showed antibacterial and antifungal efficacies *in vitro* [3, 1].

For the reasons mentioned above, this study aimed to evaluate the activity of adding clove EO or thyme EO in the beef sausage's industry for controlling its content of residual nitrite content. Also, studying the effect of adding these EOs during storage for three months at-18°C on the chemical composition, microbial growth, and lipid oxidation of beef sausage formulas that were treated with or without 100 ppm nitrite. Moreover, this study aimed to estimate the influence of the studied EOs on the nitrite activity if they possess a synergistic effect on nitrite or not.

2. Materials & Methods

2.1. Materials

2.1.1. Essential Oils

Two essential oils of clove and thyme were attained from the Agricultural Research Center, Giza, Egypt.

2.1.2. Beef's Fat and Meat Tissues

Fresh beef's fat and meat were purchased from butcher's shop in Mansoura city, Egypt.

2.2. Preparing Beef Sausage's Formulas

2.2.1. Preparation of Spices Mixture

Spices were obtained from the local market in Mansoura city, Egypt. They were grounded separately, then the mixture of spices was made according to Osheba's method (2003) that was described in El-Zainy *et al.* [12], as follows: 4.74% bay leaf, 10.52% Arab yeast, 25.23% cubeb, 7.05 % clove, 1.84% cardamom, 9.91% cinnamon, 10.22% white pepper, 8.22% corengan, 4.97% thyme, 2.69% nutmeg, and 14.61% rosewood.

2.2.2. Preparing Beef Sausage's Formulas Without Adding Nitrite

Three sausage's formulas without adding nitrite were prepared in this study. Control's formula (A) was made according to Hassan's method [13] that was described in El-Zainy *et al*. [12], as follows: minced beef meat (65.66%), animal fat (20%), spices (1.8%), fresh garlic (0.55%), sodium chloride (1.8%), ascorbic acid (0.03%), and iced water (10.16%). Two sausage's formulas (B and C) were prepared by substituting 0.02 g% iced water from the control formula's ingredients by 200 ppm clove EO or thyme EO, respectively. These EOs were dissolved in the used animal fat before their adding during preparing sausage's formulas.

2.2.3. Preparing Beef Sausage's Formulas with Adding Nitrite

For preparing beef sausage's formulas with nitrite, 0.01 % iced water from the ingredients of formula (A)- which is mentioned above- was substituted by 100 ppm sodium nitrite [14]. Two sausage's formulas with nitrite (Y and Z) were prepared by substituting 0.02 g% iced water from the ingredients of formula (X) by 200 ppm clove EO or thyme EO, respectively.

2.3. Chemical Composition

Estimating the contents of moisture, crude lipids, crude protein, and ash was done in the studied sausage's formulas, according to AOAC [15]. This estimation was accomplished on the studied sausage's formulas at zero time and after 1, 2 & 3 months from frozen storage at-18°C.

2.4. Gas Chromatography/Mass Spectrometry Analysis

The constituents of the studied EOs were detected by the GC/MS technique (Focus/DSQ II, Thermo Scientific, USA), as mentioned in Uddin *et al*. [16].

2.5. Estimation of Thiobarbituric Acid Reactive Substances (TBARS)

The values of TBARS were evaluated spectrophotometrically in the tested sausage's formulas according to the mentioned method by Siu and Draper [17]. Values of TBARS were expressed in the tested sausage's formulas by mg malonaldehyde/Kg sausage's formula (=ppm).

2.6. Estimation of Residual Nitrite

Ten grams from each sausage's formula were homogenized in 100 ml distilled water. After that, the centrifugation process was carried out on each homogenate for 15 min at 3000 rpm. Nitrite's estimation was performed according to Montgomery and Dymock's [18] method.

2.7. Estimation of Total Bacterial Count

Total Bacterial Count (TBC) was evaluated in each sausage's formula according to the method of Difco Manual [19] by using the nutrient agar media.

2.8. Identification of Bacterial and Fungal Strains

The Gram-Negative bacteria's strains were recognized by the rapid biochemical tests (API 20 E, Api 20 NE). At the same time, Gram-Positive bacteria's strains were identified by the API Staph and automated system (Vitek 2 compact) according to the instructions of the manufacturer (Api Strips, Biomerieux, France). Identification of Bacterial Species in the tested sausage's formulas was carried out according to the mentioned method in Ahmed and Sabiel [20]. The identification of molds and yeast species was carried out according to the method described by Anwer *et al.* [21].

2.9. Sensory Evaluation

Sensory evaluation was done using a hedonic test that was mentioned in El-Abasy *et al.* [22]. For overall acceptability's evaluation of the tested sausage's formulas, a nine-point hedonic scoring scale was applied (9=Like extremely, 8=Like very much, 7=Like moderately, 6=Like slightly, 5=Neither like nor a dislike, 4=Dislike slightly, 3=Dislike moderately, 2=Dislike very much, 1=Dislike extremely).

2.10. Statistical Analysis

Data was analyzed by GraphPad Prism [23] version 3.0 for Windows, and a comparison of means was made at a 5% level of Tukey's Multiple Comparison.

3. Results

3.1. GC-MS Analysis of the Tested EOs

GC-MS analysis's results for the tested EOs displayed that Eugenol and Thymol are the main components in clove EO and thyme EO, respectively.

3.2. Chemical Composition of the Studied Sausage Formulas

From table 1, it is clear that adding 100 ppm nitrite to sausage resulted in lowering in its contents of moisture and crude lipid when comparing with their corresponding in the control sample, which was made without nitrite. Meanwhile, adding nitrite to sausage increased its ash and crude protein contents. With increasing the storage time at-18°C from zero time to three months, it was found that nitrite's presence in sausage decreased the reduction of moisture and crude lipid contents. Besides, nitrite's presence in sausage reduced the rise in protein and ash contents of sausage.

Protein and moisture contents were lowered in the sausage's control formula with increasing the storage period. At the same time, adding either clove EO or thyme EO resulted in reducing the reductions in these contents. In addition to that, it found that these tested EOs had a synergistic influence on nitrite action. For example, adding thyme EO with nitrite to sausage's formula lessened the reduction in protein and moisture than the control sausage's formula only treated with nitrite.

Table 1. *Chemical composition of the tested sausage's formulas during storing period at-18°C.*

Sample	Storage months	Moisture %	Dry Matter %	Crude Protein %	Crude Lipids %	Ash %	Carbohydrates %
A	Zero	65.66	34.34	12.49	15.94	5.11	0.79
	1	64.41	35.59	12.04	16.96	5.78	0.81
	2	63.52	36.48	11.23	17.60	6.05	1.60
	3	62.18	37.82	10.65	18.10	6.59	1.60
B	Zero	65.59	34.41	12.95	15.32	5.41	0.73
	1	64.49	35.51	12.45	16.36	6.14	0.56
	2	63.78	36.22	11.57	17.17	6.43	1.06
	3	62.50	37.50	11.28	17.63	6.97	1.06
C	Zero	65.36	34.64	13.18	15.03	5.57	0.86
	1	64.22	35.78	12.60	16.08	6.31	0.80
	2	63.53	36.47	11.72	16.86	6.58	1.31
	3	62.21	37.79	11.08	17.38	7.16	1.31
X	Zero	65.10	34.90	13.82	14.15	6.07	0.86
	1	64.27	35.73	13.09	15.10	6.79	0.76
	2	63.57	36.43	12.23	15.90	7.13	1.17
	3	62.52	37.48	11.49	16.62	7.68	1.17
Y	Zero	65.07	34.93	14.33	13.61	6.36	0.62
	1	64.43	35.57	13.39	14.51	7.14	0.53
	2	63.87	36.13	12.52	15.34	7.45	0.82
	3	62.82	37.18	11.77	16.12	8.02	0.82
Z	Zero	64.83	35.17	14.57	13.36	6.58	0.66
	1	64.06	35.94	13.57	14.16	7.28	0.93
	2	63.73	36.27	12.75	15.03	7.68	0.81
	3	62.74	37.26	11.87	15.86	8.17	0.81

+ Where:
A: A control sausage formula without any additives. B: A sausage formula with clove EO. C: A sausage formula with thyme EO.
X: A sausage formula with nitrite. Y: A sausage formula with nitrite & clove EO. Z: A sausage formula with nitrite & thyme EO.

The crude lipids' content increased in the sausage's control formula with increasing the storage's period of sausage. Adding either clove EO or thyme EO to sausage's formula reduced this rise in the sausage's lipids content. Adding thyme EO was better in reducing the increase in sausage's lipid content than adding clove EO. Adding thyme EO or clove EO with nitrite to sausage's formula lessened the increase in lipids compared with the control sausage's formula only treated with nitrite.

Ash content raised in the sausage's control formula with increasing the period of frozen storage. However, this elevation in ash content was increased by adding one of the tested EOs. Also, it was observed that the tested EOs and nitrite had a synergistic influence. For example, adding nitrite with clove EO caused elevating ash content increase compared with the control formula that was only treated with nitrite.

3.3. Thiobarbituric Acid Reactive Substances Value (TBARS) in the Studied Sausage Formulas

From displayed data in the table 2, it is clear that TBARS values in all the sausage's formulas rose nearly throughout the study. Adding sodium nitrite to sausage's formulas decreased significantly (P < 0.05) the sausage's TBARS values. TBARS values were not influenced at zero time by adding the tested EOs to sausage. However, the TBARS values in the sausage formulas which contained clove EO or thyme EO were less (p < 0. 001) than corresponding values in the control formula, especially after 2 & 3 months from storing at-18°C. About the combined influence for nitrite with clove EO or thyme EO, it observed that adding nitrite with clove EO or thyme EO decreased the TBARS values in sausage. These values were less than their corresponding values in the sausage formula that treated with nitrite only. In addition to that, clove EO or thyme EO reduced TBARS values in all sausage formulas, that were made with or without adding nitrite when comparing with the control formula that did not contain any additives.

Table 2. *% Change in thiobarbituric acid reactive substances value (TBARS*) of the tested sausage formulas during storing period at-18°C.*

Sausage formula	Zero time	One month	Two months	Three months
A	0.278	0.340	0.513	0.905
B	0.263	0.318	0.451	0.760
C	0.251	0.299	0.422	0.720
X	0.233	0.255	0.400	0.652
Y	0.212	0.228	0.352	0.562
Z	0.202	0.217	0.329	0.523

+ where: *mg MDA/kg: mg malonaldehyde/Kg fresh sausage formula. A: Control formula without any additives. B: Formula with clove EO.
C: Formula with thyme EO X: Formula with nitrite. Y: Formula with nitrite & clove EO. Z: Formula with nitrite & thyme EO.

3.4. Residual Nitrite in the Studied Sausage Formulas

Data in the table 3 showed the percentage change in residual nitrite of the tested sausage formulas during the frozen storage period at-18°C. At zero time, residual nitrite content in all tested and control sausage formulas is a tiny fraction from the quantity added. However, its content gradually increased with increasing the storing period from zero time to three months.

At zero time, the residual nitrite content in sausage formulas that contained clove EO or thyme EO was lower (p< 0. 001) than its corresponding in the control formula. With the increasing period of the storing from zero time to three months, the residual nitrite content in sausage formulas containing clove EO or thyme EO elevated gradually. However, it was less (p < 0. 001) than its corresponding in the control formula. The thyme-contained sausage formula was less than the clove included formula in residual nitrite content throughout the storing time. When adding clove EO or thyme EO with nitrite while making sausage, the residual nitrite content lessened.

Table 3. *% Change in residual nitrite* of the tested sausage samples during the storing period at-18°C.*

Sausage formula	Zero time	One month	Two months	Three months
A	0.89	1.21	1.49	1.76
B	0.73	0.94	1.22	1.49
C	0.64	0.90	1.12	1.34
X	3.05	3.93	4.87	5.73
Y	2.45	3.12	3.76	4.57
Z	2.17	2.75	3.31	3.98

+ * mg $NaNO_2$/ 100g fresh sausage formula. A: Control formula without any additives. B: Formula with clove EO.
C: Formula with thyme EO. X: Formula with nitrite. Y: Formula with nitrite & clove EO. Z: Formula with nitrite & thyme EO.

3.5. Microbial Examination in the Tested Sausage Formulas

Data in the table 4 displays microbial examination of beef meat, fat, and the tested sausage's formulas during the storing period at-18°C. It is clear that TBC values decreased with increasing the period of storing from zero to three months in sausage formulas that contained with clove EO or thyme EO. Noticeable reducing in the total bacterial count was caused by adding sodium nitrite, clove EO, or thyme EO. However, adding sodium nitrite to sausage showed the least TBC values, followed by thyme EO and then clove EO. TBC values in sausage formulas treated with adding sodium nitrite with thyme EO or with clove EO were lower than their corresponding values in the sausage formula treated with nitrite only. The addition of sodium nitrite with thyme EO showed the least TBC values in sausages throughout the storage period, followed by adding sodium nitrite with clove EO.

Clostridium, Listeria monocytogenes, *Staphylococcus aureus*, *Salmonella*, *Vibrio*, and *Pseudomonas* were not found in beef meat, fat, and all the tested sausage's formulas during the storing period at-18°C. Meanwhile, *E. coli*, *Klebsiella*, and *Proteus* were detected at zero time in beef meat, fat, and all the tested formulas. However, at zero time, adding nitrite with thyme EO resulted in hindering the growth of these bacterial strains in sausage.

Table 4. *Microbial examination of beef meat, fat and the tested sausage's formulas during the storing period at-18°C. (At fresh basis).*

Sample	Storage period (months)	TBC* cfu/g	*E. coli*	*Klebsiella*	*Proteus* spp.	*Clostridium spp.*	*Listeria monocytogenes*
Meat	Zero	4×10^3	+	+	+	-	-
Fat	Zero	5×10^3	+	-	+	-	-
A	Zero	3.6×10^3	+	+	+	-	-
	1	3.3×10^3	+	+	+	-	-
	2	3.1×10^3	+	+	+	-	-
	3	2.9×10^3	+	+	+	-	-
B	Zero	2.4×10^3	+	+	+	-	-
	1	1.8×10^3	+	+	-	-	-
	2	1.4×10^3	+	-	-	-	-
	3	1.0×10^3	-	-	-	-	-
C	Zero	2.1×10^3	+	+	+	-	-
	1	1.5×10^3	+	+	-	-	-
	2	1.1×10^3	+	-	-	-	-
	3	9.8×10^2	+	-	-	-	-
X	Zero	5.2×10^2	+	+	+	-	-
	1	4.6×10^2	+	+	-	-	-
	2	3.6×10^2	+	-	-	-	-
	3	3.1×10^2	+	-	-	-	-
Y	Zero	4.3×10^2	+	+	-	-	-
	1	3.6×10^2	+	-	-	-	-
	2	1.9×10^2	+	-	-	-	-
	3	9.9×10	-	-	-	-	-
Z	Zero	3.8×10^2	-	-	-	-	-
	1	3.0×10^2	-	-	-	-	-
	2	1.3×10^2	-	-	-	-	-
	3	4.8×10	-	-	-	-	-

Table 4. *Continued.*

Sample	Storage period (months)	*Pseudomonas* spp.	*Salmonella* spp.	*Staphylococcus aureus*	*Vibrio spp.*	*Aspergillus niger*	*Candida albicans*
Meat	Zero	-	-	-	-	+	+
Fat	Zero	-	-	-	-	-	+
A	Zero	-	-	-	-	+	+
	1	-	-	-	-	+	+
	2	-	-	-	-	+	+
	3	-	-	-	-	+	+
B	Zero	-	-	-	-	+	+
	1	-	-	-	-	+	+
	2	-	-	-	-	+	+
	3	-	-	-	-	+	-
C	Zero	-	-	-	-	+	+
	1	-	-	-	-	+	+
	2	-	-	-	-	-	-
	3	-	-	-	-	-	-
X	Zero	-	-	-	-	+	+
	1	-	-	-	-	+	+
	2	-	-	-	-	-	-
	3	-	-	-	-	-	-
Y	Zero	-	-	-	-	+	+
	1	-	-	-	-	-	-
	2	-	-	-	-	-	-
	3	-	-	-	-	-	-
Z	Zero	-	-	-	-	-	-
	1	-	-	-	-	-	-
	2	-	-	-	-	-	-
	3	-	-	-	-	-	-

+ Where: * TBC: Total bacterial count. cfu: Colony forming unit.-=Negative +=Positive
A: A control sausage formula without any additives. B: A sausage formula with clove EO. C: A sausage formula with thyme EO.
X: A sausage formula with nitrite. Y: A sausage formula with nitrite & clove EO. Z: A sausage formula with nitrite & thyme EO.

The antibacterial effect for either nitrite or the tested EOs in all the studied sausage- formulas increased with increasing the storing period. After one month of storing, *Proteus* was not found in all the tested sausage formulas except the control formula that was not treated with nitrite. However, *E. coli* and *Klebsiella* were resistant to adding either nitrite, clove EO, or thyme EO. Adding nitrite with thyme EO inhibited the growth of *E. coli* and *Klebsiella* in sausage. Also, adding nitrite with clove EO caused in inhibition of the *Klebsiella* growth in sausage. Two months after the storage, *Klebsiella* was not found in all the tested sausage formulas except the control, which did not prepare by adding nitrite. Meanwhile, *E. coli* was detected in all the tested sausage except the sausage formula that was treated with adding nitrite with thyme EO. After three months of storing, adding thyme EO or nitrite did not influence *E. coli* in sausage. However, adding clove EO with or without nitrite inhibited the growth of *E.coli.* Also, adding thyme EO with nitrite was effective in hindering the *E.coli* growth.

Candida albicans and *Aspergillus niger* were found in beef meat, fat, and all the studied sausage formulas at zero time. These fungal strains were resistant to nitrite. While adding nitrite with thyme EO to sausage resulted in the growth inhibition of these fungi. After one month from storage, the tested fungi were detected in all the tested sausage formulas except that treated by adding nitrite with thyme EO or clove EO. After two months from storage, the tested fungi were not detected in all the tested sausage formulas except which were treated with clove EO or control. After three months of the storage at-18°C, *Candida albicans* was sensitive to thyme EO and clove EO, while *Aspergillus niger* was sensitive to thyme EO only.

3.6. Sensory Evaluation

Data in the table 5 displays organoleptic properties of the studied sausage's formulas at zero time, and after three months from the storing at-18^0 C. It is clear that the control formula showed the highest hedonic evaluation. The formula that was treated with nitrite and clove EO had slightly more scores in all organoleptic properties except odor than the formula that was treated with nitrite and thyme EO. The lowest odor scores were in the sausage formula that was treated by adding clove EO because of its aroma intensity in sausage.

Table 5. *Organoleptic properties of the studied sausage's formulas at zero time and after storing at-18°C for three months.*

Sausage formula	Storage period (months)	Color	Texture	Taste	Odor	Overall acceptability	Composition score	Average
X	Zero	8.90	8.95	8.95	8.90	8.90	44.60	8.92
	After 3 months	8.80	8.85	8.90	8.80	8.80	44.15	8.83
Y	Zero	8.85	8.90	8.60	7.90	8.45	42.70	8.54
	After 3 months	8.70	8.75	8.50	7.70	8.30	41.95	8.39
Z	Zero	8.85	8.90	8.40	8.15	8.30	42.60	8.52
	After 3 months	8.70	8.75	8.25	8.00	8.20	41.9	8.38

+ Where: Scores: 1=Dislike extremely, 2=Dislike very much, 3=Dislike moderately, 4=Dislike slightly, 5=Neither like nor dislike, 6=Like slightly, 7=Like moderately, 8=Like very much, 9=Like extremely.

Each record is a mean value of twenty replicates.

X: A sausage formula with nitrite. Y: A sausage formula with nitrite & clove EO. Z: A sausage formula with nitrite & thyme EO.

4. Discussion

Adding nitrite is essential for the sausage's protection from severe chemical and microbial changes. This result approves with that reported by El-Zainy *et al.* [12]. This influence of nitrite is due to its antimicrobial and antioxidative activity. Adding thyme EO and clove EO while making sausage resulted in reducing the changes in the sausage's chemical composition through the frozen storage period. This effectiveness of these EOs may be consequent to their antimicrobial and antioxidative properties.

Adding clove EO or thyme EO to sausage's formulas, which contained nitrite, reduced the changes in the sausage's chemical composition through the frozen storage period. The nitrite's effect with them was more strength than the nitrite's effect alone. So, the EOs for thyme and clove had a synergistic effect with nitrite.

Changes in the protein and lipids contents of sausage were lowered by adding one of the studied EOs, nitrite, or together. This special effect on sausage's protein content may be due to lessening hydrolysis and protein oxidation. Simultaneously, the special effect on crude lipids' sausage content may be due to a reduction in crude lipids' oxidation.

TBARS value is an indicator of the lipid oxidation. Egyptian Organization for Standardization and Quality (EOS) [24] stated that TBARS value equals 0.9 mg of malonaldehyde (MDA)/kg considered the threshold of organoleptic perception of lipid oxidation in meat products. Because, TBARS values which are high than 0.9 mg MDA/kg, produce "off-odors". So, the values of TBARS in this study display appropriate values from an organoleptic viewpoint. TBARS values in all the tested sausage's formulas raised nearly over the entire study period. This result concurs with that reported by Aminzare *et al.* and El-Zainy *et al.* [25,12].

When adding 100 ppm sodium nitrite to sausage, TBARS values were less than those of the control that made without $NaNO_2$ at zero time and through the storage period (for three months at-18°C). So, adding nitrite to sausage significantly reduced ($P< 0.05$) the lipid oxidation. This influence is owing to nitrite possess antioxidant activity. This result accedes with that reported by Oliveira *et al.* [14]. They found that adding $NaNO_2$ to mortadella-type sausages lessened the TBARS values significantly than their corresponding values in the sausage control that made without adding $NaNO_2$. Also, this result agrees with that stated by El-Zainy *et al.* [12]. They observed that adding $NaNO_2$ to beef sausage decreased the TBARS values significantly during the storage times at-18°C (for three months). Besides that, this result accedes with that obtained by Wójciak *et al.* [26]. They found that 100 ppm sodium nitrite addition would be enough to preserve minced roasted beef meat without appearance any significant impact on oxidative stability, flavor, color, and microbiological safety of minced roasted meat. Meanwhile, the low level from adding nitrite (50 and 75 ppm) caused an acceleration of the lipid oxidation's rate (high TBARS values) in minced roasted beef through the chilling storing for 21 days.

The antioxidant efficiency of nitrite is owing to the following: a) The nitric oxide ability, which results from nitrite, to combine with and settle heme iron in meat pigments and consequently lessening free iron released quantity, and chelates free radicals that fast the happening of lipid oxidation. Besides, NO can speedily oxidize in oxygen's presence to NO_2, producing oxygen's depletion. Consequently, the rancidity's speed is retarded in cured meat products. b) The nitrite's ability to combine with free iron and stabilize the heme iron. This ability results in a decrease in the lipid oxidation's rate by limiting the pro-oxidative effect of iron. c) The nitrite ability to form other antioxidant compounds, such as S-nitrosocysteine [26-27].

Adding clove EO displayed decreased TBARS values in sausage. The result of the influence of clove EO on the TBARS values of sausage's formula, which was made without nitrite, agrees with that stated by Zengin and Baysal [28]. They found that adding clove EO to ground beef showed decreased TBARS values throughout nine days of storing at 4°C. This result also accedes with that is stated by Sharma *et al.* [29]. They observed that TBARS values in the raw chicken sausage's control sample were significantly higher than their corresponding values in the raw chicken sausage sample that is treated with clove EO throughout the storing period for 45 days at-18°C. This decrease in the TBARS values by adding clove EO may be due to its antioxidant effect. Ghadermazi *et al.* and Purkait *et al.* [7-8] reported that clove EO possesses an antioxidant effect *in vitro*.

Adding thyme EO demonstrated decreased TBARS values in sausage. This result accedes that it is stated by Viuda-Martos *et al*. [30]. They observed that adding thyme EO to bologna sausage lessened TBARS values. Also, this result matches what is found by El Adab and Hassouna [31]. They found that adding thyme EO decreased the TBARS values significantly in a Tunisian dry fermented poultry meat sausage during its ripening. This result agrees with what is stated by Blanco-Lizarazo *et al*. [32]. They found that adding 100 ppm thyme EO to sausage lowered TBARS values compared to the control during storing at 8°C for 41 days. Also, this result agrees with what is observed by Boskovic *et al*. [10]. They found that adding thyme EO to minced pork decreased TBARS values throughout the storage period. The decrease in the TBARS values by adding thyme EO may be due to its antioxidant effect. Boskovic *et al.* and Gedikoğlu *et al.* [10-11] observed that thyme EO possessed antioxidant impact *in vitro*.

The antioxidant influence for clove EO or thyme EO may be owing to their high phenols contents. The antioxidant activity of their phenolic constituents is related to one or more hydroxyl groups that bind with one or more aromatic rings, which can award hydrogen atoms or electrons and consequently reduce free radicals [33-34]. So, adding clove EO or thyme EO may hinder lipid oxidation during the storing period by inhibiting the malonaldehyde formation.

Adding thyme EO showed a marked higher decrease in TBARS value in sausage than adding clove EO. This result does not accede with what is stated by Zengin and Baysal [28]. They found that clove EO resulted in a remarkable more reduction in TBARS value than thyme EO in ground beef during the storing period at 4°C.

About the combined influence for nitrite with clove EO or thyme EO, it observed that adding nitrite with clove EO or thyme EO decreased the TBARS values in sausage. These values were less than their corresponding values in the sausage formula that treated with nitrite only. This result pointed to both clove EO and thyme EO have a synergistic influence on nitrite.

During making sausage, the nitrite's quantity added lessened rapidly and converted to various compounds such as nitrous acid, nitric oxide (NO), and nitrates [35]. This decline in the quantity of nitrite added may be due to the influence of the endogenous reducing constituents in meat, like sulfur amino acids, or additives like ascorbate. At zero time, residual nitrite content in all tested and control sausage formulas is a small fraction from the quantity added. This result concurs with that is stated by El-Zainy *et al.* and Merino *et al.* [12, 36]. They found that residual nitrite's content in the cured meat products is a tiny fraction of the quantity of nitrite added.

The residual nitrite content in the control or tested sausage formulas increased gradually with increasing the storing period at-18°C. This result concurs with that is reported by El-Zainy *et al.* [12]. They found that residual nitrite's content increased gradually in beef sausage during the storing period at-18°C. This increase in residual nitrite content during the storing period may be due to consuming some of the chemical compounds in sausage formulas, which are linked to previously with nitrite and consequently caused in an increase of the residual nitrite.

Thyme EO or clove EO lowered the residual nitrite in the sausage formula. This decrease in the residual nitrite content by these EOs could be an agreeable alternative for decreasing the quantity of adding nitrite in processed meats, such as sausage. This decrease in the residual nitrite content by these EOs helps avoid forming the carcinogenic N-nitrosamines. And consequently, the potential hazard of cancer happening by taking sausage may be alleviated [2].

The maximal permissible limit of TBC is 10^5 cfu/g in fresh sausages [37]. The results showed that TBC in all the tested sausage formulas remained below 10^5 cfu/g. TBC was decreased remarkably by adding nitrite. This result concurs with what is stated by Wójciak *et al.* [26]. They found that the Enterobacteriaceae count increased by decreasing the adding sodium nitrite quantity in minced roasted meat. And they reported that 100 ppm sodium nitrite was enough for minced roasted meat, without happening significant influences on the microbiological safety, color, and oxidative stability of minced roasted meat.

The adding nitrite in the sausage manufacture inhibits the pathogenic and spoilage- induced bacteria growth. The anti-bacterial impact of nitrite occurs in a bacterial cell by some mechanisms, like breaking the proton gradient, limiting oxygen uptake, and inhibiting metabolic enzymes. Nitrite also inhibited some enzymes which are necessary for bacterial metabolism, for example, aldolase. Furthermore, nitrite is highly linked with iron and limits iron availability, which is essential for enzymes' actions and metabolism and growth of bacteria [38].

Adding thyme EO or clove EO lowered the TBC values in sausage compared to TBC values in the control formula. So, the studied EOs bettered the microbial quality of beef sausage relative to the control formula. This result agrees with what is reported by El Adab & Hassouna, and Shaltout *et al.* [31,6]. This decrease in the TBC values proved that thyme EO or clove EO has antimicrobial activities. These antimicrobial influences of thyme EO or clove EO may be owing to the hydrophobicity characteristic of these EOs. This property is responsible for the change in the microbe's cell membrane's permeability and the destruction of its cells. The antimicrobial influence of these EOs may also be due to cytoplasm coagulation and its diffusion between the double lipid layer of the microbial cell membrane [39].

Both adding either sodium nitrite or one of the tested EOs lowered TBC values when comparing with the control formula. This result concurs with what is stated by Blanco-Lizarazo *et al.* [32]. They found that the *Listeria monocytogenes* growth was inhibited by adding either 100 ppm thyme EO or 200 ppm sodium nitrite. Adding thyme EO to sausage showed more decreased TBC values compared to what occurs when adding clove EO. This result agrees with what is informed by Ibrahim *et al.* [40]. They reported that thyme EO showed a noticeable more antibacterial impact than clove EO in ground beef and chicken sausage, respectively.

TBC values in sausage samples treated by nitrite with thyme EO or clove EO were less than their corresponding values in sausage formula that treated with nitrite only throughout the storage period at-18°C. So, either thyme EO or clove EO has a synergistic effect on nitrite. Hence, the EO for clove or thyme increases nitrite's impact in decreasing TBC values in sausage during the storage period.

Nitrite inhibits the growth of *Clostridium botulinum*'s spores in the cured meat products [41] and consequently controls entirely in the occurrence of botulism. Nitrite also hinders the growth of some pathogenic bacteria that produces lethal neurotoxins such as *Salmonella typhimurium*, and *Listeria monocytogenes* [26, 32]. Besides, nitrite hinders the growth of several other pathogens such as *E. coli O157*: *H7*, *Staphylococcus aureus*, *Bacillus cereus* and *Clostridium perfringens* [26-27].

Proteus was sensitive to adding either nitrite or EO, followed by *Klebsiella*, whereas *E. coli* was resistant. This result corresponds to what is reported by De Alba *et al.* [42]. They found that *E. coli* displayed resistance to nitrite's influence, particularly at pH 5.0 or pH 7.0. Also, this result concurs what is stated by Badhe *et al.* [43]. They found that *Klebsiella pneumoniae* was moderately sensitive, while *E. coli* was comparatively resistant to the clove EO impact in the chicken.

Both thyme EO and clove EO showed an antibacterial impact in sausage. These EOs displayed a synergistic effect on nitrite because they rose the antibacterial effect of nitrite.

Aspergillus niger and Candida albicans were resistant to adding nitrite or EO in sausage at zero time. This result concurs with what is observed by Tompkin [44], who found that both molds and yeasts are resistant to nitrite's impact. However, adding nitrite with thyme EO or clove EO resulted in the absence of these fungal species. So, both clove EO and thyme EO have a synergistic influence on nitrite. This effect increases the antifungal impact of nitrite in sausage.

This result corresponds to that is observed by Ozturk [45]. He found that when thyme EO was added to the ripened sucuk (a fermented dry-cured sausage), the yeast and mold count was less than its corresponding count in the control sample. Also, this result concurs what is stated by Sakkas *et al*. and Gonçalves *et al.* [46-47]. They found that thyme EO showed a remarkable antifungal influence on *Candida albicans* and Aspergillus niger, respectively, *in vitro*.

Concerning organoleptic properties, it is clear that adding 100 ppm sodium nitrite only or with 200 ppm EO from clove or thyme to sausage formula had higher color and texture scores compared with the control sausage sample, which did not treat with any additives. The high color and texture scores are due to the nitrite's action, and the tested EOs had no inhibitory effect on nitrite action. The lowest odor scores were in the sausage formula that was treated by adding clove EO because of its aroma intensity in sausage.

5. Conclusion

Thyme EO and clove EO have antioxidant, antibacterial, and antifungal influences. Also, they have a synergistic impact on nitrite. In addition to that, these oils were able to decrease the ratio of residual nitrite. Therefore, it is recommended to use these EOs as a natural additive for increasing the nitrite's impact with diminishing the percentage of nitrite's added to sausage for lessening the rate of carcinogenic N-nitrosamines formation in sausage. The obtained results may help the sausage industry select the more appropriate EOs to guarantee the sausage safety and prolong its shelf life.

To complement this research that has been done, the researchers suggest evaluating the antimicrobial and antioxidant activities of the main components of the tested EOs in sausage and other meat products, especially if combined with a low percentage from nitrite.

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