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A comparative study between the performance of thymol-nanoemulsion and thymol-loaded nanostructured lipid carriers on the textural, microbial, and sensory characteristics of sausage

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ABSTRACT

The objective of this research was to compare the function of thymol-loaded nanostructured lipid carriers (NLC) and a thymol-nanoemulsion (NE) with nitrite (120 mg/kg) on quality parameters of sausage. The droplet size of the NLC and NE was 140 and 86.39 nm with encapsulation efficiency of 97 and 94%, respectively. The results on sausage showed that all samples containing NLC and NE exhibited the lowest increase in peroxide value, total volatile base-nitrogen, and TBA with the highest inhibitory effect on the growth of *E. coli*, *C. perfringens*, lactic acid bacteria, psychrophilic bacteria, mold and yeast, and total viable counts as well as good texture and sensory attributes with the best results in the NLC + nitrite and NE + nitrite samples. The L* and *a** values were relatively higher in the samples treated with nitrite, NLC + nitrite, and NE + nitrite after 4-week storage. This increase in redness was associated with the maintenance of oxymyoglobin levels and a decrease in metmyoglobin production. The results of this study indicated that the combined use of NLC/NE (particularly NE) with 60 mg/kg nitrite significantly improved the oxidative and color stability, and delayed the spoilage and off-flavor in sausage.

1. Introduction

Sausage is categorized as a highly perishable meat product due to the present of high amount of ground meat in its formulation, leading to the product spoilage and the proliferation of pathogenic microorganisms. The inherent characteristics of sausage, such as its high moisture content and nutrient-rich composition, have created a proper environment for bacterial growth. Additionally, sausage contains a significant amount of fat, which makes it susceptible to lipid oxidation (Alirezalu et al., 2020, 2021; Hugo and Hugo, 2015). Nitrate and nitrite have indeed been extensively used in processed meats as preservatives to stabilize and enhance the color and inhibit lipid oxidation (Glorieux et al., 2019; Smaoui et al., 2017), therefore, preventing the development of off-flavors and maintaining product quality. Additionally, these preservatives have antimicrobial properties that can inhibit the growth of various pathogenic bacteria, including *Clostridium perfringens*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella enteritidis*. By controlling microbial spoilage, nitrate and nitrite ensure the safety of meat products like sausages (Peighambardoust et al., 2022; Saggiorato et al., 2012; Sepahvand et al., 2022; Smaoui et al., 2017; Tosati et al., 2017). However, it is important to use these additives within regulatory limits to ensure food safety and minimize the potential health risks associated with their excessive consumption (Sepahvand et al., 2021). Although nitrite has numerous benefits, there are concerns regarding its utilization due to the formation of nitrosamine derivatives (Sepahvand et al., 2021; Hojati et al., 2024). The reaction between sodium nitrite (or sodium nitrate) and amines (or amides) can lead to the formation of N-nitrosamines, which are known to be as carcinogenic and toxic compounds. The meat industry is actively seeking natural additives or preservatives with natural origin to replace or reduce the use of nitrites or nitrates in order to meet the consumer demands. The utilization of plant-based extracts for their antimicrobial and antioxidant properties in food products has received considerable attention in recent times (Hojati et al., 2022).

Recent studies have demonstrated that thyme essential oil (EO) exhibits a potent antimicrobial effect (Hashemi et al., 2023). This effect

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can be attributed to the presence of thymol, a highly effective compound found in thyme EO. Despite its strong antimicrobial effect, the practical application of thymol is limited due to its high volatility, water insolubility, and poor oxidative stability. These factors restrict its usage in various practical applications (Almasi et al., 2021a,b).

To manage challenges such as the distinct odor and bitterness of thymol, which can adversely affect the organoleptic quality of food products, numerous studies have been conducted over the past two decades. One emerging technology that has shown promise results in overcoming these issues is encapsulation. Encapsulation involves entrapping active agents, such as thymol, within a carrier material to protect them and extend their shelf life. This technique helps to mask the undesirable sensory characteristics of thymol while preserving its antimicrobial properties (Sepahvand et al., 2022).

Nanoemulsions (NEs) have verified to be effective in enhancing the utilization of EOs in food formulations. They achieve this by improving the dispersibility of EOs within the food systems, minimizing the impact of EOs on the product quality attributes, and enhancing their antimicrobial activity. NE act as carriers for EOs, allowing for better incorporation and dispersion throughout the food matrix. This technology helps to mitigate any negative effects on taste, odor, or appearance that EOs may have on the final product, as well as to enhance their antimicrobial efficacy (Ghadimi et al., 2024; Sepahvand et al., 2021; Talesh et al., 2024).

Colloidal dispersions that contain lipid nanoparticles, such as nanoemulsions, nanostructured lipid carriers (NLCs), and solid lipid nanoparticles (SLNs), are commonly employed for encapsulating hydrophobic bioactive compounds like EOs (Abedi et al., 2023a). These lipid-based delivery systems provide an effective means of protecting and delivering hydrophobic compounds, such as EOs, in various applications (Mozaffar et al., 2021). The lipid nanoparticles act as carriers, enabling the encapsulation of EOs, and improving their stability, solubility, and bioavailability. This encapsulation approach enhances the utilization of EOs in different fields, including food, pharmaceuticals, and cosmetics (Sepahvand et al., 2022).

To the best of our knowledge, there have been no published studies on the utilization of NLCs as a carrier of thymol in the sausage formulations. Additionally, there is a lack of comparative research between NLC and NE with and without the use of nitrite in the sausage formulations. Therefore, the aim of this study was to investigate and compare the effects of different sausage formulations incorporating NLC and NE, with thymol or reduced nitrite (60 mg/kg), on various parameters such as oxidative indices (peroxide value and thiobarbituric acid reactive substances), microbial count, color values (L^*, a^*, b^*), and sensory attributes during storage. These findings will be compared to a commercial sausage formulation containing 120 mg/kg nitrite.

2. Materials and methods

2.1. Materials

Thymol (purity 98.5%) was obtained from Sigma-Aldrich. Sulfadiazine Polymyxin Sulfite agar (SPS agar), Tween 60 (T60), Tween 80 (T80), triphenyl tetrazolium chloride, violet red bile agar (VRBA), nutrient broth, plate count agar (PCA), sulfadiazine polymyxin sulfite agar (SPS agar), De Man, Rogosa and Sharpe agar (MRS), and yeast extract glucose chloramphenicol agar (YGC) were supplied by Merck Chemical Co., Limited (Darmstadt, Germany). All of the reagents used were of analytical grade. Deionized water was used for the experiments.

2.2. Formulation of NLC

For the preparation of NLCs, thymol was dissolved in the melted purified edible tallow (30% w/w) at 85 °C and the thymol-loaded lipid (2%) was dispersed in a 5% (w/w) hot T60 aqueous solution. The mixtures were stirred using an ULTRA-TURRAX (T18, IKA, Germany) for 5 min at 8000 rpm. The pre-emulsion that was obtained was then homogenized (Lab 60 high pressure homogenizer, APV Gaulin, AxFlow, Durham, UK) for three cycles at 90 °C and a pressure of 800 bars. The final aqueous concentration of NLC that was obtained was 2%, containing 600 mg/L thymol (Mozaffar et al., 2021).

2.2.1. Scanning electron microscopy (SEM)

After diluting a 1% thymol-loaded NLC sample to 1000 times with distilled water, a drop of the sample was placed on a glass lamella. The sample let dry at ambient temperature and then sputter-coated with gold to take images by using a field emission scanning electron microscope (WEGA3 SB, TSCAN, Czech Republic, 20 kV, and the magnification of 35000X) (Akhavan et al., 2021).

2.3. Formulation of NE

Six hundred milligram thymol was added to 1000 mL distilled water containing 20 g T80, and then stirred for 30 min at 1200 rpm to form a coarse emulsion. A NE was then produced by sonicating (Q700, Q-Sonica, USA) the coarse emulsion for 4 min at an intensity of 100 W cm^{-2}. A jacketed glass container and a cold-water circulation device (TC502D, Brookfield Engineering, MA, USA) were used to prevent temperature rise during sonication (Hojati et al., 2022).

2.4. Determination of particle size distribution and zeta potential

The particles size of the thymol nanocapsules in the NE and NLC systems were measured. The measuring instrument was equipped with a 4 mW He Ne laser. The particle size measurement was performed at 633 nm with a dynamic viscosity of 8.76 mPa s (at 25 °C) and detection angles of 70 and 90° (Nano ZS, Malvern Instruments Ltd., UK). A DTS software (5.02 version, Malvern Instruments Ltd., UK) was used to analyze the z-average, hydrodynamic droplet size, and polydispersity index (Sepahvand et al., 2022).

2.5. Encapsulation efficiency

To evaluate the encapsulation efficiency (EE) of the NE and the NLCs, an ultraviolet–visible spectroscopy method was used (Pivetta et al., 2018). For this aim, all samples were analyzed at the wavelength of maximum absorption of thymol in a T80+ spectrophotometer (PG Instruments, LTD., UK). Thereafter, the free thymol in the dispersion was quantified after centrifugation of the NE or the NLC dispersion using a microfilter (10,000 g/mol cutoff size, Millipore). The filtrate was diluted in ethyl alcohol (1:1) and then analyzed at a wavelength of 276 nm using the method previously described. The amount of thymol was calculated according to the following equation:

$$\mathrm{EE}\ (\%) = [(\mathrm{Thymol}_{\mathrm{total}} - \mathrm{Thymol}_{\mathrm{not\ encapsulated}}) / \mathrm{Thymol}_{\mathrm{total}}] \times 100 \quad (1)$$

2.6. Sausage preparation

A mixture of 70% beef (from the semimembranosus (inside round)), soy protein isolate (20%), sodium chloride (1%), corn starch (1%), sodium phosphates (0.2%), water (5%), oil (2%), and dry milk (0.8%) was used for the sausage preparation. Spices were deleted from the sausage formula to remove any interference effect with the antimicrobial effect of thymol, its NE and NLCs. The crude materials were mixed (Robokit 2154, BEKO, Istanbul, Turkey) well to produce a batter. The produced batter was divided into 8 parts and each part was well homogenized (Robokit 2154, BEKO, Istanbul, Turkey) with 600 mg/kg thymol, 600 mg/kg thymol-loaded NLC, NE containing 600 mg/kg thymol + 60 mg/kg nitrite, 600 mg/kg thymol-loaded NLC + 60 mg/kg nitrite, 60 mg/kg nitrite, NE containing 600 mg/kg thymol, 120 mg/kg nitrite, separately. A sample without nitrite, thymol, NE and NLC was considered as control. Afterward, the batter of sausages was packaged in plastic wraps using a

Bush filler (MFW68640, Stuttgart, Germany). Then, baking was carried out at a temperature of 70 °C for 40 min using a water bath (SHZ-82, Aria teb, Tehran, Iran). After baking, the samples were transferred into a refrigerator (4 °C), and the effect of treatments was determined every week for 28 days. A completely randomized design was used for the experiment. The sausages were packaged in 100 g plastic wraps and 5 plastic wraps containing 100 g sausage were placed in a tray to be used as a replicate for each measurement time. Regarding the presence of 5 measurement times (throughout the storage time), 25 plastic wraps were considered for the total measurement time of 30 days for one replicate and a total of three replicates were considered for each treatment (Sepahvand et al., 2021).

2.7. Chemical analysis

The moisture, protein (total nitrogen × 6.25), ash, and lipid contents of different prepared sausage samples were determined according to Sayadi et al., (2021). The total volatile base nitrogen (TVB-N) was measured using the macro-distillation method. The oxidative indices namely peroxide value (PV; meq kg^{-1} sausage) and thiobarbituric acid values (TBA; mg MDA/kg) as well as pH of different sausage samples were determined according to the methods described by Naveena et al. (2013) and Sayadi et al. (2021).

2.8. Sausages microbial counts

To count the microbial population, 10 g of each sample was aseptically weighed, diluted, and homogenized (Seward Stomacher 400) with 50 mL of sterile saline solution (0.9%). After serial dilution, 0.1 mL of the desired dilutions prepared from the relevant treatments was cultured on MRS for determination of lactic acid bacteria (LAB), PCA for the total bacterial count (TVC), VRBA for *E. coli*, SPS agar for *C. perfringens*, and YGC for the total mold and yeast. The plates were then incubated for 24 h at 37 °C for PCA and VRBA, 48 h at 37 °C for SPS, 24–72 h at 37 °C for MRS agar, and 4–7 days at 37 °C for YGC (Sepahvand et al., 2022).

2.9. Color measurement

The sausage samples were cut into slices and the color of slices was measured using a digital colorimeter (CR-400, Konica Minolta Sensing Inc., Osaka, Japan), which was calibrated using a standard white plate ($L^* = 95.44$, $a^* = -0.47$, $b^* = 2.51$). The slices were placed on the standard white plate and light reflectance was used to measure the L^* (lightness), a^* (green–red), and b^* (blue–yellow) values (Abedi et al., 2015).

2.10. The oxymyoglobin (OxyMb) and metmyoglobin (MetMb) measurements

The OxyMb and MetMb content of the sausages were evaluated according to the method described by Abedi et al. (2023b). The OxyMb and MetMb contents were calculated using Eqs. (2) and (3):

$$\text{OxyMb}\ (\%) = (0.882R_1 - 1.267R_2 + 0.809R_3 - 0.361) \times 100 \quad (2)$$

$$\text{MetMb}\ (\%) = (-2.541R_1 + 0.777R_2 + 0.800R_3 + 1.098) \times 100 \quad (3)$$

where R_1, R_2, and R_3 are the absorbance ratios of A_{572}/A_{525}, A_{565}/A_{525}, and A_{545}/A_{525}, respectively.

2.11. Sensory evaluation

The sensory evaluation was determined by warm serving of different prepared sausage samples after cooking using 5-point hedonic scale, scoring from like extremely (score 5) to dislike extremely (score 1). The sensory quality of sausages was evaluated for taste, color, texture, odor, and overall acceptability. Eighty subjects (40 males and 40 females) with the age range of 18–40 years and the average age of 25 years among the Food Science students of Yasuj Azad University were selected to evaluate the sensory attributes of sausages every 7 days for 28 days. The aim in sensory assessing of the samples were performing a qualitative test. The same panelists were used throughout the 5 sessions hold during 28 days of storage and all treatments were tested by the panelists in one session. The taste attribute was not tested by the panelists on day 28 due to health issues. It should be mentioned that participants were informed about the general aim of the study and the evaluation procedure, and the appropriate protocols for protecting the rights and privacy of all participants including full disclosure of study requirements and risks, no coercion to participate, participants consent, ability to withdraw from the study at any time, and no release of participant data without their knowledge, were utilized during the execution of the research. The samples were coded with three-digit random numbers and the order of presentation was made using random permutation. All necessary precautions were taken to ensure that each panelist made an independent judgment.

2.12. The measurement of texture firmness

In order to determine textural profile analysis, sausage pieces of 1 × 1 × 2.5 cm (height × width × length) were compressed at a crosshead speed of 3.33 mm/s in a texture analyzer (TA.XTplus, Stable Micro Systems, Vienna Court, UK). The test was developed according to the methodology proposed by Abedi et al. (2018). Textural parameters were measured by compressing to 80% using a compression probe with 19.85 cm^2 of surface contact.

2.13. Statistical analysis

All experiments were performed in triplicate and the average values were recorded. IBM SPSS version 22 (IBM Armonk, NY, USA) was used for all statistical analyses, and an alpha level of $P < 0.05$ was set as a threshold using Duncan's multiple range test.

3. Results and discussion

3.1. Zeta potential, dynamic light scattering (DLS), and scanning electron microscopy (SEM)

The zeta potential was measured using a nanoPartica SZ-100 instrument (Horiba Ltd., Japan), and it was −0.86 mV for NE and −0.52 mV for NLC (Fig. 1). The mean droplet size of thymol-NE and thymol-loaded NLC were 86.39 and 140 nm (Fig. 1), respectively, and their polydispersity indices (PDI) were 0.28 and 0.37, respectively.

It has been reported that zeta potentials lower than −30 mV indicates the presence of a strong electrical charge for droplets which keeps the droplets stable due to the repulsive forces between them. Therefore, NEs have higher stability than NLC (Salvia-Trujillo et al., 2015). On the other hand, the polydispersity indexes indicated nanosystems with good homogeneity. The polydispersity index shows the particle homogeneity and ranges between 0 and 1. When the index approaches to zero, the system is more homogenous (Radi and Amiri, 2013). Hojati et al. (2022) found a diameter of 146.1 nm for their cinnamaldehyde NE. Meanwhile, Radi et al. (2023) reported a diameter of 125 nm for *Thymus vulgaris* EO-NLCs.

Fig. 1 displays the SEM image of thymol-loaded NLC. The SEM analysis revealed that the thymol-loaded NLC contained smooth surface spherical nanoparticles. This conformation allows for the even distribution of EOs within a food system, as mentioned by Khorrami et al. (2021). These findings were also supported by Mozaffar et al. (2021) and Akhavan et al. (2021).

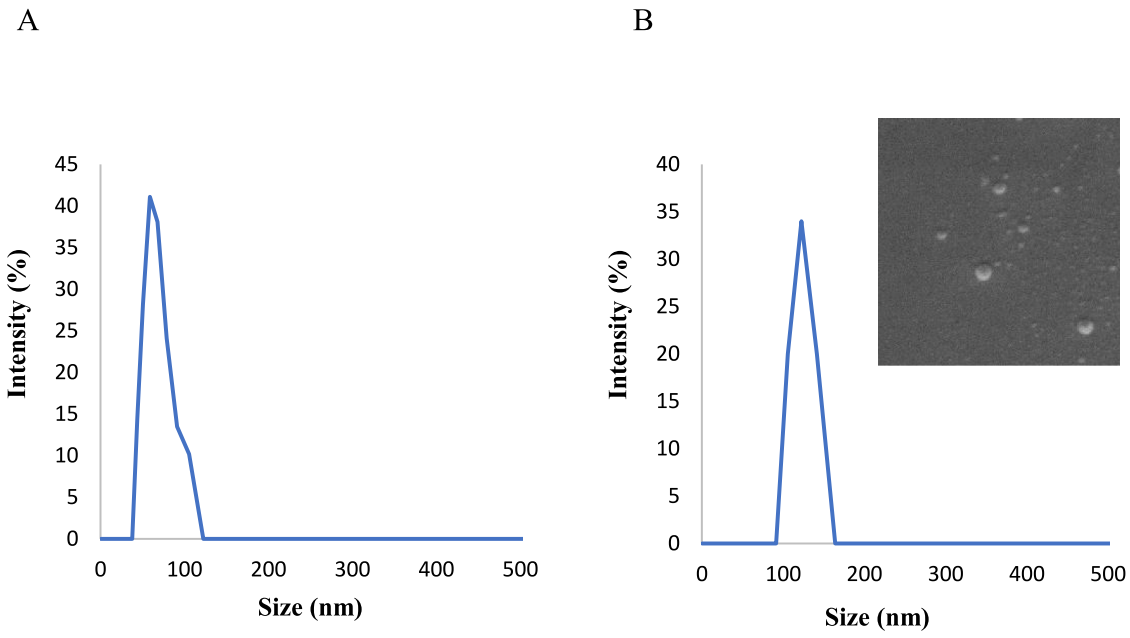

Fig. 1. The particle size distribution of thymol -NLC (A) and thymol-NE (B) and scanning electron microscopy of NLC.

3.2. The encapsulation efficiency

The EE of NLC and NE were estimated to be 97 and 94%, respectively. Thymol exhibits a high EE in NLC and NE systems, likely attributed to its robust interaction with the lipid matrix. Presence of liquid lipids prevented the full crystalline structure and increased the loading capacity in the NLC nanocapsules rather than the NE system (Sepahvand et al., 2022). Similar results were conducted by Piran et al. (2017) and Pivetta et al. (2018) on menthol and thymol-loaded NLCs, respectively.

3.3. Proximate chemical composition

Table 1 presents the approximate chemical composition results of various sausage formulations that were prepared with the addition of thymol, nitrite, thymol + nitrite, NE, NLC, NLC + nitrite, and NE + nitrite. The sausages were stored at a temperature of 4 °C ± 2 for a duration of 28 days.

The addition of thymol, nitrite, NE, and NLC resulted in a significant decrease ($P < 0.05$) in the moisture content of the prepared sausage samples. As the storage period increased, the moisture content of the different samples showed a significant decrease ($P < 0.05$). At the beginning of the storage period, the moisture content of different samples ranged from 63.4 to 63.7% that decreased to 44.6, 51.4, 51.4, 55.3, 55.1, 55.5, 56.8, 56.2% and 56.6% in the control, nitrite-120, nitrite-60, thymol, thymol + nitrite, NLC, NLC + nitrite, NE, and NE + nitrite samples, respectively, after 4 weeks. There was no significant difference among the sausages treated with free or encapsulated thymol. The decrease in moisture content during the cold storage of sausages can be attributed to moisture vapor migration from the surfaces of the samples. This is due to the difference in water vapor pressure between the sausages and the surrounding cold air (Feng et al., 2019). Based on the observed results, the addition of thymol, NE, and NLC to the sausage samples resulted in a lower rate of moisture loss. This indicates an improvement in the water holding capacity of the treated sausage samples through stimulating the growth of muscle fibers and improving the connective tissue structure. Moreover, the amount of moisture in a substance can be influenced by various factors, including the amount of protein present. As the amount of protein decreases, it can lead to a decrease in the amount of moisture in the substance. The correlation between protein denaturation, protein content, and oxidative reactions with moisture loss is well-established (Hughes et al., 2014). When proteins undergo denaturation, changes in their structure and charge can affect the binding of water and solubility of the protein. These changes, naturally, occur during the sausage production. However, moderate oxidation can contribute to the water-holding capacity (WHC) of proteins, which refers to their ability to retain water. Oxidation can lead to the formation of protein cross-links, enhance protein structure, and inhibit water loss (Hughes et al., 2014). Such situation might exist in the thymol treated sausages.

Our findings are consistent with the results reported by other researchers who have used natural preservatives in the sausage product. Studies utilizing natural preservatives such as oregano EO (Ozaki et al., 2021a,b) and beetroot and radish powders (Ozaki et al., 2021a,b) have also shown similar trends in the moisture content of their sausage samples.

As the storage period lengthened, the protein content of the different sausage samples showed a significant decrease ($P < 0.05$). This trend was as the same as the pattern of moisture content change in the prepared sausage samples during the storage period. The control sample showed the greatest decrease in the protein content (from 17.2 to 13.4%) after 4 weeks of storage. However, this reduction was from 17.4 to 15.4% (nitrite-120), 17.3 to 15.3% (nitrite-60), 17.2–15.3% (NLC), 17.3–15.1% (thymol), 17.3–15.2% (thymol + nitrite), 17.2–15% (NLC + nitrite), 17.3–14.9% (NE), and 17.5–15.1% (NE + nitrite) for other samples at the end of the storage. As it is shown in Table 1, there was no significant difference among the treated sausages with nitrite or thymol (in the free or encapsulated form).

The ash content of the sausage treatments showed a significant increase as the storage duration increased (2.8–4.4%, $P < 0.05$). This may relate to the loss of moisture and subsequently some mineral accumulation during storage. However, the inclusion of nitrite, thymol, NE, and NLC did not have a significant impact on the ash content of the sausage samples. This implies that these additives did not contribute to any significant alteration in the mineral content of the samples. It is important to note that the lack of significant changes in ash content could be attributed to various factors such as the specific concentrations of the additives used, the composition of the sausage samples, and the storage conditions (Alirezalu et al., 2021).

Table 1
Chemical composition of different sausages formulated with thymol, nitrite, NLC, and NE.

Week	Fat					Moisture					Ash					Protein				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Control	17.3 ± 0.3 aA	17.4 ± 0.4 aA	17.5 ± 0.3 aA	17.7 ± 0.4bA	17.9 ± 0.4 cA	63.5 ± 0.6 aA	61.5 ± 0.7abB	53.7 ± 0.3 cC	50.3 ± 0.8cD	44.6 ± 0.5 cE	2.6 ± 0.3 aC	3.0 ± 0.1acBC	3.3 ± 0.2 cB	3.8 ± 0.2bcA	4.2 ± 0.3 aA	17.2 ± 0.5 aA	16.1 ± 0.2bB	15.2 ± 0.4 dC	14.1 ± 0.2eD	13.4 ± 0.3bE
Nitrite-120	17.4 ± 0.3 aB	17.5 ± 0.4 aB	17.9 ± 0.2aAB	18.1 ± 0.2 aA	18.3 ± 0.2bA	63.7 ± 0.4 aA	61.3 ± 0.8abB	55.7 ± 0.8bC	55.5 ± 0.4bD	51.4 ± 0.5bE	2.7 ± 0.2aD	3.1 ± 0.1 cC	3.5 ± 0.1abB	3.7 ± 0.3 cA	4.3 ± 0.2 aA	17.4 ± 0.2 aA	17.1 ± 0.2aAB	16.8 ± 0.2 aB	16.1 ± 0.3 aC	15.4 ± 0.3aD
Nitrite-60	17.4 ± 0.4 aB	17.4 ± 0.6 aB	17.9 ± 0.3aAB	18.0 ± 0.2 aA	18.2 ± 0.3bA	63.7 ± 0.5 aA	61.2 ± 0.5abB	55.6 ± 0.6bC	55.6 ± 0.7bD	51.5 ± 0.3bE	2.8 ± 0.4aD	3.0 ± 0.5 cC	3.4 ± 0.7abB	3.6 ± 0.8 cA	4.3 ± 0.4 aA	17.3 ± 0.6 aA	17.2 ± 0.3aAB	16.8 ± 0.4 aB	16.2 ± 0.7 aC	15.3 ± 0.5aD
Thymol	17.5 ± 0.4aD	17.4 ± 0.2aD	17.8 ± 0.3 aC	18.4 ± 0.2 aB	19.2 ± 0.3 aA	63.7 ± 0.5 aA	62.1 ± 0.4 aB	58.3 ± 0.8 aC	57.7 ± 0.8aD	55.3 ± 0.8 aE	2.8 ± 0.3aD	3.2 ± 0.2 aC	3.4 ± 0.2bC	3.8 ± 0.2bcB	4.3 ± 0.2bcdA	17.3 ± 0.3 aA	16.8 ± 0.3aAB	16.7 ± 0.3 aB	15.9 ± 0.3 aC	15.1 ± 0.3aD
Thymol + nitrite	17.3 ± 0.3aD	17.2 ± 0.3aD	17.6 ± 0.2 aC	18.3 ± 0.3 aB	19.0 ± 0.3 aA	63.5 ± 0.4 aA	62.0 ± 0.3 aB	57.8 ± 0.9 aC	57.5 ± 0.7aD	55.1 ± 0.9 aE	2.7 ± 0.2aD	3.0 ± 0.1 aC	3.2 ± 0.3bC	3.6 ± 0.3bcB	4.5 ± 0.3bcdA	17.5 ± 0.2 aA	16.7 ± 0.2abB	16.6 ± 0.3 aB	15.8 ± 0.3 aC	15.2 ± 0.5 aC
NLC	17.6 ± 0.3aD	17.2 ± 0.3aD	17.6 ± 0.3 aC	18.3 ± 0.2 aB	19.1 ± 0.4 aA	63.4 ± 0.4 aA	62.5 ± 0.6 aB	58.8 ± 0.4 aC	57.8 ± 0.7aD	55.5 ± 0.7 aE	2.8 ± 0.2aD	3.2 ± 0.2 aC	3.6 ± 0.2abB	4.2 ± 0.4 aA	4.5 ± 0.3abcdA	17.2 ± 0.3 aA	16.9 ± 0.3aAB	16.5 ± 0.4 aB	16.0 ± 0.3 aB	15.3 ± 0.3 aC
NLC + Nitrite	17.6 ± 0.3aD	17.4 ± 0.2aD	17.9 ± 0.3 aC	18.2 ± 0.3 aB	19.2 ± 0.3 aA	63.7 ± 0.5 aA	62.2 ± 0.4 aB	58.4 ± 0.4 aC	57.5 ± 0.4aD	56.8 ± 0.9 aE	2.8 ± 0.3 aC	3.2 ± 0.3 aB	3.5 ± 0.3abB	4.0 ± 0.2abA	4.4 ± 0.3abcdA	17.2 ± 0.3 aA	16.6 ± 0.4abB	16.5 ± 0.5 aB	15.7 ± 0.4 aC	15.0 ± 0.3aD
NE	17.4 ± 0.3aD	17.6 ± 0.2aCD	17.9 ± 0.3 aC	18.2 ± 0.3 aB	18.9 ± 0.3 aA	63.5 ± 0.9 aA	62.1 ± 0.4 aB	58.8 ± 0.5 aC	57.3 ± 0.5aD	56.2 ± 0.6 aE	2.7 ± 0.2aD	3.4 ± 0.2 aC	3.7 ± 0.2 aC	4.2 ± 0.2 aB	4.7 ± 0.2abA	17.3 ± 0.3 aA	16.9 ± 0.3aAB	16.4 ± 0.4 aB	15.9 ± 0.5 aB	14.9 ± 0.4 aC
NE + Nitrite	17.5 ± 0.2aD	17.7 ± 0.3aCD	17.9 ± 0.2 aC	18.5 ± 0.2 aB	19.1 ± 0.4 aA	63.7 ± 0.8 aA	62.3 ± 0.5 aB	58.3 ± 0.7 aC	57.2 ± 0.8aD	56.6 ± 0.8 aE	2.8 ± 0.3aD	3.4 ± 0.2aCD	3.8 ± 0.2 aC	4.3 ± 0.3 aB	4.8 ± 0.3 aA	17.5 ± 0.3 aA	16.8 ± 0.4 aB	16.5 ± 0.4 aB	15.8 ± 0.5aBC	15.1 ± 0.5 aC

Values are the average of triplicates as mean values ± standard error. Different lowercase letters in each column and capital letters in each row indicate significant statistical difference ($p < 0.05$).

3.4. Physicochemical and chemical quality criteria

3.4.1. Oxidation indexes

The oxidative quality of the products was assessed by measuring the peroxide value (PV) and thiobarbituric acid (TBA) values, which are well-known indicators of primary and secondary lipid oxidation products (Sayadi et al., 2022). Fig. 2 represents the PV (meq O2/kg fat) and TBA (malonaldehyde/kg sample) of the sausage samples as chemical quality criteria.

In this study, all of the samples showed an increasing trend in the PV during the first 21 days of storage and then a decreasing trend from the 21st day onwards. This indicates the accumulation of peroxide compounds as the indicators of oxidative rancidity during the first 21 days. After the 21 days of storage, PV began to decrease due to the hydroperoxide breakdown. This breakdown could be attributed to various factors, such as the presence of antioxidants (thymol or nitrite) or other protective mechanisms within the sausage samples, which effectively reduced the accumulation of hydroperoxides and prevented further lipid oxidation. The control sample had a remarkable faster rate of increase to 27.83 meq kg^{-1} after 21 days and then reached a peroxide value of 23.73 meq kg^{-1} on the 28th day of storage, which was followed by nitrite-60 (12.00 meq kg^{-1}), thymol (5.69 meq kg^{-1}), nitrite-120 (5.34 meq kg^{-1}), thymol + nitrite (5.14 meq kg^{-1}), NLC (3.36 meq kg^{-1}), NLC + nitrite (2.89 meq kg^{-1}), NE (3.02 meq kg^{-1}), and NE + nitrite (2.65 meq kg^{-1}). It is important to note that consumers typically perceive rancidity in PV when it exceeds 25 meq O_2/kg of meat (Abedi et al., 2023a,b). The PV values of the sausage samples were all below this threshold (except of the control), indicating that they are within an

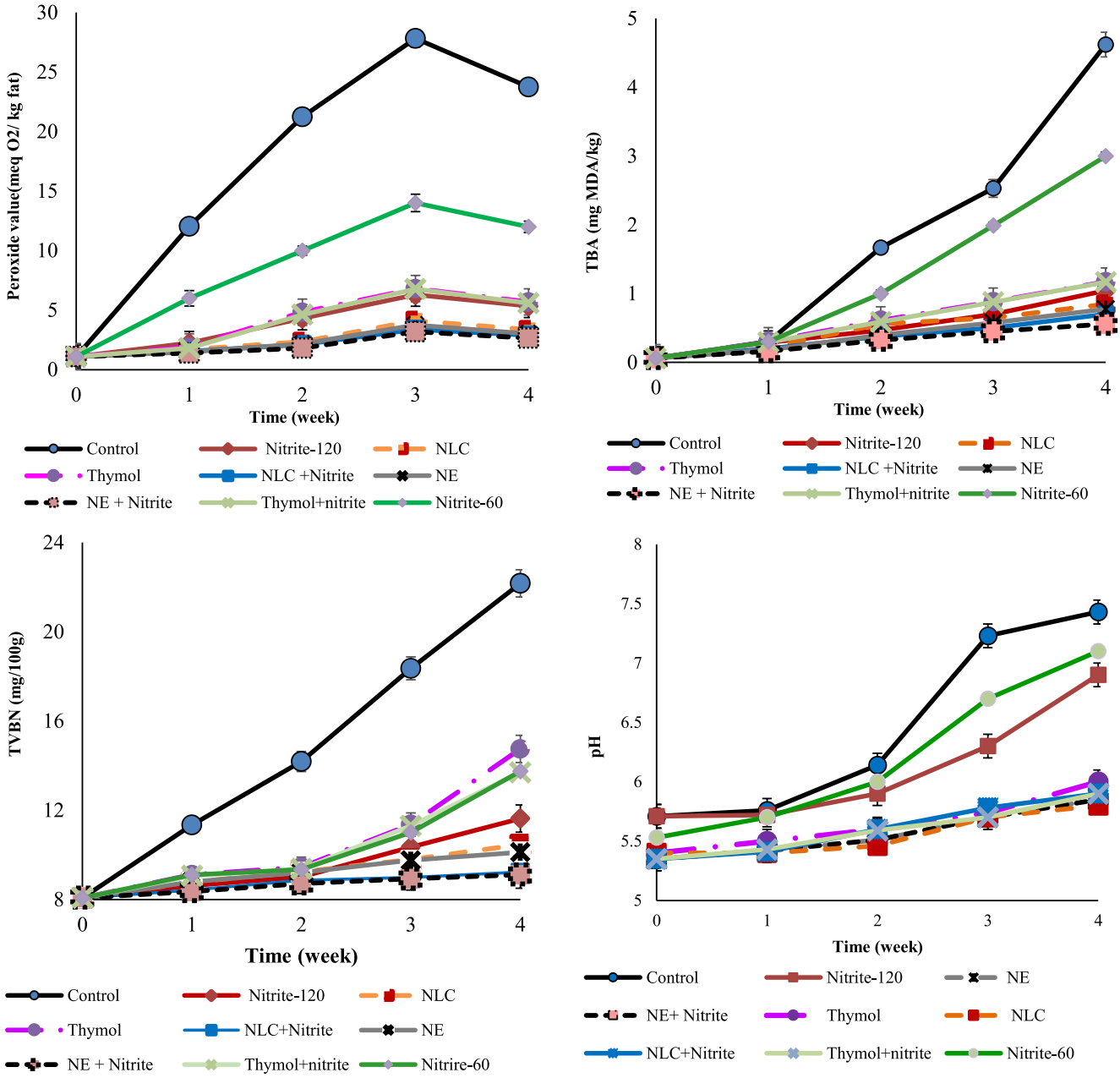

Fig. 2. Changes in pH, peroxide value, thiobarbituric acid, and total volatile base nitrogen of formulated sausages with nitrite, thymol, nitrite, thymol-NE, and thymol-NLC at 4 °C during 28 days of storage content.

acceptable range. A statistically significant difference ($P < 0.05$) was found in the PV between the commercial (nitrite 120 mg/kg)/free thymol-containing samples and the samples containing nanocapsulated thymol (NE and NLC). Lower peroxide values are indicative of reduced levels of lipid oxidation in NE- and NLC-containing samples, resulting in better preservation of the samples quality. This indicates that the use of thymol-NE or thymol-NLC may have a significant impact on reducing the PV of sausages compared to the commercial additive (nitrite). The PVs in samples containing NE + nitrite and NLC + nitrite was lower than the NE and NLC samples. This suggests that the use of nitrite besides the NE and NLC systems, effectively, delayed lipid oxidation in the sausage samples. The stronger antioxidant activity of thymol when incorporated into the NE or NLC in fat-containing meat products like sausages can be attributed to their increased liposolubility. This means that NE and NLC can enhance the stability and bioavailability of thymol. For an antioxidant agent to be effective, it needs to diffuse into the medium. The diffusion of thymol is significantly affected by its encapsulation. NEs and NLCs allow for effective delivery of the EOs to a specific location, enhancing the antioxidant activity (Moghimi et al., 2016). Regarding the antioxidant activity, NE and NLC showed the same performance.

During the 28-day cold storage period, the TBA increased regardless of the treatments applied.

The observed increase in TBA values during chilled storage may relate to the primary oxidation compounds present in the sausages, which converts into the secondary oxidation compounds over time. This conversion could contribute to the increase in TBA values, indicating the occurrence of lipid oxidation (Taheri et al., 2018). Control had the highest rate of TBA increase, reaching 4.62 mg MDA/kg after 4 weeks of storage. The TBA content of control was followed by nitrite-60 (3.21 mg MDA/kg), plain thymol-containing sausages (1.17 mg MDA/kg), nitrite-120 (1.04 mg MDA/kg), NLC (0.84 mg MDA/kg), NE (0.77 mg MDA/kg), NLC + nitrite (0.69 mg MDA/kg), and NE + nitrite (0.55 mg MDA/kg). Therefore, the TBA content of all of the treated samples were significantly lower than the control. This can be due the antioxidant function of the used additives. It is reported that myoglobin cause to undergo partial or complete denaturation during the sausage production. This denaturation leads to loss of the supportive effect of globin on Fe^{2+} ions. Consequently, ferrous metal ions (Fe^{2+}) and free radicals can act as reactive compounds that initiate lipid peroxidation. Numerous documents have proven that antioxidant components have the ability to bind with metal ions, such as Fe^{2+}, and inhibit the formation of free radicals (Aloqbi et al., 2016). On the other hand, the TBA values for the sausage samples treated with NE/NLC and nitrite (60 mg/kg) were significantly lower ($P < 0.05$) compared to samples treated with NE and NLCs. This suggests that the combination of nitrite (60 mg/kg) with NLCs and NEs can effectively mitigate lipid oxidation, possibly through mechanisms such as chelation of iron or reaction with lipid peroxyl radicals (Abedi et al., 2023b). Meanwhile, it is possible that during the storage time at 4 °C, some decomposition occurred for thymol. Thymol degradation rate in NLC and NE were less than the free form. This decomposition could also contribute to the increase in TBA values, as these compounds may have antioxidant properties which inhibit lipid oxidation. Meanwhile, the lower TBA values of NE versus NLC may refer to higher thymol release from NE than that of NLC, resulting in higher concentration of thymol, as confirmed by Karimi-Khorrami et al. (2022). An increase in TBA values of sausages formulated with pomegranate peel extract (Hugo and Hugo, 2015) and pomegranate and sage (Abedi et al., 2023b; Aloqbi et al., 2016) were observed during chilled storage time. This suggests that lipid oxidation, as indicated by TBA values, can occur over time in both treated and untreated samples with different rate, potentially leading to a deterioration in product quality.

3.4.2. pH

According to Fig. 2, the pH levels of the sausage samples increased significantly over time of storage ($P < 0.05$). After 24 days of storage, the pH values of the control sample showed the highest increase from 5.68 to 7.39 over time. The nitrite-60 and then nitrite-120 samples placed after the control, respectively, and the pH rate increase in the sausages formulated with thymol, NLC, and NE was significantly lower than that of the control and nitrite samples. Meanwhile, no significant difference was observed among samples containing thymol, NE, NLC, NLC + nitrite, and NE + nitrite respectively. The activity of bacterial enzymes might cause an increase in the pH and TVB-N compounds during the decomposition of protein compounds. As explained in the microbial section, the addition of thymol and nitrite effectively delayed the growth of microbes, inducing lower activity of bacterial enzymes in the samples (Alizadeh-Sani et al., 2020a,b), and therefore, slowing down the increase trend of pH. The ultimate pH on day 28 was 5.85 (NE), 5.89 (NE + nitrite), 6.0 (thymol), 5.80 (NLC), 5.90 (NLC + nitrite), and 5.90 (thymol + nitrite). The ultimate pH did not exceed the upper limit value (5.8) for pH in thymol treated samples even on day 28, in contrast to the control (7.5) and nitrite (6.9) samples. Taheri et al. (2018) found lower pH values for turkey breast meat coated with chitosan containing cumin EO (Taheri et al., 2018). Similar results were also obtained by using oregano or thyme EO in the lamb meat (Karabagias et al., 2011) and chicken coated with gelatin containing cumin EO (Sayadi et al., 2021).

3.4.3. Total volatile base-nitrogen (TVB-N)

TVB-N serves as a widely used indicator to gauge protein breakdown during the storage of meat products. This breakdown can be attributed to the activity of tissue proteolytic enzymes or microbial processes, resulting in the degradation of proteins into amino acids, leading to the development of undesirable flavors, putrefaction, and sourness in sausages (Dehghani et al., 2018; Sayadi et al., 2021).

The data presented in Fig. 2 indicates that the TVB-N content of different prepared sausage samples exhibited a gradual and significant increase ($P < 0.05$) throughout the storage period. Notably, the control sausage consistently had the highest TVB-N content (22.17 mg/100 g, $P < 0.05$) across all storage period, which aligned with the observed increase in microbial count. The TVB-N value of control was followed by thymol, nitrite-60, thymol + nitrite (13.74 mg/100 g), nitrite-120 (11.63 mg/100 g), NLC (10.46 mg/100 g), NE (10.12 mg/100 g), NLC + nitrite (9.23 mg/100 g), and NE + nitrite (9.11 mg/100 g) after 28 days of storage. The TVB-N content of the treated samples complied with the permissible limit of 20 mg/100 g set by the Egyptian Standard Specifications for TVB-N levels in the meat products. This ensures that the samples meet the meat safety and quality standards. Therefore, the addition of thymol, nitrite, NE, and NLC in the treated samples resulted in a notable reduction in bacterial growth, particularly proteolytic microorganisms that are responsible for breaking down proteins and releasing volatile nitrogen compounds. In this regard, the efficiency of encapsulated thymol was higher than the free thymol-containing samples. Meanwhile, in encapsulated systems, the function of NE/NLC + nitrite samples were more pronounced than those of NE/NLC samples. Other researchers, such as Gutiérrez-Cortés and Suarez Mahecha (2014), Ali et al. (2010) and Çoban et al. (2019) have also supported these findings.

3.4.4. Microbiological criteria

Table 2 provides information about the antimicrobial effects of incorporating thymol, NE, and NLC as natural preservatives in the formulation of sausage samples. For this purpose, the total population of bacteria, mold and yeast, LAB, psychrophiles, *C. perfringens*, and *E. coli* were counted in the sausage samples during 28 days of storage. At the beginning of the storage period, the total viable count (TVC) values of the various prepared sausages were zero, whereas, during the storage period, an increase in the TVC of all the samples was observed, reaching 5.88 log CFU/g in the control sample, 5.55 log CFU/g in the nitrite-60 sample, 5.33 log CFU/g in the nitrite-120 sample, 4.14 log CFU/g in the NLC, 3.98 log CFU/g in the NLC + nitrite sausages, 4.93 log CFU/g in the thymol sample, 4.89 log CFU/g in the thymol + nitrite sample, 4.08 log CFU/g in the NE sample and 3.84 log CFU/g in the NE + nitrite

Table 2
Changes in microbiological counts (Log CFU/g) of different sausages formulated with thymol, nitrite, NLC, and NE during cold storage.

Week	*E. coli*					*Cl. perfringens*					Lactic acid bacteria				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Control	ND	ND	ND	3.62 ± 0.08 aB	4.54 ± 0.09 aA	0	2.42 ± 0.08aD	3.49 ± 0.07 aC	3.59 ± 0.08 aB	4.62 ± 0.07 aA	0	3.90 ± 0.08aD	4.00 ± 0.07 aC	4.13 ± 0.08 aB	4.80 ± 0.12 aA
Nitrite-120	ND	ND	ND	ND	4.10 ± 0.08bA	0	2.02 ± 0.08cD	2.13 ± 0.09 dC	2.51 ± 0.12 dB	3.37 ± 0.12 cA	0	2.60 ± 0.08cD	3.50 ± 0.09 cC	3.77 ± 0.07 cB	4.16 ± 0.12bA
Nitrite-60	ND	ND	ND	3.52 ± 0.07bB	4.40 ± 0.08abA	0	2.22 ± 0.06bD	3.13 ± 0.08bC	3.22 ± 0.05bB	4.15 ± 0.06bA	0	3.40 ± 0.08bD	3.80 ± 0.07bC	4.01 ± 0.05bB	4.20 ± 0.02bA
Thymol	ND	ND	ND	ND	3.42 ± 0.12 cA	0	2.32 ± 0.12bD	2.39 ± 0.11 cC	3.04 ± 0.09 cB	4.12 ± 0.08bA	0	2.45 ± 0.12 cC	3.38 ± 0.11cdC	3.93 ± 0.09bcB	4.24 ± 0.09bA
Thymol + nitrite	ND	ND	ND	ND	3.38 ± 0.10 cA	0	2.30 ± 0.11bD	2.36 ± 0.08 cC	3.01 ± 0.13 cB	4.09 ± 0.09bA	0	2.43 ± 0.08 cC	3.31 ± 0.12 dC	3.85 ± 0.09 cB	4.21 ± 0.13bA
NLC	ND	ND	ND	ND	3.04 ± 0.08eA	0	1.53 ± 0.08dD	1.85 ± 0.12eC	2.12 ± 0.10eB	2.24 ± 0.13 dA	0	2.02 ± 0.11dD	2.47 ± 0.09eC	3.32 ± 0.11 dB	3.46 ± 0.12 cA
NLC + Nitrite	ND	ND	ND	ND	0f	0	1.45 ± 0.13eD	1.76 ± 0.13fgC	1.96 ± 0.09efB	2.02 ± 0.08eA	0	1.96 ± 0.10eD	2.14 ± 0.09 fC	2.89 ± 0.08eB	3.17 ± 0.08 dA
NE	ND	ND	ND	ND	3.18 ± 0.11 dA	0	1.35 ± 0.12fgD	1.64 ± 0.11fgC	1.82 ± 0.13fgB	2.14 ± 0.09eA	0	1.91 ± 0.12fD	2.27 ± 0.12efC	2.63 ± 0.09 fB	3.32 ± 0.07 dA
NE + Nitrite	ND	ND	ND	ND	0f	0	1.24 ± 0.07gD	1.57 ± 0.12gC	1.74 ± 0.11gB	1.97 ± 0.12eA	0	1.82 ± 0.09gD	2.04 ± 0.11 fC	2.58 ± 0.08 fB	3.01 ± 0.08eA
	Total Mold and yeast					**Psychrophilic bacteria**					**Total viable counts**				
Week	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**
Control	0	0	0	3.90 ± 0.09 aB	4.18 ± 0.10 aA	0	0	0	4.19 ± 0.08 aB	5.88 ± 0.09 aA	0	2.98 ± 0.08aD	3.68 ± 0.11 aC	4.55 ± 0.08 aB	5.88 ± 0.09 aA
Nitrite-120	0	0	0	3.54 ± 0.07 cB	3.72 ± 0.09 cA	0	0	0	3.90 ± 0.09bB	5.20 ± 0.12 cA	0	2.53 ± 0.09cD	3.13 ± 0.08 cC	4.18 ± 0.06 cB	5.33 ± 0.07 cA
Nitrite-60	0	0	0	3.72 ± 0.06bB	4.01 ± 0.06bA	0	0	0	4.00 ± 0.08bB	5.52 ± 0.07bA	0	2.73 ± 0.08b	3.55 ± 0.04bC	4.33 ± 0.05bB	5.55 ± 0.06bA
Thymol	0	0	0	3.48 ± 0.09 cB	3.91 ± 0.12bA	0	0	0	3.46 ± 0.10 cB	4.28 ± 0.09 dA	0	2.38 ± 0.07dD	2.65 ± 0.13 dC	3.54 ± 0.07 dB	4.96 ± 0.08 dA
Thymol + nitrite	0	0	0	3.40 ± 0.12 cB	3.89 ± 0.08 cA	0	0	0	3.43 ± 0.09 cB	4.19 ± 0.12 dA	0	2.37 ± 0.08dD	2.52 ± 0.11deC	3.47 ± 0.08 dB	4.89 ± 0.09 dA
NLC	0	0	0	3.23 ± 0.08 dB	3.42 ± 0.12 dA	0	0	0	3.15 ± 0.08 dB	3.81 ± 0.12eA	0	2.05 ± 0.09hD	2.31 ± 0.08 fC	3.26 ± 0.12eB	4.14 ± 0.12eA
NLC + Nitrite	0	0	0	3.20 ± 0.07 dB	3.39 ± 0.09 dA	0	0	0	3.04 ± 0.12gB	3.57 ± 0.11fgA	0	2.08 ± 0.09gD	2.37 ± 0.07 fC	3.21 ± 0.08 fB	3.98 ± 0.08 fA
NE	0	0	0	3.13 ± 0.13 dB	3.31 ± 0.07 dA	0	0	0	3.18 ± 0.11eB	3.75 ± 0.08efA	0	2.13 ± 0.10fgD	2.42 ± 0.09efC	3.15 ± 0.06 fB	4.08 ± 0.07eA
NE + Nitrite	0	0	0	3.06 ± 0.12 dB	3.17 ± 0.09eA	0	0	0	3.09 ± 0.08 fB	3.51 ± 0.09gA	0	2.19 ± 0.13eD	2.48 ± 0.10efC	3.04 ± 0.09 fB	3.84 ± 0.11gA

Values are the average of triplicates as mean values ± standard error. Different lowercase letters in each column and capital letters in each row indicate significant statistical difference ($p < 0.05$).

sample after 28 days of storage. Therefore, the highest and lowest TVC were observed in the control and NE + Nitrite samples, respectively. In the other samples including the nitrite and thymol-containing sausages, the TVC decreased significantly compared to the control with no significant differences among them, indicating that all these treatments were equally effective against the mesophilic bacteria. A similar increasing trend was also observed for the LAB and psychrophilic bacteria, whereas, the lowest and highest population belonged to the control and NE + Nitrite samples, respectively, throughout the storage time.

The total mold and yeast did not grow until the second week of storage in all of the samples and after that, there was a significant increase in their population, reaching up to 4.18 log CFU/g in the control. However, when preservatives were added to the sausages, the total mold and yeast counts showed a slower rate of increase compared to the control. In this regard, the thymol + nitrite and NLC + nitrite samples were more effective than the other treatments and showed the lowest populations. However, no significant difference was observed between the other thymol- and nitrite-containing samples. This indicates that the incorporation of preservatives, effectively, inhibited fungal growth and slowed down fungal spoilage during the storage period.

The count of pathogenic bacteria such as *E. coli* (Gram-negative) and *C. perfringens* (Gram-positive), in different sausage formulations, during storage, is also shown in Table 2. In the control sample, the population of *E. coli* increased from 0 to 4.54 log CFU/g after 4 weeks of storage. However, *E. coli* was not detected in all treated samples until the 3rd week of storage and in the NE + nitrite and NLC + nitrite samples, *E. coli* was not identified even after 4 weeks. However, in the control sample, a population of 3.62 log CFU/g on day 21 and a population of 4.54 log CFU/g on day 28 were observed.

The same increasing pattern was identified for *C. perfringens*. The sausage samples that contained NE + nitrite had the lowest count of *C. perfringens*, with a count of 1.97 log CFU/g and the control showed the highest count (4.62 log CFU/g) after 4 weeks of storage. In regard to *C. perfringens,* the effectiveness of samples containing NLC and NE against *C. perfringens* was significantly higher than those of the samples contained 120 mg/kg nitrite or free thymol (thymol and thymol + nitrite).

Results showed that the sausages treated with thymol (in the plain or nanocapsulated form) exhibited enhanced antibacterial properties. This could potentially explain why there was a slower increase in the total bacteria count, total mold and yeast, LAB, psychrophilic bacteria, *C. perfringens*, and *E. coli* population during the storage period. The presence of thymol, especially in the form of NE or NLC and in combination with nitrite, likely contributed to the observed antibacterial effects. The decline in the protein content (section 3.3.1) of the produced sausage samples during storage might also be ascribed to the microbial growth and production of proteolytic enzymes as well as loss of soluble and volatile amino compounds linked to the protein, along with the loss of

water content (Alirezalu et al., 2021; Feng et al., 2019).

The antibacterial and antifungal activities of thymol was reviewed by Marchese et al. (2016). The antimicrobial activity of thymol may be attributed to its ability to disrupt the lipid fraction of the bacterial membrane. Thymol contains a phenolic hydroxyl group on its phenolic ring, which enhances its hydrophilicity and enables it to dissolve the microbial membrane, thereby causing damage to the membrane (Sepahvand et al., 2021; Xu et al., 2008). Additionally, thymol has been

Table 3
Changes in color parameters, oxymyoglobin (%) and metmyoglobin (%) of different sausages formulated with thymol, nitrite, NLC, and NE.

Week	*L**					*a**					*b**				
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Control	55.1 ± 0.6bA	52.3 ± 0.5 cB	49.2 ± 0.6 cC	43.1 ± 0.8eD	38.3 ± 0.7 dE	15.1 ± 0.8 aA	13.4 ± 0.4 dB	11.8 ± 0.5 dC	10.2 ± 0.5cD	7.3 ± 0.6 dE	20.2 ± 0.8 aA	14.8 ± 0.8 cB	9.4 ± 0.9eC	6.7 ± 0.6dD	3.8 ± 0.7eE
Nitrite-120	56.2 ± 0.5 aA	56.1 ± 0.4 aA	55.8 ± 0.6aAB	54.1 ± 0.6 aB	53.1 ± 0.8 aB	15.3 ± 0.5 aA	15.1 ± 0.6 aA	14.8 ± 0.8aAB	14.2 ± 0.9aBC	13.4 ± 0.7 aC	20.4 ± 0.6 aA	19.8 ± 0.7 aB	19.1 ± 0.6 aB	18.3 ± 0.8aBC	17.4 ± 0.4 aC
Nitrite-60	56.1 ± 0.4 aA	56.1 ± 0.6 aA	55.4 ± 0.5aAB	54.0 ± 0.5 aB	53.5 ± 0.7 aB	15.3 ± 0.4 aA	14.5 ± 0.5bAB	14.2 ± 0.7cAB	13.9 ± 0.8bBC	13.5 ± 0.7 aC	20.3 ± 0.7 aA	18.3 ± 0.8bB	16.5 ± 0.5 dC	15.3 ± 0.6bCD	14.3 ± 0.5cD
Thymol	56.4 ± 0.4 aA	54.5 ± 0.5bB	53.1 ± 0.5bC	51.7 ± 0.4dD	49.6 ± 0.7 cE	15.2 ± 0.4 aA	13.7 ± 0.7 cB	13.2 ± 0.4cBC	12.9 ± 0.6bC	10.7 ± 0.4cD	20.5 ± 0.5 aA	17.9 ± 0.7bB	16.9 ± 0.4 dB	13.7 ± 0.6 cC	11.1 ± 0.8dD
Thymol + nitrite	56.1 ± 0.3 aA	55.7 ± 0.5bB	55.3 ± 0.4bC	53.8 ± 0.5bD	52.8 ± 0.6 cE	15.2 ± 0.3 aA	14.7 ± 0.7 cB	14.2 ± 0.6cBC	13.9 ± 0.5bC	12.8 ± 0.7cD	20.4 ± 0.4 aA	18.1 ± 0.8bB	16.6 ± 0.5 dC	15.5 ± 0.6bCD	14.2 ± 0.8cD
NLC	56.1 ± 0.3 aA	54.4 ± 0.4bB	53.7 ± 0.5bC	52.7 ± 0.6cD	51.7 ± 0.7bE	15.2 ± 0.5 aA	14.8 ± 0.4abA	14.5 ± 0.6abA	13.2 ± 0.7bAB	12.1 ± 0.4bB	20.5 ± 0.5 aA	18.8 ± 0.7abB	17.4 ± 0.8bcB	15.8 ± 0.7bC	14.3 ± 0.6 cC
NLC + Nitrite	56.1 ± 0.3 aA	56.3 ± 0.4 aB	55.3 ± 0.4 aC	54.1 ± 0.5aD	53.3 ± 0.6 aE	15.3 ± 0.4 aA	15.2 ± 0.5 aA	14.7 ± 0.4 aA	14.2 ± 0.5aAB	13.4 ± 0.5 aB	20.3 ± 0.6 aA	18.9 ± 0.5abB	18.2 ± 0.6abB	17.1 ± 0.9 aC	16.4 ± 0.5abC
NE	56.0 ± 0.5 aA	54.1 ± 0.4bB	53.6 ± 0.3bC	52.8 ± 0.6cD	51.2 ± 0.4bE	15.2 ± 0.5 aA	14.5 ± 0.5bAB	14.1 ± 0.7bB	12.8 ± 0.5bC	11.7 ± 0.6bC	20.1 ± 0.5 aA	18.5 ± 0.6bB	17.0 ± 0.6cdC	15.4 ± 0.6bD	14.1 ± 0.5 cE
NE + Nitrite	56.1 ± 0.4 aA	56.6 ± 0.4 aB	55.2 ± 0.5 aC	54.5 ± 0.6aD	53.3 ± 0.5 aE	15.1 ± 0.7 aA	14.7 ± 0.4abB	14.4 ± 0.5abB	14.0 ± 0.7aBC	13.5 ± 0.5 aC	20.4 ± 0.4 aA	19.2 ± 0.6 aB	18.7 ± 0.9abBC	17.2 ± 0.4 aC	16.7 ± 0.7 aC
Week	**Oxy (%)**					**Met (%)**									
	0	**1**	**2**	**3**	**4**	**0**	**1**	**2**	**3**	**4**					
Control	85.2 ± 0.8bA	74.3 ± 0.5eB	54.6 ± 0.5eC	42.1 ± 1.2fD	37.3 ± 1.8 fE	22.1 ± 0.8 aE	30.7 ± 1.1aD	38.0 ± 1.3 aC	44.2 ± 1.1 aB	59.7 ± 1.1 aA					
Nitrite-120	87.7 ± 0.5 aA	86.8 ± 0.6 aA	79.4 ± 1.0 aB	74.4 ± 1.3 aC	70.6 ± 1.2aD	21.4 ± 0.9aD	22.7 ± 1.2deD	25.9 ± 0.4 cC	28.3 ± 0.4eB	31.3 ± 0.6fgA					
Nitrite-60	87.4 ± 0.4 aA	86.3 ± 0.7 aA	77.4 ± 0.8bB	71.4 ± 0.9bcC	63.4 ± 1.4bcD	21.3 ± 0.8aD	21.5 ± 0.9eD	26.0 ± 0.5bcC	28.8 ± 0.7eB	31.7 ± 0.7fgA					
Thymol	86.4 ± 0.6abA	78.4 ± 0.4 dB	69.3 ± 1.1 dC	63.4 ± 0.9eD	56.3 ± 0.8eE	21.3 ± 0.8 aE	25.1 ± 0.8bD	26.8 ± 0.6bcC	34.3 ± 0.7bB	39.6 ± 0.5bA					
Thymol + nitrite	87.5 ± 0.6 aA	86.2 ± 0.5 aA	76.4 ± 1.0bB	71.4 ± 1.4bcC	63.9 ± 1.2bcD	21.5 ± 0.8aD	21.7 ± 1.2eD	26.9 ± 0.4bcC	30.3 ± 0.4 dB	31.8 ± 0.6fgA					
NLC	86.5 ± 0.6abA	83.5 ± 0.9 cB	73.4 ± 1.0 cC	65.3 ± 1.3deD	56.9 ± 1.3 dE	21.4 ± 0.6 aE	23.9 ± 0.3cdD	27.8 ± 0.5bC	31.2 ± 0.4 cB	35.3 ± 0.7 cA					
NLC + Nitrite	86.6 ± 0.9abA	85.7 ± 0.7bcA	76.4 ± 0.8bB	70.5 ± 1.3 cC	63.5 ± 1.2bcD	21.4 ± 1.1 aE	21.5 ± 0.3eD	26.2 ± 1.3bcC	30.6 ± 0.5 dB	32.7 ± 0.8efA					
NE	86.6 ± 0.8abA	84.8 ± 1.1 cA	73.9 ± 0.7 cB	65.8 ± 0.5 dC	61.4 ± 0.6cD	21.6 ± 0.7 aE	23.3 ± 1.2cdD	27.3 ± 0.6bC	30.5 ± 0.8 dB	33.3 ± 0.8 dA					
NE + Nitrite	87.2 ± 0.7 aA	85.6 ± 1.1bBA	77.8 ± 1.2bB	72.7 ± 1.3bC	64.7 ± 1.2bD	21.4 ± 0.8aD	21.6 ± 0.6eC	27.2 ± 0.9bB	30.1 ± 0.8 dA	31.6 ± 0.6fgA					

Values are the average of triplicates as mean values ± standard error. Different lowercase letters in each column and capital letters in each row indicate significant statistical difference ($p < 0.05$).

found to increase membrane permeability and to destabilize the bilayer, leading to the leakage of intracellular materials (Marchese et al., 2016). When thymol disrupts the integrity of the bacterial membrane and increases its permeability, it induces the leakage of protons and potassium, leading to the loss of membrane potential. The hydroxyl group on thymol is important in depolarizing the membrane potential and decreasing it (Sepahvand et al., 2021; Xu et al., 2008). The antimicrobial activity of thymol has been demonstrated by Ma et al. (2016) and Robledo et al. (2018). According to the results, the NE + nitrite and sometimes NLC + nitrite showed greater antimicrobial activity. NEs and NLCs are formulated to have smaller droplet sizes, which can facilitate their penetration through the porin proteins of the outer membrane of Gram-negative (G-) bacteria. This allows for effective delivery of the EO to the bacterial cell membrane, enhancing their antimicrobial activity (Moghimi et al., 2016). Meanwhile, the sustained release behaviors of NEs and NLCs may improve the antimicrobial activity throughout the storage (Karimi-Khorrami et al., 2022; Khorrami et al., 2021). In this regard, the higher release rate of NE rather than NLC (Karimi-Khorrami et al., 2022) may result in NE better antimicrobial function.

Similar findings have been reported for other EOs, such as D-limonene and oregano oil, when encapsulated in the NE (Bhargava et al., 2015; Zhang et al., 2014). In another study, chitosan NE loaded with thyme EO or thymol exhibited inhibitory effects against both *S. aureus* and *E. coli*. In a study conducted by Almadiy et al. (2016), the antibacterial activity of *Achillea* species EOs was significantly enhanced when formulated as NEs against both Gram-positive (G^+) foodborne bacteria (*S. aureus* and *Listeria monocytogenes*) and Gram-negative (G^-) species (*E. coli, Pseudomonas aeruginosa,* and *Salmonella enteritidis*).

3.4.5. Color measurement, oxymyoglobin and metmyoglobin content

One crucial aspect of sausage quality is the ability to produce and maintain a pink color. Table 3 displays the color coordinates (L^*, a^*, and b^*) for different sausage formulations. According to Table 3, there was a gradual decrease in the lightness of sausage samples during the 4 weeks of storage period. Although this decrease was statistically significant, it was relatively small. There are some reasons to declare the sausages darkening during storage: (1) the water loss over time and (2) the oxidation, enzymatic, microbial, or chemical alterations that take place in the product throughout storage (Alizadeh-Sani et al., 2020a,b; El Adab and Hassouna, 2016).

As it is shown in Table 3, no significant difference ($P \geq 0.05$) was observed between the L^* values of nitrite-120, nitrite-60, NLC + nitrite, thymol + nitrite, and NE + nitrite samples (56.2–53.1). Meanwhile, the L^* values of nitrite-containing samples were higher than the samples without nitrite during the storage period, indicating that a decrease in nitrite content from 120 to 60 mg/kg, in the thymol + nitrite, NE + nitrite, and NLC + nitrite samples, didn't have a significant impact on the color parameter of the 120 mg/kg nitrite samples.

The "redness" of sausage products is expressed by the color parameter of a^*. Along with the L^* outcomes, the a^* values also experienced a significant decrease over time, which can be attributed to the reduction in the oxymyoglobin concentration of the sausages.

Throughout the storage period, the samples containing nitrite, thymol + nitrite, NLC + nitrite, and NE + nitrite had higher a^* values compared to the other samples. This can be attributed to nitrite effect on the production of the pink-red nitrosomyoglobin pigment. In this regard, there was no statistically significant difference between the a^* values of the nitrate-containing samples (nitrite, thymol + Nitrite, NLC + nitrite, and NE + Nitrite). Therefore, the addition of thymol (in the plain or encapsulated forms) significantly increased the redness of sausages. It has been reported that thyme oil possess antioxidant and reducing properties (conversion of Fe^{3+} to Fe^{2+}). Therefore, thymol, as an antioxidant, prevents the oxidation of pigments responsible for the red hue. When thymol was incorporated into a NLC or NE system, its dispersibility, solubility, and bioavailability was improved allowing for better incorporation and distribution within the sausage matrix. Meanwhile, due to degradation, oxidation, and interaction between thymol with functional groups in protein, the preserving ability of thymol reduced over the storage time (Abedi et al., 2023b). Meanwhile, incorporation of thymol in NLC and NE postpones these reactions caused by thymol.

A small but significant decrease in b^* values throughout storage can be attributed to the formation of MetMb in the sausages during storage. The reduction in the oxymyoglobin content occurred due to the consumption of oxygen by microorganisms (El Adab and Hassouna, 2016). In this regard, the samples containing nitrite showed lower b^* values than control, thymol, and NE/NLC which showed less yellowness of these samples. No significant difference was observed among the b^* values of the control, thymol, and NE/NLC samples.

The results of color parameters variations in this study were in good agreement with the results of El Adab and Hassouna (2016) on the addition of oregano and thyme EOs in fermented poultry meat sausage and the application of TiO_2 and rosemary EO in ground meat (Alizadeh-Sani et al., 2020a,b).

The appealing red color of meat is often attributed to the proportional balance of red myoglobin, bright-red OxyMb, and gray-brown MetMb. Consumer preference dictates that the percentage of MetMb on the surface of fresh meat should ideally remain below 30–40% of the total pigments present (Abedi et al., 2023b). As the storage time increased, the level of OxyMb in all sausages decreased, while there was an increase in the content of MetMb (Table 3). The decline in the level of OxyMb was more pronounced in the sausage samples with no nitrite content. In addition, the rate of OxyMb conversion to MetMb was lower in the nitrite-120, nitrite-60, thymol + nitrite, NLC + nitrite, and NE + nitrite samples. In other words, the OxyMb content of the nitrite-containing samples was significantly ($p < 0.05$) higher than the other formulations and higher protection of OxyMb was observed in sausages that were incorporated with 120 mg/kg nitrite or lower amount of nitrite besides free or nano-encapsulated thymol. It is reported that the reduction of ferric-MetMb (Fe^{3+}) to ferrous-OxyMb (Fe^{2+}) is facilitated by the addition of nitrites or thyme EO (Abedi et al., 2023b). According to the study of Ghaderi-Ghahfarokhi et al. (2016), the combination of thyme EO and ascorbic acid delayed the reduction of OxyMb in minced beef burgers during cold storage.

3.4.6. Sensory properties

The sensory attributes of the sausage samples, namely color, odor, taste, texture, and overall acceptability, are depicted in Fig. 3. During the four-week storage time, all sensory attributes of the sausages exhibited a significant decrease. The control and thymol samples followed by thymol + nitrite and nitrite-60 consistently received the lowest sensory scores throughout the storage period and the nitrite-120 sample obtained the highest sensory scores.

The results indicate that there were significant differences among the treated samples in terms of the color parameter. The nitrite-120 sample received the highest color scores, followed by the NE + nitrite and NLC + nitrite samples (with no significant difference between them) and then the NE and NLC sausages (with no significant difference between them). The lowest color scores were obtained by the control and thymol samples ($P < 0.05$).

A similar trend was also obtained for other sensory attributes including odor, texture, taste, and overall acceptability.

The thymol and thymol + nitrite samples had a taste-altering effect, the factor which was effective in low sensory scores of these samples. These samples had changed the taste of the sausages in an unfavorable way. However, the NE and NLC systems, to some extent, showed a masking effect on thymol flavor.

However, the antimicrobial properties of nitrite, NE + nitrite, and NLC + nitrite were also effective in the improvement in sensory attributes of these samples. By effectively reducing microbial growth, spoilage may have decreased in these treatments and the quality of the sausages were better maintained, leading to a more favorable overall

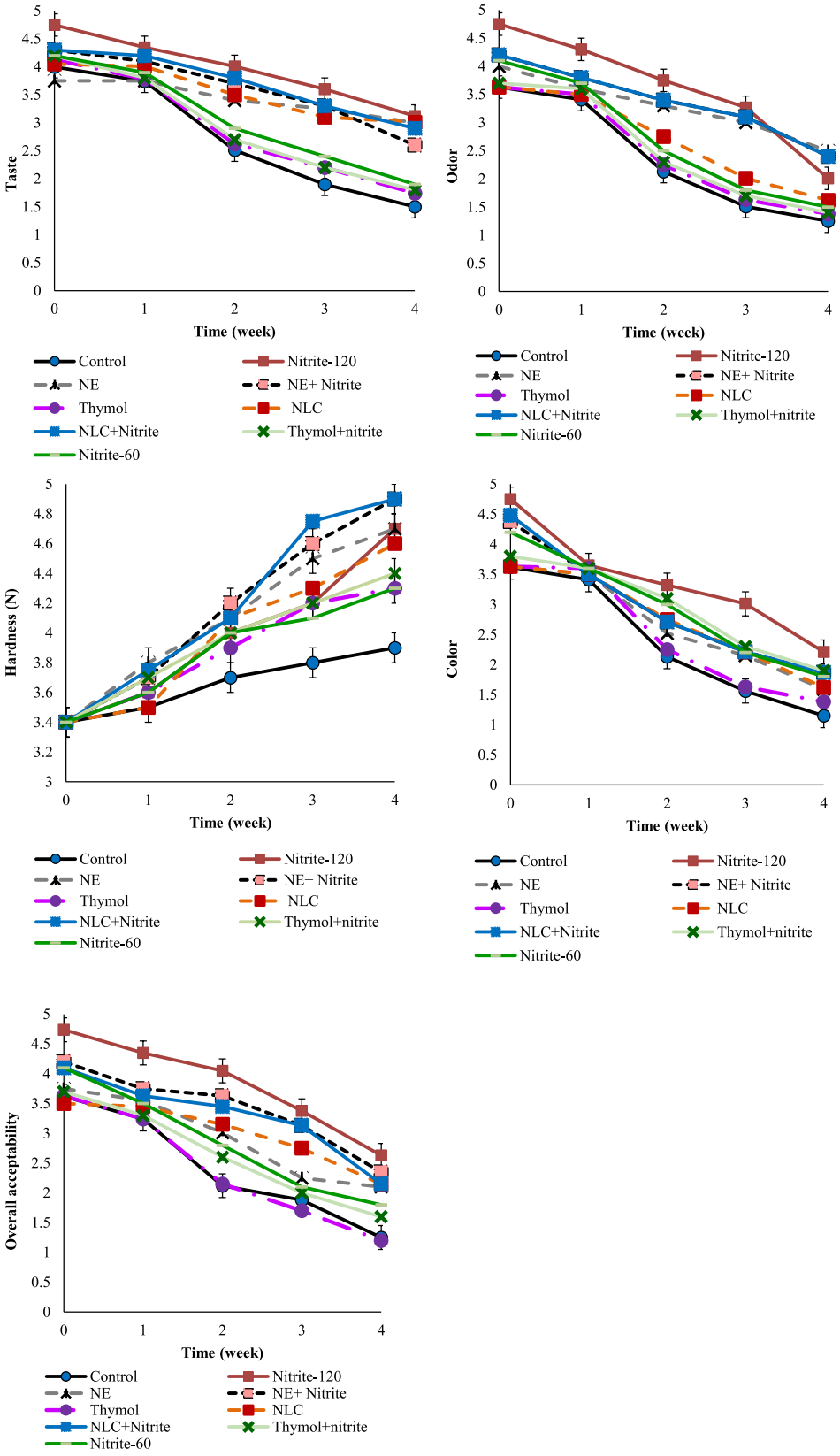

Fig. 3. Sensory attributes including taste, odor, texture color, and overall acceptability of formulated sausages with nitrite, thymol, nitrite, thymol-NE, and thymol-NLC at 4 °C during 28 days of storage content. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

acceptance. In contrast, the control samples showed signs of off-odor and discoloration. These undesirable characteristics in the control samples were attributed to their high levels of lipid oxidation and microbial growth. Mohammadpourfard et al. (2021) demonstrated the effects of different concentrations of thymol and astaxanthin on organoleptic properties of common and probiotic beef cooked sausages for up to 45 days of storage. Treatments with high concentrations of thymol (more than 250 mg/kg) received low sensory scores due to the strong taste of thymol at these levels. However, formulations with lower level of thymol (125 mg/kg), a higher level of nitrite (120 mg/kg) received the highest scores from the panelists.

3.4.7. The texture firmness

The effects of NLC and NE incorporated with thymol on the hardness of formulated sausages after 28 days of refrigerated storage are depicted in Fig. 3. The harness of all sausage samples increased slightly but significantly due to the moisture loss during storage. However, this increase in treated sausages was significantly higher than that of the control sample, which may contribute to the higher microbial growth and the higher protein reduction in the control. However, the lower microbial count of nitrite and thymol-treated samples resulted in harder texture of these samples beside the moisture loss. Statistical analysis indicated that the addition of nanoparticles (NE, NLC, NE + nitrite, and NLC + nitrite) resulted in higher firmness of these samples than the nitrite and free thymol samples. Karim et al. (2021) also reported an increase in hardness of their sausage samples during storage. These researchers stated a higher firmness and a better chewiness in the sausages contained zein-nanofibers than the control.

4. Conclusion

Lowering the levels of nitrites can effectively reduce the formation of N-nitrosamines, which are undesirable compounds. Application of NEs or NLCs as thymol delivery systems, particularly in accompanied with reduced amounts of nitrite enhanced the oxidative stability of sausages, preserved their color, and maintained their sensory characteristics during storage. Furthermore, NEs and NLCs decreased the growth of microorganisms and improved the microbiological quality of sausages and their safety. Results confirmed that the controlled release mechanism of NEs and NLCs ensures the gradual and sustained release of encapsulated thymol, maintaining their effectiveness over time. This study suggests that the combination of NLC or NE with reduced amounts of nitrite (60 mg/kg), particularly NE + nitrite, were significantly effective in maintaining the overall quality of the sausages.

Author contribution

Elahe Abedi and Sedigheh Amiri wrote the manuscript, conceived and designed the research, and analyzed the data. Mohsen Radi and Somayeh Sepahvand conceived and designed the research and analyzed the data. Somayeh Sepahvand and Sedigheh Amiri conducted the experiments. All the authors read and approved the manuscript.

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The present paper, which is original, has not been published before and is not currently being considered for publication elsewhere.

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This article does not contain any studies with human participants or animals performed by any of the authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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