Effect of Thymol and Nanostructured Lipid Carriers (NLCs) Incorporated with Thymol as Antimicrobial Agents in Sausage

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Abstract: The aim of this study was to evaluate the antimicrobial activity of thymol and thymol-loaded nanostructured lipid carriers (NLCs) on inoculated sausages at 4 °C over a period of 28 days. To this end, sausage samples containing 600 mg/kg thymol, 600 mg/kg thymol-loaded NLC, 600 mg/kg thymol + 60 mg/kg nitrite, and 600 mg/kg thymol-loaded NLC + 60 mg/kg nitrite were prepared, and each treatment was divided into three portions to be inoculated with S. aureus, E. coli, and C. perfringens (10^5.5 CFU/g). The mean diameter and zeta potential of thymol-NLCs were 140 nm and −0.52 mV, respectively. Thymol-NLCs showed (two-fold) higher values for MIC and MBC than that of thymol, but similar halo diameters were detected for both against all bacteria examined in the agar well diffusion test. The control and nitrite-containing sausages showed an increasing trend in bacterial growth and the bacterial population was the largest in these samples. The bacterial growth within samples treated with thymol or thymol-NLCs was around 3.90–4.67 log CFU/g lower in comparison with the control. In this regard, no significant differences were detected between the thymol and thymol-NLC samples against each bacterium. A first-order reaction was detected for bacterial growth in all sausages. Overall, the higher antimicrobial property of thymol and its NLC compared with nitrite makes thymol a good alternative to nitrite with regards to its antimicrobial capability.

Keywords: thymol; antimicrobial activity; nitrite substitute; nanoencapsulation; meat products

1. Introduction

Meat and meat products are considered important sources of protein, fat, essential amino acids, minerals, vitamins, and other nutrients [1], but these factors also make these products susceptible to chemical deterioration (such as lipid oxidation) and microbial spoilage. Meat spoilage creates significant changes in meat quality parameters (color, flavor, odor, and nutritional value) and reduces product shelf life [2]. To tackle these challenges, the meat and meat products industry has long utilized nitrites and nitrates to stabilize meat color and inhibit the growth of microorganisms which lead to meat spoilage and diseases [3]. Nitrates are used when the slow release of nitrite is desired. Nitrate is released into the tissue, where it is transformed into nitrite. Nitrate also undergoes chemical changes in the mouth and stomach to turn into nitrite. In the stomach, this nitrite reacts with amides and amines and forms to carcinogenic nitrosamines. These compounds may cause maternal hemoglobin disease in children [4]. In Iran, the upper limits of nitrite and nitrate that are permitted to be added to meat products are 120 and 500 mg/kg, respectively. Sausage is one of the meat products for which nitrite is used. This product is also one of the most popular foods for consumption, especially by children. It is therefore important to reduce the usage of nitrite in these products. Numerous compounds, such as Aloe vera [5], green extracts [6], salicylic acid [7], etc., have been examined for their antimicrobial activity. Among these...
different compounds, essential oils (EOs) have long been attracting attention, as they are natural in origin, and are good sources of antimicrobial agents against living organisms such as fungi, bacteria, insects, and viruses [3]. Essential Oils, directly and indirectly, play crucial roles in plants’ defensive systems against herbivores and pathogens, in the plant reproduction process (through the attraction of pollinators and seed disseminators), and in plant thermo-tolerance [8]. The antioxidant, antimicrobial, anti-cancer, and anti-inflammatory properties of EOs make these compounds useful alternatives for the nitrate used in various meat products [9].

The strong antimicrobial activity of thyme (Thymus vulgaris) EO has made it a popular antimicrobial agent for many food researchers examining its use in different antimicrobial systems. Thyme EO is a source of monoterpenic phenols, such as thymol (10–64%), p-cymene (9.1–22.2%), carvacrol (0.4–20.6%), camphor (0–7.3%), 1,8-cineole (0.2–14.2%), bornel (0.6–7.5%), α-pinene (0.9–6.6%), and linalool (2.2–4.8%) [10]. The strong antimicrobial activity of thyme EO against a wide range of Gram-negative (G−) and Gram-positive (G+) bacteria, is attributed to its main component, thymol [11]. In this regard, Karam et al. [12] demonstrated the potential of thymol to control the growth of spoilage microorganisms in marinated chicken meat. However, some features of thymol, including its high volatility, its chemical instability against environmental conditions (such as heat, oxygen, light, etc.), its insolubility in aqueous mediums, and the change in its sensory properties as a result of processing or storage have resulted in low usage of this compound in the food industry [13]. Encapsulation of thymol within different coating materials is a good strategy to alleviate these problems [3]. Encapsulation is a well-defined strategy that is performed for different reasons: to protect bioactive materials against harsh conditions (such as acidic media or the gastrointestinal tract environment); to facilitate a controlled release of bioactive materials within foods or within the body; and to cover any unpleasant odor/flavor of the core material in the food product [14]. Various EOs have been encapsulated in different colloidal delivery systems such as nanoemulsions [11], microemulsions [15], solid lipid nanoparticles (SLNs) [16,17], and nanogels [18]. Many efforts have been made to improve the antimicrobial activity of thymol through nanoencapsulation. In this regard, Robledo et al. [19] reported that nanoemulsification of thymol could improve its antifungal activity against strawberry fungi. Almasi et al. [2,10] reported that the microemulsification of thyme EO could significantly enhance the antimicrobial activity of thyme in beef, strawberries, and cucumbers. Lui and Lui [20] confirmed the positive effect of chitosan nanoemulsions containing thymol or thyme EO in increasing the shelf life of refrigerated pork. Meanwhile, nanoemulsification of curcumin-cinnamon EOs increased the shelf life of chilled chicken fillets [21]. These findings show that emulsions and dispersions are very suitable systems within the food industry [22] and suggest that nanoencapsulation utilizing these systems is a viable strategy for using EOs in the desired aqueous food mediums, as well as for increasing the antimicrobial activity of the EOs against a certain range of microorganisms.

Nanostructured lipid carriers (NLCs) are colloidal delivery systems with nanoscale-size particles. These systems are made of different kinds of lipids (solid or liquid), surfactants, and water. In these systems, water surrounds the lipid core and helps to form a solid lipid matrix. The formation of NLCs is a popular tool for controlling the release of lipophilic bioactive compounds in water-based food formulations, including a wide range of drinks [23]. Food products containing NLCs can be more easily fortified with bioactive agents such as vitamins, minerals, phytochemicals, and functional lipids, amino acids, and proteins [24].

Although there are comprehensive studies of NLC systems in the pharmaceutical literature, there have been few studies within the field of food science, which can be related to the restrictions placed on the ingredients that can be legally used in NLC formulations. In the limited research available, however, we can refer to the studies of Bagheri et al. [25] and Akhavan et al. [26] on the application of NLCs containing thymol and cinnamaldehyde to sesame paste/date syrup and dates, respectively. To a similar end, this study was conducted to assess the antimicrobial function of free thymol in comparison with thymol-loaded NLCs.
in a sausage product, and to evaluate the potential of these different applications of thymol as a nitrite substitute in sausage manufacture.

2. Materials and Methods

2.1. Materials

E. coli (ATCC 1276), S. aureus (ATCC 1337), and C. perfringens (ATCC) were provided from the Persian Type Culture Collection (PTCC). Thymol (purity 98.5%) was obtained from Sigma-Aldrich. Sulfadiazine Polymyxin Sulfite agar (SPS agar), Baird-Parker agar (BPA), Tween 60 (T60), triphenyl tetrazolium chloride, Violet Red Bile Agar (VRBA), nutrient broth, Muller-Hinton agar (MHA), and Muller-Hinton broth (MHB) 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. The Formulation of NLCs

The Mozaffar et al. [23] method was used to prepare the NLCs. 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 6 g/L thymol. This dispersion was diluted using distilled water to a concentration of 1% NLC which corresponded to 3 g/L thymol.

2.3. Particle Size Distribution

To measure the particle size of thymol-loaded NLCs, dynamic light scattering (Nano ZS, Malvern Panalytical Ltd., Malvern, UK) was conducted using a 4 (mW) He–Ne laser at a wavelength of 633 nm, at 70 and 90° detection angles, at a temperature of 25 °C. The instrument software (5.02 version, Malvern Panalytical Ltd., UK) was used to calculate the polydispersity index, hydrodynamic droplet size, and size distribution [27].

2.4. Encapsulation Efficiency

To estimate the encapsulation efficiency (EE) of NLCs, an ultraviolet–visible spectroscopy method was used according to the method described by Pivetta et al. [28]. For this purpose, 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 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:

\[
EE(\%) = \frac{[\text{Thymol}_{\text{total}} - \text{Thymol}_{\text{not encapsulated}}]}{\text{Thymol}_{\text{total}}} \times 100
\] (1)

2.5. Determination of Minimum Inhibitory Concentration (MIC)

The microdilution broth method was used to measure MIC [10]. For this purpose, an overnight culture was diluted using sterilized peptone water to obtain the approximate number of 10^8 CFU/mL in the bacterial suspension. A 96-well microplate was used to perform the experiment. In the first row of wells, MHB culture and 10 μL of the bacterial suspension (C. perfringens, E. coli, or S. aureus) were added. In the next rows, 100 μL of the culture medium and 10 μL of the bacterial suspension were mixed with 6.25, 12.5, 25, 50, 100, and 200 mg/L of thymol or with NLC containing equal amounts of thymol (the initial concentration of the NLC dispersion was 6000 mg/L). The turbidity of the wells was assessed after incubation of the microplates at 37 °C for 24 h and the lowest concentration
of thymol or thymol-loaded NLC that was found to inhibit the growth of bacteria was assumed as the MIC.

2.6. Determination of Minimum Bactericidal Concentration (MBC)

The non-turbid wells of the MIC test were used for the determination of MBC and 100 µL of their content was cultivated on MHA. After incubation at 37 ± 2 °C for 24 h, the lowest concentration of thymol or thymol-loaded NLC in which no bacterial growth occurred was assumed as the MBC [10].

2.7. Agar Diffusion Method

The agar well diffusion method was used to evaluate the antimicrobial activity of thymol in both the free and thymol-loaded NLC forms [3]. For this purpose, 0.1 mL microbial suspension (10⁶ CFU/mL) was cultivated on VRBA, BPA, and SPS agar plates with three wells to be inoculated with *S. aureus*, *E. coli*, and *C. perfringens*, respectively. Thereafter, 50 µL solutions of thymol or its NLC, containing 6.25, 12.5, 25, 50, 100, and 200 mg/L thymol, were poured into each well. After incubation of plates at 37 ± 2 °C for 24 to 48 h, the diameter of inhibition zones was measured for each bacterium. In addition, a gentamicin disk comprising 10 µg gentamicin was put in one well so as to compare the antimicrobial property of thymol and its NLC with gentamicin.

2.8. Sausage Preparation

Before the experiment, different concentrations of thymol and thymol-loaded NLCs were sensorially evaluated in the sausage formulation, and 600 mg/kg thymol was selected. Afterward, the sausages were prepared by mixing 70% beef, 20% soy protein isolate, 0.2% sodium phosphates, 1% corn starch, 5% water, 2% oil, 1% sodium chloride, and 0.8% dry milk [3]. To avoid any interference with the antimicrobial agents being tested (thymol or thymol-loaded NLCs), spices were not used in the formulation of the sausages.

The sausage mixture was prepared by thoroughly mixing (Robokit 2154, BEKO, Istanbul, Turkey) all raw materials. After dividing the mixture into six portions, each portion was thoroughly mixed with 600 mg/kg thymol, 600 mg/kg thymol-loaded NLC, 600 mg/kg thymol + 60 mg/kg nitrite, and 600 mg/kg thymol-loaded NLC + 60 mg/kg nitrite, respectively. A control sample was also prepared without the addition of thymol, nitrite, or NLC. Thereafter, each treatment was divided into three portions and each portion was inoculated with *S. aureus*, *E. coli*, and *C. perfringens* (10⁵.5 CFU/g), respectively. Afterwards, the inoculated mixtures were placed in plastic wraps (with a 3.50 cm diameter) using a filler (Bosch MFW68640, BSH Home Appliances Ltd., Stuttgart, Germany) and then heated in a water bath (SHZ-82, Aria Teb, Tehran, Iran) at 75 °C for 40 min (the internal temperature in the coldest part of the samples reached 72 °C). Then, the samples were refrigerated at 4 °C, with subsequent sampling performed once per week during 4 weeks.

2.9. The Microbial Counts of Sausages

Ten grams of each of the sausage samples was mixed with 50 mL peptone water, serially diluted, then cultured (using about 0.1 mL) on the surface of the desired culture medium (BPA for *S. aureus*; VRBA for *E. coli*; and SPS agar for *C. perfringens*). The incubation was performed at 37 °C for 48 h (for *S. aureus* and *C. perfringens*), and 37 °C for 24 h (for *E. coli*) [3].

2.10. Mathematical Modeling

The first-order model was used for calculating the growth rate (k value) of *E. coli*, *S. aureus*, and *C. perfringens* in the sausage product. The Lack-of-fit and Pearson R² tests were used for the evaluation of model validity using IBM SPSS version 22 (IBM, Armonk, NY, USA).

\[
\ln(C) = \ln(C_0) - (k \times t),
\]
C: the population of the bacterium at time t (CFU/g), C0: the population of the bacterium at time zero (CFU/g), k: the growth rate of the bacterium (per week), and t: the storage time (week) [29].

2.11. Statistical Analysis

The SPSS 16.0 software (Team EQX, USA) was used for data analysis. The average values from at least three replicates were calculated and reported as the mean ± standard deviation (SD). The data were analyzed using a one-way analysis of variance (ANOVA) and using Duncan’s multiple range test, with differences at p < 0.05 considered significant.

3. Results and Discussion

3.1. NLC Characterization

The particle size distribution of NLCs is shown in Figure 1. The mean particle size of NLCs was 140 nm with a zeta potential of −0.52 mV, indicating stable droplets due to the presence of repulsive forces among them [3]. The appearance of only one narrow peak in the particle size distribution diagram indicates the homogeneity of the particle size within the thymol-NLCs. The homogeneity of a system is indicated by the polydispersity index ranging between 0 to 1. The index of zero or close to zero indicates that the system is homogenous [14]. The results from this portion of the study are highly consistent with Karrimi Khorrami et al.’s [13] results on thymol-loaded NLC, Bagheri et al.’s [17] report on thymol-NLC, Almasi et al.’s [11] results on microemulsions containing thyme EO, Akhavan et al.’s [26] report on cinnamaldehyde-loaded NLC, and Sepahvand et al.’s [3] results on thymol-loaded nanoemulsions. Meanwhile, Radi et al. (2021) obtained an average diameter of 94.9 nm for the cinnamaldehyde-loaded NLCs they analyzed [30].

![Figure 1. The particle size distribution of thymol-NLC.](image)

Another parameter to consider was the measurement of EE after NLC preparation, which was estimated to be around 97%. The high affinity of thymol with the lipid matrix of the NLCs may be the cause of the high EE of thymol in the NLC systems. Meanwhile, the presence of liquid lipids prevents the formation of a fully crystalline structure, which results in a higher loading capacity [28]. Similar results were obtained by Piran et al. [31] on menthol-loaded NLCs and by Pivetta et al. [28] on thymol-loaded NLCs.

3.2. MIC, MBC, and Agar Well Diffusion Test Results

Table 1 shows the MIC and MBC values of thymol and NLCs against E.coli, S. aureus, and C. perfringens. As shown in Table 1, free thymol exhibited MIC values of 110, 180, and 196.67 mg/L and MBC values of 200, 406, and 406.6 mg/L against S. aureus, E. coli, and...
C. perfringens, respectively. The MIC and MBC values of thymol-loaded NLCs against all of the bacteria studied were significantly higher than those exhibited by free thymol. This shows that the NLCs could successfully entrap thymol in the solid matrix of nanoparticles, which can act as a reservoir to supply the required concentration of thymol to inhibit the growth of microorganisms throughout the storage time. Lai et al. [32] have also reported that the encapsulation of Artemisia arborescens L. EO in an NLC matrix resulted in a reduction in the rapid evaporation of this EO. Similarly, Shi et al. [9] have reported that frankincense and myrrh EO-loaded NLCs efficiently reduced the evaporation loss of these EOs.

Table 1. MIC and MBC values of thymol and its NLCs against S. aureus, E. coli, and C. perfringens.

| Samples                  | E. coli       | S. aureus    | C. perfringens |
|--------------------------|---------------|--------------|----------------|
|                          | MIC (mg/L)    | MBC (mg/L)   | MIC (mg/L)     | MBC (mg/L)   | MIC (mg/L)     | MBC (mg/L)   |
| Thymol                   | 180 ± 0.0 a   | 406 ± 0.0 a  | 110 ± 0.0 a    | 200 ± 0.0 a  | 196.67 ± 0.0 a | 406.6 ± 0.0 a |
| Thymol-loaded NLC        | 406.67 ± 0.0 b| 833.3 ± 0.0 b| 216.6 ± 0.0 b  | 406.6 ± 0.0 b| 393.3 ± 0.0 b  | 806.6 ± 0.0 b |
| NLC without thymol       | Not effective | Not effective | Not effective  | Not effective| Not effective  | Not effective |

a,b Different small letters in each column indicate statistical difference between the data (p < 0.05).

It has been reported that thymol achieves its antimicrobial activity by perturbing the bacterial membrane [33] and increasing cytoplasmic membrane permeability [34]. This, in turn, leads to the leakage of intracellular material and bacterial cell death [33]. Meanwhile, thymol penetrates the bacterial cells, and binds to bacterial cell DNA, changing its secondary structure [20]. As previously described, the entrapment of thymol within the NLC matrix and the slow-release nature of this formulation caused higher concentrations of thymol were consumed in the MIC and MBC tests in order to affect the bacteria within a short timeframe. The agar disk diffusion test results (Table 2) showed that gentamicin was a significantly stronger antimicrobial agent against the tested microorganisms, forming the largest inhibition zone diameters. The halo diameters of the different antimicrobial agents examined (Table 2) ranged 6.66–38.66 mm, depending on the bacterium and antimicrobial agent applied. The antimicrobial activity of thymol and thymol-loaded NLC was approximately the same for each of the three bacteria studied (p > 0.05), with equal inhibition zones detected for both antimicrobial agents. The effectiveness of an antimicrobial agent in the agar disk diffusion method is limited by the ability of the compound to diffuse in this medium. The results of this study showed that thymol and its NLCs can diffuse equally well in the MHA medium [3], which resulted in the same antimicrobial activity for thymol and its NLC against E. coli, S. aureus, and C. perfringens.

Among the three pathogenic bacteria, the largest inhibition zones and the lowest MIC and MBC values (MIC (110 mg/L) and MBC (200 mg/L) for free thymol and MIC (216.6 mg/L) and MBC (406.6 mg/L) for NLCs) were observed for S. aureus. This indicates that S. aureus is more sensitive than the other bacteria examined to thymol and thymol-loaded NLCs. Meanwhile, the same sensitivity was detected for C. perfringens and E. coli towards thymol and thymol-loaded NLCs, according to the results of the agar disk diffusion test, MIC, and MBC. In line with our findings, Trombetta et al. [35] reported that S. aureus exhibits a higher sensitivity to thymol compared to that of E. coli.

In general, G+ bacteria show higher sensitivity to EOs than those of G− ones [36], which is probably related to their cell wall structural differences. The outer phospholipid membrane of G− bacteria leads to better protection for these bacteria against EOs, while EOs penetrate the thicker peptidoglycan layer of G+ bacteria due to their hydrophobic nature, resulting in easier access of antimicrobial molecules to the bacterial cells [37].

Our results confirmed the substantial influence of thymol concentration (in the free or nanoencapsulated forms) on its antimicrobial activity. By increasing the concentration of thymol, higher halo diameters were obtained against the three bacteria studied. It has been reported that a higher thymol concentration leads to an increase in bacterial membrane permeability, leakage of cellular content, and bacterial morphology loss [20].
Table 2. Antimicrobial activity of thymol and its NLC against S. aureus, E. coli, and C. perfringens.

| Samples                        | Concentration (mg/L) | E. coli (mm) | S. aureus (mm) | C. perfringens (mm) |
|--------------------------------|----------------------|--------------|----------------|---------------------|
| Gentamicin                     | 10 µg                | 22 ± 1.00 b  | 30.66 ± 1.00 a | 26.00 ± 1.00 b      |
| Thymol                         | 100                  | 11.3 ± 0.5 d | 12.6 ± 1.0 de  | 10.35 ± 0.5 de      |
|                                | 50                   | 10.3 ± 0.5 de| 12.0 ± 1.0 d  | 9.30 ± 0.5 e        |
|                                | 25                   | 9.00 ± 1.0 ef| 11.0 ± 1.0 de | 9.30 ± 0.5 ef       |
|                                | 12.5                 | 7.60 ± 0.57 f| 9.3 ± 0.57 f  | 8.30 ± 0.5 f        |
|                                | 6.25                 | 6.70 ± 0.5  f| 8.0 ± 1.0  f  | 7.60 ± 0.6  f       |
| Thymol-loaded NLC              | 100                  | 10.67 ± 0.57 de| 11.33 ± 0.58 de| 11 ± 1.52 cd       |
|                                | 50                   | 10.66 ± 0.57 de| 11.33 ± 0.58 de| 9.66 ± 0.57 de      |
|                                | 25                   | 9.33 ± 0.57 ef| 10.33 ± 0.58 ef| 9.33 ± 0.57 e       |
|                                | 12.5                 | 8.33 ± 0.57 f| 8.33 ± 0.58 f| 8.67 ± 0.57 f      |
|                                | 6.25                 | 7.33 ± 0.57 f| 7.66 ± 0.57 f| 6.66 ± 0.57 h      |
| NLC without thymol             | -                    | 0.00 ± 0.00 i| 0.00 ± 0.00 i| 0.00 ± 0.00 i      |

**a–i** Different small letters in each column indicate statistical difference between the data (p < 0.05).

3.3. The Antimicrobial Activity of Thymol and Thymol-Loaded NLC in the Sausage Product

The sausage models containing thymol or thymol-loaded NLCs were inoculated with about $10^{5.5}$ CFU/g of S. aureus, E. coli, and C. perfringens in separate samples. The results are shown in Figure 2. An increase in bacterial growth was observed in the control and 120 mg/kg nitrite-containing samples throughout the storage time. After 4 weeks, the S. aureus, E. coli, and C. perfringens populations had increased to 8, 8.2, and 8.3 log CFU/g, respectively, in the control samples. These values were 7, 7.2, and 7.2 log CFU/g, respectively, in nitrite-containing samples. Therefore, after the control samples, the 120 mg/kg nitrite-containing samples showed the highest bacterial growth. The effect of nitrite on the growth of all the bacteria examined was observed from the 2nd week onward.

The use of plain thymol, NLC, thymol + nitrite, and NLC + nitrite in the sausage samples resulted in a decreasing trend in the population of all three tested bacteria over 4 weeks of storage (Figure 2), while no significant differences were observed among these samples ($p > 0.05$). These findings indicate a similar function for the plain thymol, NLC, thymol + nitrite, and NLC + nitrite against each tested bacterium.

The S. aureus population was reduced by about 0.69, 2.17, 3.92, and 3.90 log cycles in the samples containing thymol, NLC, thymol + nitrite, and NLC + nitrite when compared to the control after 1, 2, 3, and 4 weeks of storage, respectively ($p < 0.05$). These values were 1.19, 2.68, 3.58, and 4.67 log cycles for E. coli on days 7, 14, 21, and 28 ($p < 0.05$). The reduction of C. perfringens in comparison with the control sample was estimated to be around 0.33, 2.24, 2.76, and 4.45 log cycles after 7, 14, 21, and 28 days of storage, respectively ($p < 0.05$). These results indicate that thymol or thymol-loaded NLCs could effectively reduce the bacterial population in the sausage product, and as with the disk diffusion test results, there was no significant difference between the antimicrobial activity of thymol and thymol-loaded NLCs in the sausage product. It seems that the emulsified nature of the sausage resulted in the thymol becoming emulsified within the sausage mixture, leading to a homogeneous distribution of thymol. Thymol-loaded NLCs also had an even distribution throughout the sausage structure due to their nanoencapsulation. Therefore, the same performance of plain thymol and the thymol-loaded NLCs was observed. In line with the results of this study, Sepahvand et al. [3] have reported that the application of thymol-nanoemulsion did not improve the performance of thymol with regards to its reduction in the bacterial count within a sausage mixture. Mohammadpourfard et al. [38] have similarly confirmed that thymol effectively controlled the population of C. perfringens and lactic acid bacteria in cooked probiotic sausages. In addition, Wang et al. [39] have reported that chitosan nanoemulsions containing thymol reduced the proportion of spoilage organisms...
in fresh pork and reduced bacterial diversity. However, Akhavan et al. [26] reported that cinnamaldehyde-loaded NLCs could efficiently reduce the fungi and total bacteria counts in Mazafati dates in comparison with free cinnamaldehyde. Meanwhile, Radi et al. [30] found that the efficiency of cinnamon EO-loaded NLCs in inhibiting the growth of *Penicillium citrinum* and *Penicillium expansum* involved in tangerine decay was significantly higher than that of the free EO. These results show that nanoencapsulation of EOs might be a useful tool for improving their function within foodstuffs that are non-emulsified in nature. However, in foods that are intrinsically emulsified, such as sausages, nanoencapsulation may not improve the function of the EO.

Figure 2. Cont.
3.4. The Growth Kinetic of E. coli, S. aureus, and C. perfringens in the Sausage

By plotting the logarithm of E. coli, S. aureus, and C. perfringens growth versus time (Figure 3), straight lines were achieved, which were used for the calculation of k values to represent the growth rate of the examined bacteria in the sausage product during 4 weeks of storage. Data are presented in Table 3. High determination coefficients, ranging between 0.82 and 0.99, were obtained for these straight lines (Table 3). According to the results, the growth of the three bacteria obeyed the first-order reaction throughout 4 weeks. The positive k values, obtained for the control and the samples containing 120 mg/kg nitrite, showed an increasing trend in bacterial growth during the 4 weeks. However, the negative k values, obtained for thymol, NLC, thymol + nitrite, and NLC + nitrite, indicated a decreasing trend in bacterial growth within these samples, demonstrating the involvement of a new factor (the antimicrobial agent) on the trend of bacterial growth in the sausage product. The k values of all treated samples showed no significant difference with each other. This shows that the examined bacteria grew according to a similar trend in these samples.

Table 3. k values (microbial growth) of S. aureus, E. coli, and C. perfringens in different treatments of sausage stored at 4 ± 1 °C over 4 weeks of storage.

| Treatments     | C. perfringens | E. coli | S. aureus |
|----------------|----------------|---------|-----------|
|                | R²  | k (week⁻¹) | R²  | k (week⁻¹) | R²  | k (week⁻¹) |
| Control        | 0.97 | 0.162 b  | 0.98 | 0.118 a  | 0.97 | 0.128 e  |
| Nitrite        | 0.82 | 0.124 a  | 0.90 | 0.092 b  | 0.84 | 0.094 b  |
| Thymol         | 0.99 | −0.447 e | 0.92 | −0.107 c | 0.88 | −0.15 d  |
| NLC            | 0.95 | −0.116 c | 0.89 | −0.115 b | 0.96 | −0.128 c |
| Thymol + Nitrite | 0.91 | −0.115 c | 0.97 | −0.114 a | 0.91 | −0.127 c |
| NLC + Nitrite  | 0.98 | −0.229 a | 0.97 | −0.153 d | 0.86 | −0.11 e  |

Different small letters in each column indicate statistical difference between the data (p < 0.05).
Figure 3. The growth rate of bacteria in inoculated sausages plotted on a logarithmic scale.
4. Conclusions

Although nanoencapsulation of thymol as an NLC was successfully performed in this study, thymol nanoencapsulation did not improve thymol antimicrobial activity in the sausage product. This result was inconsistent with the results of our previous studies on the use of thyme microemulsion and nanoemulsion on cucumbers, strawberries [18] and ground meat [2,19] as well as on the application of cinnamaldehyde NLCs on dates [30]. In these studies, thyme nanoemulsion and microemulsion, and cinnamaldehyde-NLC reduced the microbial counts more efficiently than the free EOs. In these studies, the foods examined were not emulsions, whereas the sausage examined in the present study is already emulsified during manufacture, which may explain the equivalent antimicrobial function observed with both free thymol and its NLC form. Therefore, more investigations are needed to evaluate other quality parameters of meat products affected by thymol or its NLCs.

Author Contributions: Conceptualization, M.R. and S.A.; methodology, S.S., S.A., M.R. and M.J.A.; formal analysis, S.S., M.J.A. and S.A.; writing—original draft preparation, S.A. and S.S.; writing—review and editing, S.A. and M.R.; supervision, S.A. and M.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are available from the corresponding author on reasonable request.

Acknowledgments: This work was a part of a Ph.D. research project carried out at Islamic Azad University (Yasooj Branch).

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Sayadi, M.; Amiri, S.; Radi, M. Active packaging nanocomposite gelatin-based films as a carrier of nano TiO2 and cumin essential oil: The effect on quality parameters of fresh chicken. J. Food Meas. Charact. 2021, 16, 420–430. [CrossRef]
2. Almasi, L.; Radi, M.; Amiri, S. The release rate and antimicrobial activity of calcium-alginate films containing self-microemulsifying Thymus vulgaris essential oil against Escherichia coli and Staphylococcus aureus. J. Food Saf. 2020, 40, e12828. [CrossRef]
3. Sepahvand, S.; Amiri, S.; Radi, M.; Akhavan, H.-R. Antimicrobial Activity of Thymol and Thymol-Nanoemulsion Against Three Food-Borne Pathogens Inoculated in a Sausage Model. Food Bioprocess Technol. 2021, 14, 1936–1945. [CrossRef]
4. Ding, Z.; Johanningsmeier, S.D.; Price, R.; Reynolds, R.; Truong, V.-D.; Payton, S.C.; Breidt, F. Evaluation of nitrate and nitrite contents in pickled fruit and vegetable products. Food Control 2018, 90, 304–311. [CrossRef]
5. Amiri, S.; Akhavan, H.; Zare, N.; Radi, M. Effect of Gelatin-Based Edible Coatings Incorporated with Aloe Vera and Green Tea Extracts on the Shelf-Life of Fresh-Cut Apple. Ital. J. Food Sci. 2018, 30, 61–74.
6. Pakétrat, S.; Amiri, S.; Radi, M.; Abedi, E.; Torri, L. The influence of green tea extract as the steeping solution on nutritional and microbial characteristics of germinated wheat. Food. J. Food Sci. 2020, 332, 127288. [CrossRef]
7. Amiri, S.; Nicknam, Z.; Radi, M.; Sayadi, M.; Bagheri, F.; Khorrami, N.K.; Abedi, E. Postharvest quality of orange fruit as influenced by salicylic acid, acetic acid, and carboxymethyl cellulose coating. J. Food Meas. Charact. 2021, 15, 3912–3930. [CrossRef]
8. Pavela, R.; Benelli, G. Essential Oils as Ecofriendly Biopesticides? Challenges and Constraints. Trends Plant Sci. 2016, 21, 1000–1007. [CrossRef]
9. Shi, F.; Zhao, J.-H.; Liu, Y.; Wang, Z.; Zhang, Y.-T.; Feng, N.P. Preparation and characterization of solid lipid nanoparticles loaded with frankincense and myrrh oil. Int. J. Nanomed. 2012, 7, 2033–2043.
10. Almasi, L.; Radi, M.; Amiri, S.; Torri, L. Fully dilutable Thymus vulgaris essential oil: acetic or propionic acid microemulsions are potent fruit disinfecting solutions. Food Chem. 2020, 343, 128411. [CrossRef]
11. Almasi, L.; Radi, M.; Amiri, S.; McClements, D.J. Fabrication and characterization of antimicrobial biopolymer films containing essential oil-loaded microemulsions or nanoemulsions. Food Hydrocoll. 2021, 117, 106733. [CrossRef]
12. Karam, L.; Roustom, R.; Abiad, M.G.; El-Obeid, T.; Savvaidis, I.N. Combined effects of thymol, carvacrol and packaging on the shelf-life of marinated chicken. Int. J. Food Microbiol. 2018, 291, 42–47. [CrossRef]
13. Khorrami, N.K.; Radi, M.; Amiri, S.; McClements, D.J. Fabrication and characterization of alginate-based films functionalized with nanostructured lipid carriers. Int. J. Biol. Macromol. 2021, 182, 373–384. [CrossRef]
14. Amiri, S.; Abbasi, S.; Ezzatpanah, H.; Hosseini, E. Nanocapsulation of orange peel oil using microemulsion technique. *Agro Food Ind. Hi Tech* 2013, 24, 72–75.

15. Radi, M.; Abbasi, S.; Hamidi, Z.; Azizi, M.H. Development of a new method for extraction of canola oil using lecithin based microemulsion systems. *Agro Food Ind. Hi Tech* 2013, 24, 70–72.

16. Bagheri, F.; Radi, M.; Amiri, S. Evaluating the physical, mechanical and morphological properties of sodium alginate nanocomposite film containing solid lipid nano-particles. *J. Food Sci. Technol.* 2019, 16, 263–271.

17. Bagheri, F.; Radi, M.; Amiri, S. Drying conditions highly influence the characteristics of glycerol-plasticized alginate films. *Food Hydrocoll.* 2019, 90, 162–171. [CrossRef]

18. Cinay, G.E.; Erkoc, P.; Alipour, M.; Hashimoto, Y.; Sasaki, Y.; Akiyoshi, K.; Kizilel, S. Nanogel-integrated pH-responsive composite hydrogels for controlled drug delivery. *ACS Biomater. Sci. 2017*, 3, 370–380. [CrossRef]

19. Robledo, N.; Lope, L. Burger, A.; Tapia, C.; Abugoch, L. Effects of Antimicrobial Edible Coating of Thymol Nanoemulsion/Quinoa Protein/Chitosan on the Safety, Sensory Properties, and Quality of Refrigerated Strawberries (Fragaria × ananassa) Under Commercial Storage Environment. *Food Bioprocess Technol.* 2018, 11, 1566–1574. [CrossRef]

20. Liu, T.; Liu, L. Fabrication and characterization of chitosan nanoemulsions loading thymol or thyme essential oil for the preservation of refrigerated pork. *Int. J. Biol. Macromol.* 2020, 159–1615. [CrossRef]

21. Abdou, E.S.; Galhoum, G.F.; Mohamed, E.N. Curcumin loaded nanoemulsions/pectin coatings for refrigerated chicken fillets. *Food Hydrocoll.* 2018, 83, 445–453. [CrossRef]

22. Radi, M.; Amiri, S. Comparison of the Rheological Behavior of Solutions and Formulated Oil-in-Water Emulsions Containing Carboxymethylcellulose (CMC). *J. Dispers. Sci. Technol.* 2013, 34, 582–589. [CrossRef]

23. Mozaffar, S.; Radi, R.; Amiri, S.; McClements, D.J. A new approach for drying of nanostructured lipid carriers (NLC) by spray-drying and using sodium chloride as the excipient. *J. Drug Deliv. Sci. Technol.* 2021, 61, 102212. [CrossRef]

24. Aditya, N.; Aditya, S.; Yang, H.J.; Kim, H.W.; Park, S.O.; Lee, J.; Ko, S. Curcumin and catechin co-loaded water-in-oil-in-water emulsion and its beverage application. *J. Funct. Foods* 2018, 15, 35–43. [CrossRef]

25. Bagheri, F.; Nejatian, M.; Abbaszadeh, S.; Taghdir, M. The effect of gelatin and thymol-loaded nanostructured lipid carrier on physicochemical, rheological, and sensory properties of sesame paste/date syrup blends as a snack bar. *J. Texture Stud.* 2020, 51, 501–510. [CrossRef]

26. Akhavan, H.-R.; Hosseini, F.-S.; Amiri, S.; Radi, M. Cinnamaldehyde-Loaded Nanostructured Lipid Carriers Extend the Shelf Life of Date Palm Fruit. *Food Bioprocess Technol.* 2021, 14, 1478–1489. [CrossRef]

27. Radi, M.; Abbasi, S. Optimization of Novel Oil Extraction Technique from Canola Seeds: Lecithin-Based Microemulsion. *Eur. J. Lipid Sci. Technol.* 2018, 120, 1700267. [CrossRef]

28. Pivettaa, T.P.; Simõesb, S.; Araújoa, M.M.; Carvalhoc, T.; Arrudad, C.; Marcatoa, P.D. Development of nanoparticles from natural lipids for topical delivery of thymol: Investigation of its anti-inflammatory properties. *Colloids Surf. B* 2018, 164, 281–290. [CrossRef]

29. Amiri, S.; Niakousari, M. Shelf life of unpasteurized sour orange juice in Iran. *Fruits* 2008, 63, 11–18. [CrossRef]

30. Radi, M.; Ahmadi, H.; Amiri, S. Effect of Cinnamon Essential Oil-Loaded Nanostructured Lipid Carriers (NLC) Against Penicillium Citrinum and Penicillium Expansum Involved in Tangerine Decay. *Food Bioprocess Technol.* 2021, 11, 1566–1574. [CrossRef]

31. Piran, P.; Kafli, H.S.; Ghanbarzadeh, S.; Saifzari, R.; Hamishekar, H. Formulation of menthol-loaded nanostructured lipid carriers to enhance its antimicrobialactivity for food preservation. *Adv. Pharm. Bull.* 2017, 7, 261–268. [CrossRef]

32. Lai, F.; Wissing, S.A.; Müller, R.H.; Fadda, A.M. Artemisia aborescens essential oil-loaded solid lipid nanoparticles for potential agricultural application: Preparation and characterization. *AAPS Pharm. Sci. Tech.* 2006, 7, E10–E18. [CrossRef]

33. Marchese, A.; Orhan, I.E.; Daglia, M.; Barbieri, R.; Di Lorenzo, A.; Nabavi, S.F.; Gortzi, O.; Izadi, M.; Nabavi, S.M. Antibacterial and antifungal activities of thymol: A brief review of the literature. *Food Chem.* 2016, 210, 402–414. [CrossRef]

34. Xu, J.; Zhou, F.; Ji, B.-P.; Pei, R.-S.; Xu, N. The antibacterial mechanism of carvacrol and thymol against Escherichia coli. *Leit. Appl. Microbiol.* 2008, 37, 174–179. [CrossRef]

35. Trombetta, D.; Castelli, F.; Sarpietro, M.G.; Venturi, V.; Cristiani, M.; Daniele, C.; Saia, A.; Mazzanti, G.; Bisignano, G. Mechanisms of Antibacterial Action of Three Monoterpenes. *Antimicrob. Agents Chemother.* 2005, 49, 2474–2478. [CrossRef]

36. Seow, Y.X.; Yeo, C.R.; Chung, H.L.; Yuk, H.-G. Plant Essential Oils as Active Antimicrobial Agents. *Crit. Rev. Food Sci. Nutr.* 2013, 54, 625–644. [CrossRef]

37. Nazzaro, F.; Fratianni, F.; De Martino, L.; Coppola, R.; De Feo, V. Effect of Essential Oils on Pathogenic Bacteria. *Pharmaceuticals* 2013, 6, 1451–1474. [CrossRef]

38. Mohammadpourfard, I.; Khandari, A.; Basti, A.A.; Herrera-Latorre, C.; Shariatifar, N.; Hosseini, H. Evaluation of microbiological, chemical, and sensory properties of cooked probiotic sausages containing different concentrations of astaxanthin, thymol, and nitrite. *Food Sci. Nutr.* 2020, 9, 345–356. [CrossRef]

39. Wang, L.; Liu, T.; Liu, L.; Yu, W. X. Impacts of chitosan nanoemulsions with thymol or thyme essential oil on volatile compounds and microbial diversity of refrigerated pork meat. *Meat Sci.* 2022, 185, 108706. [CrossRef]