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Impact of Some Essential Oils and Nanoparticles in Chitosan Films to Control Pathogenic Bacteria and Storage Keeping Quality of Meat Products

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Abstract

Beef meat is highly susceptible to microbial and chemical spoilage due to its high moisture and protein content. The use of edible coatings contains herbal extracts with antioxidant and antibacterial properties that help to extend the shelf life of meat products. In this study, the effect of chitosan film coating with zinc oxide nanoparticles at 0,4 mg and thyme and clove essential oil at 400 ppm, and their combination on chemical properties (Total volatile nitrogen (TVN) , thiobarbituric acid (TBA) and pH value) as well as, microbial (total bacterial count and coliform group count) of beef burger and beef Kofta were studied up to 12 day of refrigerated storage period compared to the control sample. The results of GC/MS revealed that thymol (46.48%) was the highest chemical compound in thyme volatile oil. While Eugenol (24.00%) was the highest one chemical compound in clove volatile oil. Essential oils and nanoparticles were examined for their antimicrobial properties against two mould strains and four bacterial strains that are most commonly found in meat and meat products. The two essential oils' inhibitory effects could be arranged as follows: thyme> clove > and followed with zinc oxide nanoparticles and chitosan nanoparticles. Statistical results showed that the rate of increase in TVN, TBA and pH of all coated treatments were lower than control in beef burger and beef Kofta. Microbial analysis results showed an decrease trend in the growth of different bacteria in chitosan film treated combined with ZnNPs/ thyme and ZnNPs/ clove compared to the control sample during chilled storage. Beef burger and beef Kofta coated by chitosan film treated with ZnNPs/ thyme or ZnNPs/ clove displayed a longer shelf life compared to other samples.

Key words: Antimicrobial, zinc oxide nanoparticles, beef burger, beef Kofta, chitosan, edible, coting, thyme, clove.

Introduction

In general, meat-based foods have very low microbial and oxidative stability and are easily exposed to microbial and chemical spoilage during the production and storage chain (**Mojaddar Langroodi *et al.*, 2021**). Therefore, improving the storage life of fresh meat is one of the important challenges of the meat industry which has attracted the attention of many researchers in recent years (**do Santos Junior *et al.*, 2020** and **Sepahvand *et al.*, 2021**). In this regard, the use of biopolymeric-based coatings and films is proposed as an important solution due to their barrier, mechanical, optical, and biodegradability characteristics (**Bagheri *et al.*, 2019** and **Molayi *et al.*, 2018**).

The use of biopolymers in the forms of coating which can restrict oxygen availability, prevent moisture loss, and thereby can increase the shelf life of many products in many researches (**Baqeri *et al.*, 2020** and **Martiny *et al.*, 2020**). The effectiveness of such coatings can be remarkably increased by incorporating antimicrobial and antioxidant compounds in their matrix to maintain high concentrations of these substances on the surface of coated products which are more susceptible to bacterial infestation (**Akhavan *et al.*, 2021**).

Essential oils (EOs) are secondary metabolites of plants, herbs and spices **Oussalah *et al.* (2006)**. EOs and plant extracts are commonly used as natural antioxidant, antimicrobial and flavoring agents to enhance the product quality in addition to extend shelf life by delaying microbial and oxidative reactions (**Ghanbari *et al.*, 2020).** However, they may have undesirable influences on sensorial properties of meat products in relatively high concentrations (**Rezaeifar *et al.*, 2020).** A promising approach to overcome this restriction is application of packaging materials as carrier of these agents (**Hashemi *et al.*, 2020).** Recently, incorporation of EOs and plants extracts into the edible coating and films, named as active packaging, has been investigated in several studies for preservation of food products (**Vasilatos and Savvaidis 2013; Langroodi *et al.*, 2018** and **Chaleshtori and Chaleshtori, 2017).**

Edible films and coating manufactured from natural components like proteins, polysaccharides and lipids, or their combination, are excellent alternatives of non-biodegradable plastics used commonly in food packaging because they are biodegradable, edible, environmental friendly and have low prices (**Esmaeili *et al.*, 2021).** Chitosan is a linear, non-toxic, biocompatible and biodegradable polysaccharide produced through deacetylation of the chitin existed in the crustacean shells (**Hashemi *et al.*, 2020)**. It is well known as an antimicrobial and

antioxidant compound that is identified as generally recognized as safe (GRAS) (**Gökmen and Gürbüz, 2011)**. This biopolymer can successfully be used for production of edible films and coatings for food packaging (**Montaño-Sánchez *et al*., 2020).**

Recently, nanotechnology invades the world and has become increasingly important in the biomedical and pharmaceutical areas. This brought great opportunities for the development of materials with new properties as antimicrobial agents **(Roco, 1999).** Most antibacterial inorganic compounds are metallic nanoparticles and metal oxide nanoparticles such as silver, copper, titanium oxide, and zinc oxide (ZnO) **(Bradley *et al.*, 2011).**

ZnO nanoparticles have been extensively used in many industrial areas such as pharmaceutical, cosmetic and food industries **(Deng *et al.*, 2008).** Recently, zinc oxide is incorporated into packaging materials as antimicrobial agent. They can play an important role in reducing the risk of pathogen contamination and extending the shelf life of food **(Espitia *et al.*, 2012).**

In meat packaging, ZnO nanoparticles effectively control both Gram-positive and Gram-negative bacteria, fungi, algae, and viruses related to electrical, catalytic properties and thermal stability, (**Carbone *et al.*, 2016).** These nanoparticles have multifunctional effects: high antimicrobial efficacy, piezoelectricity, optical transparency, and electrical conductivity. They also provide UV protection, (**Kim *et al.*, 2022).** ZnO nanoparticles are used in active packaging as an antimicrobial agent since they help maintain meat quality, especially by controlling color and fat oxidation. They may affect flavor, but are considered safe by the FDA (**Panea *et al.*, 2014).**

Therefore, the present study aimed to evaluate the antibacterial effect of ZnO nanoparticles in fresh beef burger and beef Kofta.

Materials and Methods

2.1 Material:

2.1.1. Raw material:

Thyme, rosemary, clove and cinnamon bark, pure oils were obtained from Sugar Industrial Integrated Company (SIIC) Cairo, Egypt. Fragrance and extraction factory. Fresh beef meat was obtained from local market in Kaha City, Qalyoubia Governorate, Egypt, and immediately transported in ice box to the laboratory, then carefully cut into fillets and finally weighed until use. Spices mixture, were obtained from the local market in Toukh City, Qalyoubia Governorate, Egypt. Other ingredients such as texturized Soy were obtained from Food Technology Research Institute, Agricultural Research Center, Giza, Egypt. Salt, fresh eggs, bread crust, ground onion, foam plates and polyethylene film were obtained from the local market in Qalyoubia Governorate, Egypt.

2.1.2. Chemicals:

Zinc oxide nanoparticles powder (average particle diameter of about 50 nm); Tween 20 and 2-thiobarbituric acid were procured from sigma-Aldrich Chime, Steinheim Germany, While, chitosan powder was obtained from ROTH Company, Germany. Hydrochloric acid, magnesium oxide, ethanol**,** sulfuric acid, sodium hydroxide, tripolyphosphate (TPP) and acetic acid were obtained from El-Nasser pharmaceutical Chemical Company, Egypt.

2.1.3. Bacterial strains:

Faculty of Agriculture at Ain Shams University in Egypt's Cairo Microbiological Resources Center (Cairo MIRCEN) provided the bacterial strains. The test microorganisms were *Bacillus cereus* ATCC 33221, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 12600, *Salmonella typhimurium* ATCC14028, were assessed for experiments of antimicrobial activity.

2.1.4. Microbiological media:

Nutrient agar medium and violet red bile agar medium were obtained from Biolife Company, Italy and the agent in Egypt, Al-Badr Engineering Company.

2.2. Technological processing:

2.2.1. Preparation of films:

Firstly, the chitosan solution (0.4 mg/100 mL final film solution) was prepared by the dissolution of chitosan powder in distilled water under stirring at 45 °C for 30 min. Then, chitosan were produced based on ionic gelation of tripolyphosphate (TPP). For this purpose chitosan was dissolved in acetic acid aqueous solutions at 1% (w/v). ZnO NPs (0.4 mg/100 mL final film solution) were dispersed in distilled water for 15 min. Essential oils were added in concentrations from (400ppm ; v/w). The prepared films and ZnO NPs and essential oils dispersions were mixed with the chitosan solution and stirred at 25 °C for 30 min in 1000 rpm. Then, glycerol as plasticizer (1 g/100 mL final film solution) was added into the prepared mixture. At the same time, the chitosan solution was mixed and stirred for 1 h at room temperature. After that, the obtained mixtures were cast on plastic Petri dishes and dried at room temperature for 3 days. Prior to testing, the dried films were placed in a desiccator with 55% relative humidity at 25 °C for 4 days as modified by (**Sahraee, *et al.*, 2017 and Jebel and Almasi, 2016)**.

2.2.2. Treatment of beef burger and beef Kofta samples:

The beef burger formula consisted of 63% lean meat, 12% rehydrated texturized soy (it was rehydrated by water at a ratio of 1:2 w / v) and minced through 3 mm plate twice, 7% fresh eggs, 5% fresh onion, 1.5% salt, 1.8% spices, 3.7% bread crust and 6% ice water, according to **Feiner (2006)** and **Nageb (2015).**The mixture of spices was prepared according to **Bahlol and Abd El-Aleem (2004)**. The beef burger treatments were prepared as following in **Table (I).** All beef burger samples were packaged in

a foam plates and stored at 4±1˚C up to 16 days. While beef Kofta that, beef meatballs were prepared by mixing the ingredients (70% beef meat, 12% fat, 9% flour, 2.1% common salt, 1.2% onion, 1% garlic powder and spices mixture 1.2% (black pepper, cumin, ginger powder, nutmeg and turmeric powder)] in mincer to obtain meat dough, according to **Mohamed *et al.* (2020).**The beef Kofta treatments were prepared as following in **Table (II).** The samples were taken for analysis every 3 days periodically.

Table 1. Experimental treatments of beef burger treated with different films:

Treatments	*Explantation*
Ch film	Beef burger sample with chitosan film at 0,4 mg/100 ml (control)
Ch film/ZnNPs	Beef burger sample chitosan film with zinc oxide nanoparticles at 0,4 mg/100 ml
Ch film/ thyme	Beef burger sample chitosan film with thyme essential oil at 400 ppm
Ch film/ clove	Beef burger sample chitosan film with clove essential oil at 400 ppm
Ch film/ZnNPs/ thyme	Beef burger sample chitosan film with zinc oxide nanoparticles at 0,4 mg/100 ml and thyme essential oil at 400 ppm
Ch film/ ZnNPs/ clove	Beef burger sample chitosan film with zinc oxide nanoparticles at 0,4 mg/100 ml and clove essential oil at 400 ppm

Table 2. Experimental treatments of beef Kofta treated with different films:

Treatments	*Explantation*
Ch film	Beef Kofta sample with chitosan film at 0,4 mg/100 ml (control)
Ch film/ZnNPs	Beef Kofta sample chitosan film with zinc oxide nanoparticles at 0,4 mg/100 ml
Ch film/ thyme	Beef Kofta sample chitosan film with thyme essential oil at 400 ppm
Ch film/ clove	Beef Kofta sample chitosan film with clove essential oil at 400 ppm
Ch film/ZnNPs/ thyme	Beef Kofta sample chitosan film with zinc oxide nanoparticles at 0,4 mg/100 ml and thyme essential oil at 400 ppm
Ch film/ ZnNPs/ clove	Beef Kofta sample chitosan film with zinc oxide nanoparticles at 0,4 mg/100 ml and clove essential oil at 400 ppm

2.3. Methods:

2.3.1. Gas chromatography–mass spectrometry analysis (GC-MS)

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt, according to **Abd El-Motaleb *et al.*(2021).**

2.3.2. Total volatile nitrogen and thiobarbituric acid value:

Total volatile nitrogen (T.V.N) as mg/ 100 mg according to **Harold *et al*. (1987)**. The TBA as an indication for lipid oxidation was determined as reported by **Kirk *et al.* (1991)** as mg malonaldehyde /kg sample.

2.3.3. pH value:

The pH of prepared sample was measured using a pH-meter (model Consort P107 pH meter) with the technique by **Fernández-López*et al.*(2006).**

2.3.4. Microbiological examinations:

2.3.4.1. Microbiological examination:

According to established procedures for total count, ten grams of each sample were put to a culture medium (1: 10^{-1} to 1: 10^{-6} and homogenized for two minutes in a stomacher) (**ISO 4833:2013-1 protocol**). The plates were incubated at ±4^{o}C for 5 days and coliforms group (**ISO 21528-2:2004**).

2.3.4.2. Antimicrobial activity of volatile oils:

The effect of different concentrations of volatile oils (400 and 600pm) on bacteria growth was studied using the paper-disc plate method, according to **Loo *et al.* (1945) and Hassanen *et al.*, (2015),** by gauging the inhibitory zone's diameter.

2.3.5. Sensory evaluation of beef burger and beef Kofta:

Sensory evaluation was carried out to all samples which fried in a pan containing a little sunflower oil for 2 min for each side. Fried beef burgers were left to cool at room temperature before being subjected to organoleptic evaluation. Panel members were asked to evaluate different treatments and requested to score their quality attributes: color, odor, taste, tenderness, juiciness and overall acceptability on a 10 points. The scoring scheme was established as mentioned by **Nageb (2015).**

2.3.6. Statistical analysis:

The obtained results were analyzed using comparison of variance (ANOVA) and least significant different (L.S.D) at the 5% level of probability; as reported by **Snedecor and Cochran (1994).**

3. Results and Discussion

3.1. Chemical composition of beef meat:

The obtained data showed that fresh beef meat contained 72.86% moisture, 21.14% crude protein, 4.63% crude fat, 1.06% total ash and 0.31% total carbohydrates (on wet weight basis). Also, data revealed that, total volatile nitrogen was 9.23 mg /100 g, thiobarbituric acid was 0.280 mg

malonaldhyde /kg, pH values 6.11. These results are in agreement with those obtained by **Mohammed *et al.* (2020)**, reported that chemical composition of beef meat 71.52% moisture, 20.64% crude protein, 6.83% fat and 1.53% ash .Microbiological examination could be noticed that fresh beef meat contained total viable bacterial count was 3.4×10^4 and coliform group count was not detected. These results are in agreement with those obtained by **Mohammed *et al.* (2020)**, reported that TBC of beef meat samples were 3.26 $\log_{10}$ CFU /g and *E. coli* was 0.00 $\log_{10}$ CFU /g.

3.2. GC/MS characterization of the studied essential oils:

The chemical composition of the essential oils, which were isolated using GC-MS, is shown in **Table 3,** it could be noticed that, eleven compounds were ielentified from thyme volatile oil. The identified components represented (92.76 %) from the thyme volatile oil. Thymol, p-Cymene, Linalool, Carvacrol and γ-Terpinene were the chemical elements that were most prevalent in thyme volatile oil which constitute 84.2% of the total identified compounds. Thymol (46.48%) was the highest chemical compound in thyme volatile oil. However, γ-Terpinene (3.75%) was the lowest one among the most prevalent chemical elements in volatile thyme oil. These outcomes are consistent with **Mutlu-Ingok *et al.* (2021)** which proved that, thymol was the main component (46.4%) in thyme EO. The p-cymene (23.3%), Linalool (6.3%) and carvacrol (4.6%) were also detected in significant amounts in our study. **Fadel *et al.* (2020)**; **Varga *et al.* (2015)** and **Boruga *et al.* (2014)** found thymol (47.59%) as major components in Thymus vulgaris essential oil.

GC/MS characterizations of clove volatile oil was presented in **Table 3**, 18 volatile components were identified from clove volatile oil could be seen. The identified components represented (97.55%) from the clove volatile oil. Eugenol, α-Caryophellene, β-Caryophellene, Aromadendrene oxide Eugenol acetate, Trans-Caryophellene and α-farnesene were the most prevalent chemical compounds in clove volatile oil, Eugenol(24.00 %) was the highest chemical compound in clove volatile oil. However, Trans Caryophellene (5.09 %) was the lowest one among the most prevalent chemical elements in volatile clove oil. These outcomes are consistent with **Fadel *et al.* (2020)** who, reported that the seven volatile compounds identified in the hydrodistilled oil of clove buds were representing 99.9% of the total oil and Eugenol was the major compound (89.9%) followed by eugenyl acetate (7.9%), b-caryophyllene (1.4%) and ahumulene (0.4%). On other hand, GC/MS characterizations of cinnamon volatile oil was presented in **Table 3,** 17 volatile components were identified from cinnamon volatile oil could be seen. The identified components represented (90.81 %) from the cinnamonvolatile oil. α-Cinnamaldehyde, Benzaldehyde phenyl menthanol, Cinnamaldehyde (E), Benzaldehyde, 2- hydroxyl, Styrene (cymene), Spathulenol, Nerolidol and Cinnamic alcohol were the most prevalent chemical compounds in cinnamon volatile oil, α-Cinnamaldehyde (26.11 %)of the total chemical compounds was the highest compound. However, cinnamic alcohol (3.04 %) of the total chemical compounds was the lowest one among the most prevalent chemical elements in volatile cinnamon oil.

Table 3. Chemical components (%) of thyme, clove, cinnamon and rosemary essential oil identified by GC/MS:

Essential oil components	*Thyme essential oil*		*Clove essential oil*		*Cinnamon essential oil*		*Rosemary essential oil*	
	RT*	**Area %**	**RT***	**Area %**	**RT**	**Area %**	**RT**	**Area %**
α-pinene	**10.31**	**1.82**	**7.55**	**7.45**	**9.54**	**0.92**	**5.76**	**0.21**
Myrcene	**11.34**	**1.43**	-	-	-	-	-	-
Limonene	**12.32**	**1.11**	-	-	-	-	-	-
γ-Terpinene	**12.55**	**3.75**	-	-	-	-	-	-
p-Cymene	**12.80**	**23.03**	-	-	-	-	-	-
Linalool	**15.53**	**6.33**	-	-	-	-	-	-
Terpinen-4-ol	**16.11**	**1.47**	-	-	-	-	-	-
β-Caryophyllene	**16.12**	**1.55**	-	-	-	-	-	-
Borneol	**17.19**	**1.18**	-	-	-	-	-	-
Thymol	**21.98**	**46.48**	-	-	-	-	-	-
Carvacrol	**22.39**	**4.61**	-	-	-	-	-	-
α-cubebene	-	-	**17. 11**	**1.94**	-	-	-	-
α- Caryophellene	-	-	**20. 04**	**11.08**	-	-	-	-
β-Caryophellene	-	-	**20. 91**	**10.08**	-	-	-	-
Trans-Caryophellene	-	-	**20. 02**	**5.09**	-	-		
Caryophellene oxide	-	-	**23. 18**	**2.23**	-	-	-	-
Eycolyptol	-	-	**10.98**	**0.53**	-	-	-	-

α-farnesene	-	-	**21.76**	**4.22**	-	-	-	-
Aromadendrene oxide	-	-	**21.25**	**10.11**	-	-	-	-
Eugenol	-	-	**18.83**	**24.00**	-	-	-	-
Acetic acid- phenyl-methyl esters	-	-	**13.34**	**0.72**	-	-	-	-
Eugenol acetate	-	-	**30.97**	**9.61**	-	-	-	-
Benzoic acid	-	-	**14.13**	**2.76**	-	-	-	-
Nonanone	-	-	**11.54**	**1.62**	-	-	-	-
Isoleden	-	-	**21.02**	**1.11**	-	-	-	-
Naphthalene	-	-	**21.67**	**2.12**	-	-	-	-
β -Myrcene	-	-	**9.031**	**1.37**	-	-	-	-
4-carene	-	-	**9.94**	**1.51**	-	-	-	-
Styrene (cymene)	-	-	-	-	**8.31**	**5.37**	-	-
Linalyl acetate	-	-	-	-	**8.53**	**0.83**	-	-
Benzaldehyde phenyl menthanol	-	-	-	-	**10.91**	**16.87**	-	-
Benzaldehyde	-	-	-	-	**11.52**	**0.79**	-	-
1, 8 Cineole	-	-	-	-	**12.78**	**1.52**	**8.76**	**80.13**
Benzaldehyde, 2-hydroxyl	-	-	-	-	**13.51**	**7.37**	-	-
Benzenepropanal	-	-	-	-	**17.18**	**1.14**	-	-
Cinnamaldehyde (E)	-	-	-	-	**21.63**	**14.69**	-	-
Cinnamic alcohol	-	-	-	-	**26.41**	**3.04**	-	-
Trans cinnamyl	-	-	-	-	**27.22**	**0.63**	-	-
α-Cinnamaldehyde	-	-	-	-	**30.32**	**26.11**	-	-
Δ- cadinene	-	-	-	-	**32.14**	**1.97**	-	-
Cinnamaldehyde –O-methoxy	-	-	-	-	**34.53**	**1.21**	-	-
Nerolidol	-	-	-	-	**34.82**	**3.39**	-	-
Spathulenol	-	-	-	-	**35.59**	**3.98**	-	-
Azulene	-	-	-	-	**54.63**	**0.98**	-	-
d-limonene	-	-	-	-	-	-	**8.43**	**0.65**
β-pinene	-	-	-	-	-	-	**7.03**	**0.32**
camphene	-	-	-	-	-	-	**6.45**	**3.47**
Trans -caryophyllene	-	-	-	-	-	-	**25.76**	**0.22**
Camphor	-	-	-	-	-	-	**13.56**	**11.28**
Endo borynyl acetate	-	-	-	-	-	-	**19.45**	**0.27**
1,4 pentadiene	-	-	-	-	-	-	**32.65**	**0.41**
Benzene – 1- methyl	-	-	-	-	-	-	**21.45**	**2.65**

*** RT: Retention time.**

These outcomes are consistent with **Fadel *et al.* (2020)** who reported that eleven volatile compounds were identified in the hydrodistilled EO of cinnamon bark, representing 99.1% of the total oil. Cis- Cinnamaldehyde was noticed the major identified compound (92.7%), in addition to trans-cinnamyl acetate (1.4%), trans-cinnamaldehyde (1.0%) and eugenol (0.8%) were the identified oxygenated compounds whereas the other seven compounds were sesquiterpenes. While, GC/MS characterizations of rosemary volatile oil are presented in **Table 3**, 10 volatile components were identified from rosemary volatile oil could be seen. The identified components represented (99.61 %) from the rosemary volatile oil. 1, 8 Cineole, Camphor, camphene and Benzene – 1- methyl were the most prevalent chemical compounds in rosemary volatile oil, however 1, 8 Cineole constitutes 80.13 % of the total chemical compounds and the highest compound in rosemary volatile oil. However, Benzene – 1- methyl (2.65 %) of the total chemical compounds was the lowest one among the most prevalent elements in volatile rosemary oil. These outcomes are consistent with **Fadel *et al.* (2020)** which reported that analysis of rosemary revealed presence of nineteen compounds representing 98.5% of the total oil. The 1, 8-Cineol was the predominant compound followed by camphor, a-terpineol, a-pinene, bornyl acetate and borneol.

3.3. Antimicrobial activity of nanoparticles and essential oils:

The antibacterial activity of ZnNPs, ChNPs, and essential oils at various concentrations (400 and 600ppm) were tested against *E. coli, S. typhimurium, Staph. aureus*, and *B.cereus*. The obtained results are shown in **Table 4.** Our data showed that thyme had

antibacterial activity against the studied strains of bacteria, with the highest effect against *Staph. aureus*, followed by *B. cereus, S. typhimurium* and *E. coli* compared with clove, cinnamon and rosemary essential oils. On the other hand ZnNPs had antibacterial activity against the strains of bacteria selected, with the highest effect compared with ChNPs. It was reported that ZnNPs had a bactericidal effect on different bacterial strains (**Siddiqi *et al.*, 2018**). Other researchers demonstrated that there is a size-dependent antibacterial effect of ZnNPs (**Raghupathi *et al.*, 2011**). The antibacterial effect of free form essential oils are attributed to several mechanisms. Essential oils interact with lipids in microbial cell and mitochondrial membranes, increase cell permeability, change membrane potential, cause ion loss and collapse of the proton pump, and disturb microbial metabolism leading to lysis and microbial death (**Boskovic *et al.*, 2017 and Burt, 2004).** However, the exact mode of action of nanoencapsulated EOs is still not completely elucidated. It is supposed that nanoencapsulation enhances essential oil activity due to the reduced size, allowing nano- essential oils to interact more efficiently with cell membranes (**Gupta *et al.*, 2016)** by increasing the surface area per unit of mass (**Donsì *et al.*, 2012).** Consequently, lower doses of essential oils can be used, (**Acevedo-Fani *et al.*, 2015).** Apart from the active agent, some carriers used in nanomaterial production also possess antimicrobial activity, change membrane potential, generate reactive oxygen species and affect microbial metabolism(**Álvarez-Paino *et al.*, 2017)** and **Prabuseenivasan *et al.*, 2006)** .Overall, rosemary and thyme were the most effective aromatic essential oils tested. Standards for testing antimicrobial activity of pharmaceutical drugs are codified by Clinical and Laboratory Standards Institute (**Patel *et al.*, 2015).**

Table 4. Diameter of inhibition zones (mm) of nanoparticles and essential oils against some selected bacteria strains.

	Diameter of inhibition zones (mm)											
	Thyme		**Clove**		**Cinnamon**		**Rosemary**		**ZnNPs**		**ChNPs**	
Microorganisms	**400 ppm**	**600 ppm**	**400 ppm**	**600 ppm**	**400 ppm**	**600 ppm**	**400 ppm**	**600 ppm**	**400 ppm**	**600 ppm**	**400 ppm**	**600 ppm**
Gram negative bacteria												
E. coli	**14.00**	**34.00**	**13.00**	**31.00**	**11.50**	**27.00**	**10.50**	**25.00**	**10.00**	**24.50**	**8.50**	**13.50**
S. typhimurium	**15.00**	**39.00**	**14.00**	**33.00**	**12.50**	**31.00**	**11.00**	**27.00**	**13.00**	**26.00**	**9.50**	**15.50**
Gram positive bacteria												
Staph. aureus	**18.50**	**45.00**	**17.50**	**38.00**	**16.00**	**34.00**	**15.00**	**31.00**	**24.00**	**27.50**	**13.00**	**18.50**
B. cereus	**16.50**	**41.00**	**15.50**	**34.00**	**15.00**	**32.00**	**13.50**	**29.00**	**21.00**	**25.00**	**11.50**	**17.50**

ZnNPs: Zinc oxide nanoparticles **ChNPs:** chitosan nanoparticles

4.8.2. Sensory evaluation of beef burger to choose the best percentage adding for essential oils:

Organoleptic evaluation of different beef burger treatments as affected by thyme, clove, cinnamon and rosemary at 400 ppm volatile oils was presented in **Table (5)**. From statistical analysis of these data, it could be noticed that there were significant differences at level 0.05 in all sensory properties between different beef burger treatments. The color score of beef burger prepared with volatile oils were nonsignificant differences at level 0.05 between them. Also, from the same table, it could be observed that the odor scores of different beef burger treatments were significantly at level 0.05 affected by thyme, clove, cinnamon and rosemary volatile oils. The highest or best odor score (8.35) was recorded for sample prepared with thyme 400ppm followed by that prepared with clove 400ppm, and finally control sample without additives (7.35). These results are in agreement with **Dwivedi *et al.* (2006)** evaluated ground beef (15% fat) was treated with a retail 5-spice blend and its individual components cinnamon, clove, fennel, pepper, and star anise at 0.1%, 0.5% and 1%. Statistical analysis of these data showed that, there were significant differences at level 0.05 in taste scores between different beef burger treatments. The highest taste score (8.25) was recorded for beef burger treatment prepared with thyme volatile oils 400 ppm followed by that prepared with clove 400ppm, respectively with no significant differences between them. Also, from the same Table, it could be noticed that, tenderness and juiciness scores of beef burgers were no significantly as affected by the type of additives. Average overall acceptability scores of beef burgers

significantly were affected by addition of thyme, clove, cinnamon and rosemary volatile oils as shown in **Table 5**. From these results, it could be found that, significant differences at level 0.05 were recorded in overall acceptability between different beef burger treatments. The highest overall acceptability score (8.45) was given by panelists for beef burger prepared with thyme followed by prepared with clove, when that prepared with rosemary and prepared with cinnamon and finally both of beef burgers prepared with clove and rosemary. Many researchers have recently worked on the effects of essential oils, when added alone or in combination with other essential oils and/or preservation methods to improve the sensory qualities and extend the shelf life of meat and meat products, (**Du and Li, 2008)**. Generally, addition of thyme and clove at 400 ppm volatile oils to improve the sensory properties of beef burger treatments and we use the same ratio of volatile oil in beef Kofta.

Table 5. Mean values of sensory evaluation scores of beef burger treated with essential oils.

Treatments	*Organoleptic quality*					
	Color (10)	**Odor (10)**	**Taste (10)**	**Tenderness (10)**	**Juiciness (10)**	**Overall acceptability (10)**
Beef burger control	**8.35 ^{a} ±0.263**	**7.35 ^{b} ±0.257**	**7.35 ^{c} ±0.239**	**8.00 ^{a} ±0.313**	**8.05 ^{a} ±0.175**	**7.60 ^{c} ±0.257**
Thyme	**8.55 ^{a} ±0.250**	**8.35 ^{a} ±0.248**	**8.25 ^{a} ±0.267**	**8.40 ^{a} ±0.323**	**8.30 ^{a} ±0.182**	**8.45 ^{a} ±0.266**
Clove	**8.45 ^{a} ±0.250**	**8.30 ^{a} ±0.266**	**8.05 ^{a} ±0.245**	**8.20 ^{a} ±0.188**	**8.35 ^{a} ±0.267**	**8.40 ^{ab} ±0.266**
Cinnamon	**8.30 ^{a} ±0.250**	**7.50 ^{bc} ±0.245**	**7.65 ^{b} ±0.250**	**8.05 ^{a} ±0.250**	**8.25 ^{a} ±0.182**	**8.15 ^{b} ±0.125**
Rosemary	**8.30 ^{a} ±0.263**	**7.85 ^{b} ±0.494**	**7.70 ^{b} ±0.422**	**8.25 ^{a} ±0.205**	**8.15 ^{a} ±0.274**	**8.20 ^{ab} ±0.226**
L.S.D	**0.465**	**0.380**	**0.290**	**0.425**	**0.405**	**0.250**

Where: a,b,c,d in the same column are not significantly different at levels 0.05.

LSD: Least significant differences. (Mean ± S.E.).

3.5. Freshness properties of beef burger and beef Kofta affected by nanoparticles and essential oils during cold storage at 4±1ºC:

3.5.1. Total volatile basic nitrogen of beef burger:

The activity of the endogenous enzymes of the meat as well as the bacteria enzymes produces nitrogenous compounds which are measured in the TVBN test. Therefore, the higher values of TVBN indicate the higher activity of endogenous enzymes as well as bacterial activity that in turn is an indication of the meat spoilage (**Alizadeh-Sani *et al.*, 2020**).

On zero time of cold storage period, the TVBN value of the beef burger samples was ranged from 8.47 to 8.91 mg/100g sample, indicating the good hygienic quality of beef burger. The TVBN values of all samples increased significantly with storage period (**Table 6**), which obeyed a faster trend for the control sample, followed by Ch film/ ZnNPs, Ch film/ thyme, Ch film/ clove, Ch film/ZnNPs/ thyme and Ch film/ ZnNPs/ clove treated , respectively. All beef burger treatments the total volatile nitrogen values were significantly impacted by cold storage times as storage times increased. This could be a result of bacterial breakdown linked to the production of some alkaline substances like ammonia, which was confirmed by the rapid development of total volatile bases nitrogen (**Valipour Kootenaie *et al.* 2017)**. Control sample had significantly higher total volatile nitrogen than any other treatments stored for 3 and 6 days. This may be due to high antimicrobial effect of ZnNPs, thyme and clove essential oil. After 6 th days control sample was not evaluated because it exceeded the permissible limits of TVN and showed off odor, while total volatile nitrogen values 11.65 and 12.13 mg N/100g was observed for beef burger treated with Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove, respectively were ranged of permissible level reported by **ES, (2005a)**. As for all beef burger treatments the total volatile nitrogen values were significantly impacted by cold storage times as storage period increased. Also, at 12th day beef burger with Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove had the lowest total volatile nitrogen 17.68 and 18.80 mg N/100g, these values not exceed the permissible level. These results reflex the strong antimicrobial effect of zinc oxide nanoparticle and thyme and clove essential oil. **Sayadi *et al.* (2022)** and also, **Suo *et al.* (2017)** reported an increase on TVB-N content over cold storage of fresh pork meat protected with CMC film incorporated or not with ZnO NP. These authors also concluded that the presence of nanoparticles reduced the values of TVB-N, and the content recorded for the samples protected with the active films at day 14 was smaller than for the samples coated with control films without ZnO NPs at day 6, in good agreement with

our results. Total volatile base nitrogen value of the meat samples is related to the decomposition of protein compounds to nonprotein nitrogen compounds as a result of bacteria activity and the proteolytic enzymes (**Alizadeh-Sani *et al.*, 2020**), In line with the microbial results. Similar results were obtained by **Alizadeh-Sani *et al.* (2020)** on the addition of rosemary EO and TiO_2 in refrigerated meat and **Sayadi *et al.* (2021)** on turkey meat coated with chitosan containing Berberis vulgaris extract and Mentha pulegium EO under MAP condition.

Table 6. Total volatile basic nitrogen (mg /100 g sample) of beef burger as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef burger treatments*						*LSD*
	Ch film (control)	**Ch film/ ZnNPs**	**Ch film/ thyme**	**Ch film/ clove**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**	
Zero time	**8.91^{Ca} ±0.035**	**8.70^{Da} ±0.011**	**8.61Ea ±0.015**	**8.64Ea ±0.023**	**8.47Ea ±0.017**	**8.56Ea ±0.011**	**0.570**
3	**13.79^{Ba} ±0.017**	**12.83Cb ±0.027**	**11.59Dc ±0.012**	**11.72Dc ±0.030**	**9.48De ±0.026**	**10.53Dd ±0.0233**	**0.650**
6	**20.40^{Aa} ±0.014®**	**16.52Bb ±0.023**	**12.40Cc ±0.020**	**12.65Cc ±0.168**	**11.65Cd ±0.017**	**12.13Ccd ±0.017**	**0.697**
9	®	**21.50Aa ±0.027®**	**16.50Bb ±0.020**	**16.80Bb ±0.023**	**14.52Bd ±0.031**	**15.83Bc ±0.024**	**0.710**
12	®	®	**20.21Aa ±0.021®**	**20.65Aa ±0.017®**	**17.68Ac ±0.023**	**18.80Ab ±0.026**	**0.610**
LSD	**0.660**	**0.570**	**0.530**	**0.550**	**0.580**	**0.540**	

Where: Mean values in the same column (capital letter) or row (small letter) with the same letter are not significantly different at levels 0.05. LSD: Least significant differences ®: Rejected.

3.5.2. Total volatile basic nitrogen of beef Kofta:

The results from **Table 7** showed that TVBN values of Kofta samples containing Ch film/ ZnNPs, Ch film/ thyme, Ch film/ clove, Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove were lower (from from8.90 - 9.47 mg/100g sample) than control sample (9.70 mg/100g), while the samples containing Ch film/ thyme and Ch film/ clove recorded the values from 9.18 to 9.30 mg/100g, respectively at zero time. Also the same table showed that TVBN content of all beef Kofta samples gradually increased during cold storage at 4 ˚C up to 12 days. This increase could be mainly attributed to the effect of microorganisms as well as autolysis processes (**Alizadeh-Sani *et al.*, 2020**). Control sample had significantly higher total volatile nitrogen than beef Kofta samples treated with zinc oxide nanoparticle and thyme and clove essential oil storage for 3 and 6 days. This could be due high antimicrobial effect of nanoparticle and essential oil. After 6 th days control sample was not evaluated because it exceeded the permissible limits of TVN and showed off odor, while total volatile nitrogen values 12.30 mg N/100g was observed for beef Kofta treated with Ch film/ ZnNPs/ thyme were range of permissible level reported by **ES, (2005b)**. All beef Kofta treatments' total volatile nitrogen values were significantly impacted by cold storage times as storage times increased. Also, at 12th day beef Kofta with Ch film/ ZnNPs/ thyme had the lowest total volatile nitrogen 17.68 mg N/100g, these values not exceed the permissible level. These results reflex the strong antimicrobial effect of nanoparticle and essential oil, **(Sayadi *et al.* (2022); Suo *et al.* (2017) and Alizadeh-Sani *et al.* 2020).**

Table 7. Total volatile basic nitrogen (mg /100 g sample) of beef Kofta as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef Kofta treatments*						*LSD*
	Ch film (control)	**Ch film/ ZnNPs**	**Ch film/ thyme**	**Ch film/ clove**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**	
Zero time	**9.70Ca ±0.028**	**9.47Dab ±0.014**	**9.18Eab ±0.023**	**9.30Eab ±0.023**	**8.90Eb ±0.026**	**8.94Eb ±0.023**	**0.650**
3	**14.49Ba ±0.026**	**13.59Cb ±0.020**	**12.85Db ±0.028**	**12.95Db ±0.027**	**10.59Dc ±0.014**	**10.92Dc ±0.651**	**0.740**
6	**22.96Aa ±0.014®**	**17.57Bb ±0.020**	**16.18Cc ±0.020**	**16.64Cc ±0.023**	**12.30Ce ±0.026**	**13.00Cd ±0.031**	**0.640**
9	®	**21.99Aa ±0.009®**	**18.70Bc ±0.028**	**19.11Bb ±0.037**	**15.60Bd ±0.028**	**16.39Bd ±0.023**	**0.790**
12	®	®	**21.05Aa ±0.025®**	**21.25Aa ±0.017®**	**18.70Ab ±0.029**	**19.29Ab ±0.020**	**0.650**
LSD	**0.680**	**0.610**	**0.580**	**0.620**	**0.640**	**0.660**	

Where: Mean values in the same column (capital letter) or row (small letter) with the same letter are not significantly different at levels 0.05. LSD: Least significant differences ®: Rejected.

3.5.3. Thiobarbituric acid of beef burger:

In the TBA test, MDA as an aldehyde compound is measured. Aldehydes are the secondary products of lipid oxidation, and their increase is an indication of lipid rancidity (**Heydari-Majd *et al.*, 2019**).

TBA values of beef burger as affected by type of Ch film/ thyme, Ch film/ clove, Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove during cold storage at 4 ˚C up to 12 days are presented in **Table 8**. It could be noticed that significant ($P < 0.05$) differences were recorded in TBA values among the tested samples either at zero time or throughout of cold storage periods. Whereas, TBA values of beef burger samples containing different Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove ranged from0.210 to 0.254 mg malonaldehyde/kg, respectively. While, TBA contents of control sample was 0.300 mg malonaldehyde/kg sample at zero time. These results are in line with those obtained by **Sayadi *et al.* (2022)** who reported that the active films significantly induced the reduction in lipid oxidation, microbial growth, and TVBN values, improved the sensory attributes of treated samples, maintained the redness of meats for a longer time, and increased the shelf life of beef from 4 to 16 days. Beef burger with Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove sample has significantly lower TBA value than control sample at any time of storage 3 and 6 days. This might be due to the antioxidant effect of ZnNPs and essential oil. At 6th day of cold storage, the highest TBA value was 0.961 mg malonaldehyde / kg for control sample. Similar results were obtained by other researchers on turkey breast meat coated with chitosan containing 1% CEO (**Taheri *et al.*, 2018**), O,ruber fillets packaged with nanocomposite film based on PLA/zinc oxide nanoparticle/ZEO and MEO (**HeydariMajd *et al.*, 2019**), fresh pork and meat loins coated with alginate based film produced with turmeric (**Bojorges *et al.*, 2020**), and beef wrapped with citric acid, corn starch, and linear LDPE active films (**Júnior *et al.*, 2015**). Also, at the end of cold storage at the 12th day, TBA values of beef burger with Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove sample was 0.740 and 0.763 mg malonaldehyde / kg sample. TBA value of beef burger treated with ZnNPs and essential oil not exceed the range of permissible level reported by the **ES, (2005a)**, being not more than 0.9 mg malonaldehyde / kg sample. Also, **Table 8** indicated that TBA values of all tested samples gradually increased during cold storage up to 12 days.

Table 8. Thiobarbituric acid values (mg malonaldehyde/kg) of beef burger as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef burger treatments*						*LSD*
	Ch film (control)	**Ch film/ ZnNPs**	**Ch film/ thyme**	**Ch film/ clove**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**	
Zero time	**0.300Ca ±0.002**	**0.295Da ±0.002**	**0.266Ea ±0.002**	**0.278Ea ±0.153**	**0.210Ea ±0.002**	**0.254Ea ±0.002**	**0.174**
3	**0.607Ba ±0.003**	**0.490Cab ±0.164**	**0.379Db ±0.003**	**0.420Dab ±0.002**	**0.321Db ±0.010**	**0.358Db ±0.003**	**0.187**
6	**0.961Aa ±0.002®**	**0.610Bb ±0.002**	**0.525Cc ±0.002**	**0.549Cbc ±0.002**	**0.456Cd ±0.003**	**0.479Ccd ±0.003**	**0.067**
9	®	**0.952Aa ±0.003®**	**0.730Bbc ±0.002**	**0.751Bb ±0.001**	**0.659Bc ±0.003**	**0.690Bbc ±0.002**	**0.078**
12	®	®	**0.905Aa ±0.001®**	**0.920Aa ±0.002®**	**0.740Ab ±0.003**	**0.763Ab ±0.002**	**0.067**
LSD	**0.006**	**0.007**	**0.008**	**0.009**	**0.006**	**0.007**	

Where: Mean values in the same column (capital letter) or row (small letter) with the same letter are not significantly different at levels 0.05. LSD: Least significant differences ®: Rejected.

3.5.4. Thiobarbituric acid of beef Kofta:

TBA values of beef Kofta as affected by type of Ch film/ thyme, Ch film/ clove, Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove during cold storage at 4 ˚C up to 12 days are presented in **Table 9**. It could be noticed that significant ($P < 0.05$) differences were recorded in TBA values among the tested samples either at zero time or throughout of cold storage periods. Whereas, TBA values of beef Kofta samples containing different Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove ranged from0.251 to 0.333 mg malonaldehyde/kg respectively sample. While, TBA contents than control sample was 0.333 mg malonaldehyde/kg sample at zero time. These results are in line with those obtained by **Sayadi *et al.* (2022)**. beef Kofta with Ch film/ ZnNPs/ thyme and Ch film/ZnNPs/ clove sample has significantly lower TBA value than control sample at any time of storage 3 and 6 days. This might be due to the antioxidant effect of ZnNPs and essential oil. At 6th day of cold storage, the highest TBA value was 1.030 mg malonaldehyde / kg for control sample. Similar results were obtained by other researchers on turkey breast meat coated with chitosan containing 1% CEO (**Taheri *et al.*, 2018**; **HeydariMajd *et al.*, 2019** and **Bojorges *et al.*, 2020**). Also, at the end of cold storage at the 12th day, TBA values of beef Kofta with Ch film/ ZnNPs/

thyme and Ch film/ ZnNPs/ clove sample was 0.875 and 0.890 mg malonaldehyde / kg sample. TBA value of beef Kofta with ZnNPs and essential oil not exceed the range of permissible level reported by **ES, (2005b)**, being not more than 0.9 mg malonaldehyde / kg sample. Also, **Table 9** indicated that TBA values of all tested samples gradually increased during cold storage up to 12 days.

Table 9. Thiobarbituric acid values (mg malonaldehyde/kg) of beef Kofta as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef Kofta treatments*						*LSD*
	Ch film (control)	**Ch film/ ZnNPs**	**Ch film/ thyme**	**Ch film/ clove**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**	
Zero time	**0.333Ca ±0.011**	**0.321Da ±0.043**	**0.295Ea ±0.026**	**0.307Ea ±0.049**	**0.251Ea ±0.015**	**0.260Ea ±0.020**	**0.149**
3	**0.650Ba ±0.030**	**0.521Cab ±0.043**	**0.481Db ±0.030**	**0.499Db ±0.011**	**0.450Db ±0.043**	**0.462Db ±0.026**	**0.146**
6	**1.030Aa ±0.049®**	**0.837Bb ±0.015**	**0.736Cc ±0.020**	**0.750Cbc ±0.040**	**0.597Cd ±0.025**	**0.616Cd ±0.020**	**0.060**
9	**®**	**0.961Aa 0.026±®**	**0.855Bb ±0.014**	**0.879Bb ±0.012**	**0.709Bc ±0.023**	**0.724Bc ±0.020**	**0.073**
12	**®**	**®**	**0.910Aab ±0.018®**	**0.934Aa ±0.012®**	**0.875Ab ±0.016**	**0.890Aab ±0.015**	**0.055**
LSD	**0.007**	**0.004**	**0.007**	**0.005**	**0.008**	**0.009**	

Where: Mean values in the same column (capital letter) or row (small letter) with the same letter are not significantly different at levels 0.05. LSD: Least significant differences ®: Rejected.

3.5.5. pH value of beef burger:

Data presented in **Table 10** shows the changes in pH values of beef burger samples as affected by Ch film/ ZnNPs, Ch film/ thyme, Ch film/ clove, Chfilm/ZnNPs/ thyme, Ch film/ ZnNPs/ clove treated during cold storage at 4 ˚C up to 12 days. From these results, it could be noticed that significant differences were recorded in pH values among the samples at zero time or throughout of cold storage periods. pH values of all beef burger at zero time were ranged from 5.63 to 5.80, respectively. As shown in **Table 10** , the addition of ZnNPs, thyme and clove at 400ppm essential oils into burger formulations resulted in a slight increase in pH values of tested samples when compared with that of control samples after 6 days. **Afshar Mehrabi *et al.* (2021)** showed that, over time, pH values of the samples increased slightly during all days of storage ($p < .05$). It is worth mentioning, that pH values of beef burger slightly increased by increasing during cold storage at 4 ˚C up to 12 days, this might be due to this rising trend is in line with the report of **Hassanzadeh *et al.* (2018)**. After 12th days of cold storage at 4±1ºC, there were significant differences ($p \leq 0.05$) in pH values between the control and beef burger with ZnNPs, while at the end of cold storage the pH value (5.96) was recorded for beef burger sample with Ch film/ZnNPs/ thyme. These results reflection the strong antimicrobial effect of ZnNPs.

Table 10. pH values of beef burger as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef burger treatments*						*LSD*
	Ch film (control)	**Ch film/ ZnNPs**	**Ch film/ thyme**	**Ch film/ clove**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**	
Zero time	**5.80Ca ±0.466**	**5.75Ca ±0.503**	**5.70Ca ±0.230**	**5.72Ca ±0.466**	**5.63Ba ±0.655**	**5.67Ba ±0.416**	**0.345**
3	**6.30Ba ±0.208**	**6.05Bab ±0.642**	**5.78Cb ±0.466**	**5.97Cb ±0.378**	**5.68Bb ±0.305**	**5.72Bb ±0.466**	**0.308**
6	**6.85Aa ±0.264®**	**6.49Ab ±0.667**	**5.96BCc ±0.243**	**6.20BCc ±0.505**	**5.72ABc ±0.615**	**5.79Bc ±0.667**	**0.272**
9	**®**	**6.73Aa ±0.594®**	**6.10Bb ±0.575**	**6.30Bb ±0.214**	**5.82ABb ±0.212**	**5.88ABb ±0.323**	**0.393**
12	**®**	**®**	**6.54Aa ±0.318®**	**6.65Aa ±0.32®**	**5.96Ab ±0.323**	**6.04Ab ±0.412**	**0.360**
LSD	**0.378**	**0.301**	**0.295**	**0.313**	**0.192**	**0.219**	

Where: Mean values in the same column (capital letter) or row (small letter) with the same letter are not significantly different at levels 0.05. LSD: Least significant differences ®: Rejected.

3.5.6. pH value of beef Kofta:

Data presented in **Table 11** shows the changes in pH values of beef Kofta samples as affected by Ch film/ ZnNPs, Ch film/ thyme, Ch film/ clove, Ch film/ZnNPs/ thyme and Ch film/ ZnNPs/ clove treated during cold storage at 4 ˚C up to 12 days. From these results, it could be noticed that significant differences were recorded in pH values among beef burger samples at zero time or throughout of cold storage periods. pH values of all beef Kofta at zero time were ranged from 6.04 to 6.28, respectively. As shown in **Table 11** , the addition of ZnNPs, thyme and clove at 400ppm essential oils into burger formulations resulted in a slight increase in pH values of tested samples when compared with that of control samples after 6 days. **Afshar Mehrabi *et al.* (2021)**. It is worth mentioning, that pH values of beef Kofta slightly increased by increasing during cold storage at 4 ˚C up to 12 days, this might be due to this rising trend is in line with the report of **Hassanzadeh *et al.* (2018)** and after 12th days of cold storage at 4±1ºC, there were significant differences ($p \leq 0.05$) in pH values between the treated samples and that beef burger treated with ZnNPs, while the end of cold storage, the pH value (6.55) was recorded for beef Kofta sample with Ch film/ZnNPs/ thyme. These results reflection the strong antimicrobial effect of ZnNPs.

Table 11. pH values of beef Kofta as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef Kofta treatments*						*LSD*
	Ch film (control)	**Ch film/ ZnNPs**	**Ch film/ thyme**	**Ch film/ clove**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**	
Zero time	**6.28Ca ±0.312**	**6.20Ba ±0.419**	**6.12Ca ±0.353**	**6.13Ca ±0.342**	**6.04Ca ±0.333**	**6.10Ca ±0.359**	**0.309**
3	**6.59Ba ±0.319**	**6.46Ba ±0.267**	**6.35Bab ±0.233**	**6.41Ba ±0.267**	**6.12BCb ±0.389**	**6.15Cb ±0.288**	**0.259**
6	**6.90Aa ±0.267®**	**6.65ABab ±0.394**	**6.50Bb ±0.388**	**6.60ABab ±0.489**	**6.23Bb ±0.404**	**6.41Bb ±0.358**	**0.376**
9	**®**	**6.78Aa ±0.337®**	**6.62ABab ±0.378**	**6.71Aa ±0.351**	**6.39ABb ±0.351**	**6.48ABb ±0.435**	**0.201**
12	**®**	**®**	**6.79Aa ±0.214®**	**6.82Aa ±0.173®**	**6.55Ab ±0.205**	**6.58Ab ±0.145**	**0.200**
LSD	**0.253**	**0.263**	**0.217**	**0.238**	**0.177**	**0.168**	

Where: Mean values in the same column (capital letter) or row (small letter) with the same letter are not significantly different at levels 0.05. LSD: Least significant differences ®: Rejected.

3.4. Microbiological quality attributes of treated beef burger and beef Kofta with nanoparticles and essential oils during cold storage at 4±1°C:

Thereafter, the use of natural antimicrobial compounds in foods has gained much attention by the consumers and the food industry. This is due primarily to two major factors: First, the misuse and mishandling of antibiotics has resulted in the dramatic rise of a group of microorganisms including food borne pathogens that are not only antibiotic resistant but also more tolerant to several food processing and preservation methods. Second, increasing consumers' awareness of the potential negative impact of synthetic preservatives on health versus the benefits of natural additives has generated interest among researchers in the development and use of natural products in foods. This has prompted the food industry to look for alternative preservatives that can enhance the safety and quality of foods (**Gyawali and Ibrahim, 2014).**

3.4.1. Total bacterial count (cfu/g) of beef burger:

Total bacterial count (TBC) of any food product is significantly correlated directly with the sanitary conditions of processing, handling and storage. From the obtained results in **Table 12,** it could be noticed that, total bacterial counts of beef burgers were influenced by chitosan film with ZnNPs 400ppm, chitosan film with thyme 400ppm, chitosan film with clove 400ppm and chitosan film with ZnNPs with thyme and clove at 400ppm during cold storage period at 4˚C up to the 12 days. Total bacterial count of all beef burger treatments was ranged from 2.80×10^3 and 7.15×10^3 cfu/g at zero time. Control sample had higher total bacterial count (7.15×10^3 cfu/g) while, the lowest total bacterial count at zero time (2.80×10^3 cfu/g) was recorded for beef burger sample treated with chitosan film with ZnNPs with thyme 400ppm as compared with other beef burger samples which ranged from (3.70×10^3 to 5.15×10^3 cfu/g). On the other hand, the increase in total bacterial counts after the 3 day related to that bacteria already changes in some properties of beef burgers such as increase simple nitrogen compounds (amino acids and nucleoids) and fatty acids which were produced by hydrolysis of protein and fat during

storage by natural meat enzymes which consequently leads to suitable conditions for bacterial growth (**Abdou *et al.*, 2012**). At the 6 th days of cold storage period, the lowest total bacterial count was recorded for beef burger samples with chitosan film with ZnNPs with thyme 400ppm and chitosan film with ZnNPs with clove at 400ppm (1.09×10^4 and 2.75×10^4cfu/g, respectively) compared to the control (1.25×10^6 cfu/g), chitosan film control sample was rejected because they had counts that were greater than 10^6 cfu/g, The similar trend to those reported by **Ghaderi-Ghahfarokhi *et al.* (2016)** evaluated all samples treated with thyme essential oil significantly reduced the population of investigated microbial counts ($P< 0.05$) compared to the control during 8 days of storage. At the end of storage period, E-0.05-thyme essential oil and E-0.1-thyme essential oil , presented, respectively, 2.2 and 3 log cycles reduction of Enterobacteriaceae, along with 3.1 and 3.7 log cycles reduction of Staphylococcus aureus. **Ghaderi-Ghahfarokhi *et al.* (2017)** evaluated samples containing free and encapsulated cinnamon essential oil and ascorbic acid were analyzed for microbial growth during 8 days of storage at 4 ˚C. Both free and encapsulated cinnamon essential oil decreased the microbial population of patties compared to the control ($p < 0.05$) throughout the experiment. Also, the high bactericidal activity of ChNPs is certainly due to a change in cell permeability barrier due to interactions between the positively charged chitosan and the negatively charged microbial cell membranes as reported by **Duan *et al.*, (2019)**. Also, from the same table, it could be noticed that, total bacterial count of all beef burger treatments were also affected by cold storage period. The total bacterial counts were increased in all beef burger treatments up to the 12 th of cold storage, after that, total bacterial count of all treatments were tended to increase but with various numbers depending on the type of chitosan film with ZnNPs and thyme and clove at 400ppm. During cold storage, all beef burger Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove treatments were lower in total bacterial counts than the permissible limit of **ES, (2005a)** which stated that, total bacterial count in frozen meat should not exceed (10^6 cell/g sample), except control sample which except control sample which exceed this limit after the 6 days of cold storage (10^6 cfu/g), consequently this sample was rejected.

Table 12. Total bacterial counts (cfu/g) of beef burger as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef burger treatments*					
	Ch film (control)	**Ch film/ ZnNPs 400ppm**	**Ch film/ thyme 400ppm**	**Ch film/ Clove 400ppm**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**
Zero time	**7.15×10^3**	**5.15×10^3**	**3.70×10^3**	**4.05×10^3**	**2.80×10^3**	**3.50×10^3**
3	**4.35×10^5**	**3.95×10^4**	**8.90×10^3**	**1.15×10^4**	**6.65×10^3**	**7.90×10^3**
6	**1.25×10^6 ®**	**2.75×10^5**	**3.95×10^4**	**4.35×10^4**	**1.09×10^4**	**2.75×10^4**
9	®	**1.05×10^6 ®**	**2.20×10^5**	**3.65×10^5**	**7.40×10^4**	**9.65×10^4**
12	®	®	**1.05×10^6 ®**	**1.30×10^6 ®**	**3.75×10^5**	**4.20×10^5**

Where: ®: Rejected (cfu/ g): colony forming unit /gram

3.4.2. Total bacterial count (cfu/g) of beef Kofta:

From the obtained results in **Table 13**, it could be noticed that, total bacterial counts of beef Kofta were influenced by chitosan film with ZnNPs 400ppm, chitosan film with thyme 400ppm, chitosan film with clove 400ppm and chitosan film with ZnNPs with thyme and clove at 400ppm during cold storage period at 4˚C up to the 12 days. Total bacterial count of all beef Kofta treatments ranged from 4.85×10^3 and 8.25×10^3 cfu/g at zero time. beef Kofta with chitosan film control sample had higher total bacterial count (8.25×10^3cfu/g) while, the lowest total bacterial count at zero time (4.85×10^3 cfu/g) was recorded for beef Kofta sample treated with chitosan film with ZnNPs with thyme 400ppm as compared with other beef Kofta samples which ranged from (5.50×10^3 to 7.90×10^3 cfu/g). On the other hand, the increase in total bacterial counts after the 3 day related to that bacteria already changes in some properties of beef Kofta such as increase simple nitrogen compounds

(amino acids and nucleoids) and fatty acids which were produced by hydrolysis of protein and fat during storage by natural meat enzymes which consequently leads to suitable conditions for bacterial growth (**Abdou *et al.*, 2012**). At the 6 th days of cold storage period, the lowest total bacterial count was recorded for beef Kofta samples with chitosan film with ZnNPs with thyme 400ppm and chitosan film with ZnNPs with clove at 400ppm (3.85×10^4 and4.85×10^4 cfu/g ,respectively) compared to the control (1.95×10^6 cfu/g), beef Kofta samples with chitosan film control sample was rejected because they had counts that were greater than 106 cfu/g, The similar trend to those reported by (**Priyadarshi *et al.* 2021)** showed that the addition of ZnONPs increased the mechanical and water vapor barrier properties with potent antibacterial activity against foodborne pathogens, E. coli, and L. monocytogenes. Therefore, the CMC/ZnO3%/GSE film can be used as a sustainable material in active packaging applications of high-fat meat products such as beef. Also, from the same table, it could be noticed that, total bacterial count of all beef Kofta treatments were also affected by cold storage period. The total bacterial counts were increased in all beef Kofta treatments up to the 12 th of cold storage, after that, total bacterial count of all treatments were tended to increase but with various numbers depending on the type of chitosan film with ZnNPs and thyme and clove at 400ppm. During cold storage, all beef burger Ch film/ ZnNPs/ thyme and Ch film/ ZnNPs/ clove treatments were lower in total bacterial counts than the permissible limit of **ES, (2005b)** which stated that, total bacterial count in frozen balls should not exceed (10^6 cell/g sample), except control sample which except control sample which exceed this limit after the 6 days of cold storage (10^6 cfu/g), consequently this sample was rejected.

Table 13. Total bacterial counts (cfu/g) of beef Kofta as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef Kofta treatments*					
	Ch film (control)	**Ch film/ ZnNPs 400ppm**	**Ch film/ thyme 400ppm**	**Ch film/ Clove**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/**
Zero time	**8.25×10^3**	**7.90×10^3**	**6.75×10^3**	**7.25×10^3**	**4.85×10^3**	**5.50×10^3**
3	**6.40×10^5**	**5.50×10^4**	**1.25×10^4**	**3.40×10^4**	**7.45×10^3**	**9.40×10^3**
6	**1.95×10^6 ®**	**2.35×10^5**	**5.25×10^4**	**8.10×10^4**	**3.85×10^4**	**4.85×10^4**
9	®	**1.15×10^6 ®**	**4.45×10^5**	**6.15×10^5**	**8.60×10^4**	**1.05×10^5**
12	®	®	**1.50×10^6 ®**	**1.95×10^6 ®**	**4.20×10^5**	**6.05×10^5**

Where: ®: Rejected (cfu/ g): colony forming unit /gram

3.4.3. Coliform group count (cfu/g) of beef burger:

Since coliforms are regarded as an indicator for the evaluation of the hygienic conditions, the coliform group counts were determined in all beef burger treatments under investigation as part of cold storage at 4^{o}C and the results are shown in **Table 14**. No detected of coliform group in first storage due to hygiene and sanitation during processing samples in laboratory. The data indicated that, beef burger control counts were 7.00×10 and 1.1×10^2 cfu/g after 3 and 6 days, respectively. Whenever, in the treatment beef burger with Ch film/ZnNPs/ thyme and Ch film/ ZnNPs/ clove during over all cold storage period showed no detected of coliform group as compared to beef burger coated Ch film/ ZnNPs 400ppm , Ch film/ thyme 400ppm and Ch film/clove 400ppm duo to effect of antimicrobial for zinc nanoparticle and essential oils. It exceeded the maximal permissible limit of 10^2 cfu / gm for the coliform bacterial count (**ES, 2005a**).

Table 14. Coliform group counts (cfu/g) of beef burger as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef burger treatments*					
	Ch film (control)	**Ch film/ ZnNPs 400ppm**	**Ch film/ thyme 400ppm**	**Ch film/ Clove 400ppm**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**
Zero time	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
3	**7×10**	**ND**	**ND**	**ND**	**ND**	**ND**
6	**1.1×10^2 ®**	**3×10**	**ND**	**ND**	**ND**	**ND**
9	®	**5×10 ®**	**ND**	**1×10**	**ND**	**ND**
12	®	®	**2×10 ®**	**6×10 ®**	**ND**	**ND**

Where: ®: Rejected (cfu/ g): colony forming unit /gram

3.4.4. Coliform group count (cfu/g) of beef Kofta:

The coliform group counts were determined in all beef Kofta treatments under investigation as part of cold storage at 4±1°C and the results are shown in **Table 15**. No detected of coliform group in first storage due to hygiene and sanitation during processing samples in laboratory. The data indicated that, beef Kofta control counts were 9×10 and 1.3×10^2 cfu/g after 3 and 6 days, respectively. Whenever, the all treatments beef Kofta with Ch film/ZnNPs/ thyme and Ch film/ ZnNPs/ clove during over all cold storage period were no detected as compared to other treatments duo to effect of antimicrobial for zinc nanoparticle and essential oils . It exceeded the maximal permissible limit of 10^2 cfu / gm for the coliform bacterial count (**ES, 2005b).**

Table 15. Coliform group counts (cfu/g) of beef Kofta as affected by nanoparticles and essential oils treatments and cold storage periods at 4±1°C.

Storage time (days)	*Beef Kofta treatments*					
	Ch film (control)	**Ch film/ ZnNPs 400ppm**	**Ch film/ thyme 400ppm**	**Ch film/ Clove 400ppm**	**Ch film/ ZnNPs/ thyme**	**Ch film/ ZnNPs/ clove**
Zero time	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
3	**9×10**	**ND**	**ND**	**ND**	**ND**	**ND**
6	**1.3×10^2 ®**	**5×10**	**ND**	**ND**	**ND**	**ND**
9	**®**	**7×10 ®**	**ND**	**3×10**	**ND**	**ND**
12	**®**	**®**	**3×10 ®**	**7×10 ®**	**ND**	**ND**

Where: ®: Rejected (cfu/ g): colony forming unit /gram

Conclusions

In this study, nanocomposite chitosan - based films containing ZnNPs nanoparticles and essential oils (as antioxidant and antimicrobial agents), alone or in combination, were fabricated. The produced active packaging films could remarkably reduce the lipid oxidation and microbial spoilage, and increase the shelf life of fresh beef burger and beef Kofta. In this regard, the combination use of ZnNPs with essential oils resulted in better results compared to the sole use of ZnNPs and essential oils. The obtained data also showed that the use of Ch film/ ZnNPs/ thyme or Ch film/ ZnNPs/ clove as a novel nanocomposite film is greatly beneficial in preserving the quality parameters of fresh beef burger and beef Kofta.

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تأثير بعض الزيوت العطرية والجسيمات النانوية فى أفلام الشيتوزان على التحكم فى بعض البكتيريا المرضية والحفاظ على جودة تخزين بعض منتجات اللحوم

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أجريت هذه الدراسة بهدف الإستفادة من أغلفة الشيتوزان المحتوية على جزيئات أكسيد الزنك النانو و زيوت الزعتر والقرنفل العطرية كمضادة نمو للميكروبات. حيث تم دراسة التركيب الكيميائى للزيوت العطرية باستخدام جهاز GC/MS وتم تقدير التأثير المضاد لنمو الميكروبات لأكسيد الزنك والشيتوزان النانو و الزيوت العطرية موضع الدراسة. و كذلك دراسة تأثير إضافة زيت الزعتر والقرنفل العطرى بتركيز ٤٠٠ جزء فى المليون وجزيئات أكسيد الزنك النانو فى غلاف الشيتوزان على الجودة الميكروبيولوجية (العدد الكلي للبكتيريا و مجموعة القولون) والجودة الكيميائية (النيتروجين الكلى المتطاير (TVN) وحمض الثيوباربيتوريك (TBA) وpH) في برجر وكفته اللحم البقرى بغرض إطالة مدة الحفظ لهما أثناء التخزين بالتبريد على ٤ ± ١ °م . أوضحت النتائج أن المكون الرئيسى فى زيت الزعتر كان الثيمول (46.48٪) بينما المكون الرئيسى فى زيت القرنفل هو الأوجينول يمثل (24.00٪) ، يمكن ترتيب النشاط المضاد لنمو الميكروبات على النحو التالي: زيت الزعتر> زيت القرنفل> جزيئات أكسيد الزنك النانوية ثم جزيئات الشيتوزان النانوية. أظهرت النتائج لصفات الجودة الكيميائية أن معدل الزيادة في TVN و TBA و pH لجميع العينات المعاملة بفيلم الشيتوزان مع ZnNPs / الزعتر و ZnNPs / القرنفل مقارنة بعينة الكنترول أثناء التخزين المبرد في برجر وكفتة اللحم البقري. كذلك أظهرت نتائج الجودة الميكروبية انخفاضًا في العد الكلى للبكتيريا في العينات المعاملة بفيلم الشيتوزان مع ZnNPs / الزعتر و ZnNPs / القرنفل مقارنة بعينة الكنترول أثناء التخزين المبرد.و أخيرا يمكن التوصية باستخدام فيلم الشيتوزان مع ZnNPs / الزعتر و ZnNPs / القرنفل تجاريا في صناعة منتجات اللحوم مثل البرجر وكفتة اللحم البقري كمضادات لنمو الميكروبات طبيعة حيث أدت إلى زيادة فترة الصلاحية لهذا المنتجات لتصل إلى اليوم الثانى عشر مقارنة بمعاملة الكنترول التى فسدت فى اليوم السادس على درجة التبريد.

الكلمات الدالة : مضادات لنمو الميكروبات ، جزيئات الزنك النانوية ، برجر وكفته اللحم، زيت القرنفل ، زيت الزعتر ، أغلفة الشيتوزان.