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Original article

EFFICIENCY OF CHITOSAN-BASED EDIBLE FILMS LOADED WITH NANO-EMULSION ESSENTIAL OILS AGAINST COAGULASE POSITIVE *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CHICKEN MEAT

W. M. Elsherif[1], H. K. Abdel-Aall[2] & N. M. Abdel-Aziz[3]

[1]Certified Food Lab, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt; [2]Food Hygiene Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt; [3]Food Hygiene Department, Faculty of Veterinary Medicine, Sohag University, Egypt

Summary

Elsherif, W. M., H. K. Abdel-Aall & N. M. Abdel-Aziz, 2022. Efficiency of chitosan-based edible films loaded with nano-emulsion essential oils against coagulase positive *Staphylococcus aureus* isolated from chicken meat. *Bulg. J. Vet. Med.* (online first).

To decrease the incidence of coagulase positive *Staphylococcus aureus* (CPSA) in chicken meat, chitosan-based films incorporated with carvacrol nano-emulsion (Ch-CNE) and rosemary nano-emulsion (Ch-RNE) were used as an ideal solution to build effective antibacterial food packaging. CPSA was isolated from fresh and frozen chicken meat by using selective media. The prepared nano-emulsions were characterised using a zeta-sizer, Fourier-transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM) and investigated for anti-CPSA activity by the agar diffusion method. The filmogenic mixture was prepared at 0.78% and 1.56% concentrations and then cast, dried, and assessed for physical and mechanical properties. CPSA was isolated from fresh and frozen chicken meat in percentages of 40% and 12%, respectively. The droplet sizes of the CNE and RNE were 54.56 and 44.98 nm, respectively, whereas those by TEM were spherically shaped with average sizes of 40.33 and 48.78 nm and polydispersity indices (PDI) of 0.32 and 0.21, respectively. The minimum inhibitory concentrations of both nano-emulsions against CPSA were 0.78% and 1.56%. Incorporated nano-emulsions with chitosan-based films did not cause a great change in the film appearance and transparency except for Ch-CNE films at 1.56%, which was significantly different in comparison with the control, and enhanced the light barrier property. Additionally, it caused significantly improved changes to the film including physical (water resistance and water vapour permeability) and mechanical (tensile strength and elongation at break) properties and significantly eradicated the CPSA inoculated in chicken meat (6 $\log_{10}$ CFU/cm^2) on the 4th day of refrigerated storage (4±1 °C) with good organoleptic properties for 12 days. The Ch-CNE at concentration 1.56% could be considered a promising antimicrobial food packaging material with considerable beneficial packaging properties, substantial inhibition of foodborne pathogen growth, and extension of food shelf life.

Key words: active food packaging, carvacrol nano-emulsion, FTIR, rosemary nano-emulsion, physical and mechanical film parameters, TEM

INTRODUCTION

Chicken meat could be contaminated by coagulase-positive *Staphylococcus aureus* (CPSA) during storage, manipulation, and transportation, or from chickens known as reservoir (Okorie-Kanu *et al*., 2020). CPSA is an opportunistic, foodborne, toxic pathogen and is the third largest cause of food-related illness worldwide (Parvin *et al.*, 2021). The Centers for Disease Control and Prevention recorded that 48 million people get sick, 128,000 are hospitalised, and 3000 die every year because of foodborne diseases (Soltaninezhad *et al*., 2020).

Biopolymers (such as chitosan) have been studied extensively over the last two decades because of their potential to be a viable solution for the disposal of waste plastic food packaging materials. Furthermore, biopolymer films are excellent vehicles for incorporating a wide variety of additives, such as antioxidants, antifungal agents, antimicrobials, dyes and other nutrients; thus, these biodegradable materials can also improve food quality and extend shelf life by minimising microbial growth in the product (Dhankhar *et al*., 2021). Chitosan is considered an environmentally friendly polymer because of its impressive film-forming properties and ability to produce elastic transparent films with biodegradable, non-toxic, and nonallergic properties (Eldaly *et al*., 2018).

Due to their antibacterial and antioxidant activities, essential oils (EOs), especially carvacrol and rosemary, are considered the most powerful natural compounds with antimicrobial activity that improve food quality as they contain bioactive phenolic compounds such as flavonoids, terpenes, and carotenes. However, they are thermolabile and volatile, have a strong aroma, and are unstable because of natural fluctuations (Elshamy *et al*., 2021). To circumvent these disadvantages, EOs were converted to nanoemulsions, which are oil-in-water emulsions with droplet sizes less than 100 nm that have been recognised as a transmission channel for bioactive compounds created by nanotechnology with unique features such as transparency, stability, and high performance (Zambrano-Zaragoza *et al*., 2018). The carvacrol (CNE) and rosemary (RNE) nano-emulsions are considered a new opportunity against CPSA. Their antimicrobial efficacy returned to the active free oxygen atom syndrome; also, they are able to penetrate the bacterial cell wall and disturbing the permeability of the bacterial cell (Garavito *et al*., 2020; Abbasi *et al*., 2021). At the same time, they do not cause significant physical and mechanical modification of parameters during the packaging and manufacturing of the food products (Elshamy *et al*., 2021).

An effective, biocompatible food packaging film is regarded as an innovative and renewable key to maintaining food quality and safety; indeed, packaging can restrict undesired factors in the food industry (Abdollahi *et al*., 2012; Zambrano-Zaragoza *et al*., 2018). Additionally, such nanocomposite films based on natural biopolymers are environmentally friendly with all benefits one may expect from biopolymer and nanocomposite packaging materials (Napoli *et al*., 2020). Therefore, there is a good reason to incorporate the chitosan-based filmogenic mixture with nano-emulsions of EOs.

The present study aimed to detect the presence of CPSA in chicken meat samples and study the effect of CNE and RNE on the isolated strains. The study also aimed to investigate the physical and mechanical properties of chitosan-based

films with CNE and RNE within the filmogenic mixture, as well as their anti-CPSA activity in inoculated chicken meat during refrigerated storage.

MATERIALS AND METHODS

Materials

Baird-Parker agar base (REF: 4011162), egg yolk emulsion 50% (REF: 42111605; LOT: 3MH093), and coagulase plasma EDTA (REF: 429936: Rabbit plasma) were purchased from Biolife Italiana Srl. Carvacrol (2-methyl-5-1-methyl ethyl phenol) and rosemary (*Rosmarinus officinalis L.*) EOs were purchased from National Research Center, Egypt. Polyethylene glycol sorbitan (Tween 80) and chitosan (medium molecular weight, 190–310 kDa, 75%–85% deacetylated) were purchased from Sigma Aldrich, and 0.5 McFarland standard (8.2 log10 CFU/mL) (Cat. No. TM50) was purchased from Dalynn Biologicals Company. Glacial acetic acid and 96-well plates and nanofilters were purchased from Dar-ElHekma Company, Assiut City, Egypt. Mueller Hinton agar (M173) was purchased from HiMedia Pvt., India, and buffered peptone water (BPW, ISO), LAB204 - from Neogen Company. Deionised water was obtained from the molecular biology unit, Assiut University.

Collection of samples

One hundred and fifty samples of fresh and frozen (75 samples each) chicken meat including thigh, breast, and fillets (25 for each cut) were collected from different shops, supermarkets, and slaughterhouses in Sohag City, Egypt. Each sample was placed in a separate sterile plastic bag, identified to prevent spilling and cross-contamination. Then, within 1 h, it was transported to the laboratory in an icebox.

CPSA isolation and identification

For the enrichment method, 25 g of chicken meat sample was aseptically added to 225 mL of BPW supplemented with 7% NaCl and incubated at 37 °C/24 h. A loopful of the incubated broth was streaked onto Baird-Parker agar supplemented with egg yolk tellurite and incubated at 37 °C/24–48 h. Suspected typical colonies (black or gray, shining and convex, 1 to 1.5 mm in diameter after 24 h, 1.5 to 2.5 mm after 48 h of incubation, and surrounded by a clear zone) (Quinn *et al.,* 2011) were picked for further identification including Gram staining, catalase, oxidase, and slide and tube coagulase tests (ISO 6888-1: 2021).

Molecular identification of CPSA isolates by PCR

The PCR assay was done at the Reference Laboratory for Veterinary Quality Control, Biotechnology Unit in Animal Health Research Institute, Dokki, Giza, Egypt. The DNA extraction was executed using QIAamp DNA mini kit according to the manufacturer's instructions. The CPSA isolates were screened for *23S rRNA* (*S. aureus* species-specific determinant) at 1250 bp by T3 Thermal cycler (Biometra) using primer set FW (ACGGAG TTACAAAGGACGAC) and RV (AGC TCAGCCTTAACGAGTAC) (Straub *et al*., 1999). The PCR products were electrophoresed on 1% agarose gel, and then were transferred to an ultraviolet (UV) light cabinet. The gel was photographed by a gel documentation system (Alpha Innotech), and the data were analysed using computer software.

Preparation and characterisation of essential oil nano-emulsions

To obtain the oil-in-water (O/W) nano-emulsion (NE), the amount of EO/surfactants differed based on the oil and its viscosity to prepare the most stable NE. CNE was prepared according to Khan *et al*. (1999) with modification by adding carvacrol EO to Tween 80 in a 1:4 (v/v) proportion. RNE was prepared according to Hassanzadazar *et al*. (2019) with modification by using rosemary EO with Tween 80 and Tween 20 (5:8:2). All NEs were filtered through a filter measuring 0.22 µm (220 nm). Droplet size and PDI were analysed via dynamic light scattering using a zeta-sizer (3000 HS, Malvern Instruments, Malvern, UK) at Nanotechnology Unit, Al-Azhar University, Assiut Branch, Egypt. Fourier-transform infrared spectroscopy (FTIR, NICOLET, iS10, Thermo Fisher Scientific) was performed in the Chemistry Department, Faculty of Science, Assiut University. It was used to identify the functional groups with their means of attachment and the fingerprint of the molecule. To perform FTIR, samples were prepared by employing suitable methods such as the potassium bromide pellet method and the Nujol method, and then the sample was scanned in the FTIR spectrometer in the wavenumber range of 4000–500 cm^{-1}. The morphological identification was conducted via TEM (TEM, JEOL-100CX II) at the Electronic Microscope Unit, Assiut University, Egypt.

Minimum inhibitory concentration (MIC) determination

Bacterial suspension of CPSA was prepared by transferring a loopful of the stock culture to the Brain Heart Infusion media and incubation at a temperature of 37 °C for 24 h. Following that, the freshly prepared suspensions were compared with 0.5 McFarland standard turbidity by using McFarland standard apparatus (Scientific Device Laboratory, Inc., USA). Then, 100 µL of bacterial suspension was spread on Mueller Hinton agar plates. Wells of 4 mm diameter were made using sterile cork borer. Each well was filled with 100 µL of pure nano-emulsion and double-fold serial dilutions of up to 0.39%. One well in each plate filled with 100 µL of sterile deionised water was kept as a control well. After 45 min at room temperature, the plates were incubated at 37 °C for 24 h, and the inhibition zone was measured. Triplicate trials were conducted and kept for consideration.

Preparation of chitosan incorporated nano-emulsion films

Chitosan films were prepared according to Sugumar *et al*. (2015) with the following modification: chitosan solution (2% w/v) was prepared using 1% acetic acid and filtered with Whatman No.1 filter paper, and 0.75% glycerol was used as a common plasticiser. Different concentrations of CNE and RNE (0%, 0.78%, and 1.56%) were added according to MIC and mixed with chitosan solution. Then, the whole mixture was stirred vigorously (Hotplate stirrer, DAIHAN Scientific Co., Ltd, Korea) at 70 °C for 30 min. After cooling to room temperature, the resulting mixture was degassed under vacuum for 5 min to remove all bubbles. Finally, a clear solution was obtained and cast onto 15 cm Petri dishes. The cast film was left to stand at ambient temperature for 36 h to obtain the freestanding thin film. The dried film was peeled off carefully from the glass dish. The physical and mechanical properties of the prepared films were measured according to Abdollahi *et al*. (2012) and Rhim *et al*. (2006). Colour properties of films were measured using a

colour-meter (CR-400, Konica Minolta, Osaka, Japan) with the reference-calibrated plate. The total colour difference (ΔE) was calculated as $\Delta L_2+\Delta a_2+\Delta b_2$. Transparency of the prepared films was determined by measuring the percent of transmittance at 660 nm using a UV-visible spectrophotometer (Thermo Scientific™, Waltham, MA, USA): Transparency= Absorbance 660/Thickness.

Anti-CPSA activity of the prepared films in chicken meat

The fresh chicken meats were purchased from the local market and transported to the laboratory under refrigerated conditions within 15 min of purchase. Before the experiments, the chicken meat products were surface treated with UV light for 15 min to reduce background microflora (ASTM, 1989). The aseptic chicken meat was cut into sections (5 × 5 cm) and inoculated with the previously prepared suspension of CPSA to obtain approximately 6 log10 CFU/cm^2 on the surface. After inoculation, the samples were kept at room temperature for 20 min to allow for cell attachment. The inoculated chicken meat surfaces were packaged with the previously prepared chitosan films free of nano-emulsions (Ch-free) and those containing different concentrations of nano-emulsions. Afterward, the inoculated chicken meats were stored at 4 °C. CPSA count was determined during the refrigerated storage at different time intervals (Day 0, 1, 2, 4, 7, 9 and 12 after packaging) until the appearance of signs of spoilage. At each sampling period, the packaged samples were opened, transferred aseptically to a stomacher, and homogenised for 2 min in 10 mL of BPW. Tenfold serial dilutions were made in BPW and 100 μL were plated in duplicate onto Baird–Parker agar for 24 h at 37 °C to determine the number of remaining CPSA cells (ISO 6888-1: 2021). The pH was measured using a Crison pH meter (Model 507, Crison, Barcelona, Spain) equipped with a Crison combination electrode (Cat. no. 52, Crison, Barcelona, Spain).

Sensory analysis of packaged chicken meat during the storage time

For sensory evaluation, chicken samples (free from CPSA) were divided into 6 groups: control groups without any package film (C); group 1: chicken sample packaged with Ch-free; groups 2,3: packaged with Ch-CNE at 0.78, 1.56% and groups 4,5: free samples packaged with Ch-RNE at 0.78, 1.56%, respectively. Samples were stored at refrigeration temperature (4 ± 2°C) (Elsherif *et al*., 2021). A group of 10 staff members from the Animal Health Research Institute, Sohag Branch, were asked to evaluate the texture, odour, appearance, and overall acceptability (OAA) of the packaged chicken meat samples with clarification to normal status of chicken meat. The scale points were excellent (5); very good (4); good (3); acceptable (2) and poor (1).

Statistical analysis

The statistical microbiological data analysis was prepared by using Excel software version 2016. The statistical data of MIC, physical and mechanical properties, and sensory analysis of the prepared films, as well as their activity against CPSA inoculation in chicken meat, were analysed using the SPSS program (SPSS Inc., Chicago, IL, USA) to calculate the least significant difference for mean and standard error values at $P<0.01$.

RESULTS

CPSA was isolated in percentages of 48%, 24%, and 6% of chicken thighs, breast, and fillet, respectively. A higher incidence of CPSA was reported in fresh (40%) than in the frozen products (12%) (Table 1). The isolates were subjected to biochemical tests and confirmed via PCR analysis using the *23S rRNA* gene at 1250 bp to confirm that the isolated strains belonged to *S. aureus*. All isolates gave positive results for *23S rRNA* gene (Fig. 1).

The prepared CNE and RNE were characterised using a zeta sizer, and the PDIs were 0.32 and 0.21 with average dynamic lengths of 54.56 and 44.98 nm, respectively (Table 2). That indicated the good quality and stability of prepared nano-materials. The average size of CNE and RNE by TEM was 40.33 and 48.78 nm, respectively, with spherical shape (Fig. 2). The spherical shape made these NEs more capable to penetrate the cell wall of bacteria and increase the antimicrobial activity of prepared nano-materials. Also, FTIR was used for detection the new functional bond formed with NEs preparation. Fig. 3 & 4 show the cleared strength phenol peaks, which, in the IR spectroscopy reference, ranged from 3200 to 3550 cm^{-1} in all NEs. Car-

Table 1. Incidence of coagulase positive *Staphylococcus aureus* (CPSA) isolated from chicken meat samples

Sample type	Fresh samples		Frozen samples		Total examined samples	
	Number	CPSA (%)	Number	CPSA (%)	Number	CPSA (%)
Chicken thighs	25	17 (68)	25	7 (28)	50	24 (48)
Chicken breast	25	11 (44)	25	1 (4)	50	12 (24)
Chicken fillet	25	2 (8)	25	1 (4)	50	3 (6)
Total	75	30 (40)	75	9 (12)	150	39 (26)

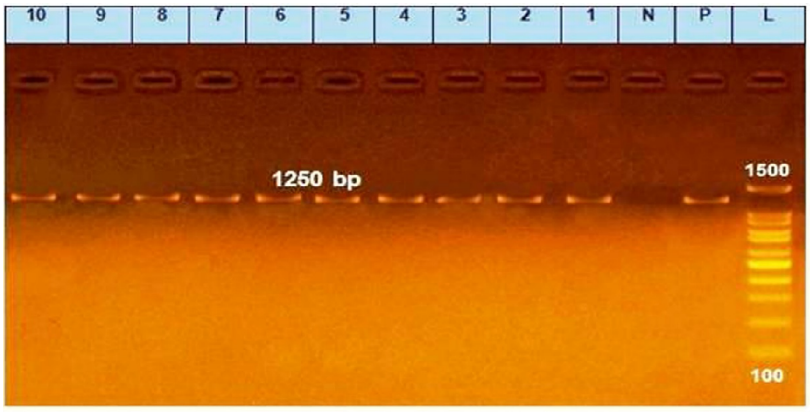

Fig. 1. PCR results for detection of *23S rRNA* gene (1250 bp) of coagulase-positive *Staph. aureus* (CPSA) isolates: lanes 1 to 10: positive isolates; lane N: negative control, lane P: positive control, lane L: marker ladder.

Table 2. Physical properties of the formulated carvacrol (CNE) and rosemary (RNE) nano-emulsions by zeta-sizer. Data are reported as means ± standard deviation

Nano-emulsions	Average droplet size (nm)	Polydispersity index (PDI)
CNE	54.56±28.70	0.32
RNE	44.98±16.40	0.21

vacrol EO had this peak at 3443.32 cm^{-1} but in NE it appeared at 3423.03 cm^{-1} and in rosemary EO, it was at 3428.4 cm–^{1}. Additionally, the CNE and RNE expressed a significant peak of C–H stretching at 2925.54 and 2869.95 cm^{-1}, present in carvacrol and rosemary EOs, that shifted and were observed at 2962.21 and 2869.99 cm^{-1}, respectively. Furthermore, the characteristic peaks for the aromatic ring, ranging from 1400 to 1600 cm^{-1}, were detected from 1421.86 to 1621.33 for carvacrol and rosemary EOs at different peaks, compared with 1421.47 to 1618.45 cm^{-1} for their NEs.

As shown in Table 3, the increasing order of concentrations showed that CNE and RNE had higher inhibitory activity without any bacterial growth around the well. The MIC for CNE was detected at concentrations of 1.56% and 0.78% with an inhibitory zone of 15.77 ± 0 and 12.17 ± 0.14 mm, whereas that for RNE was at concentrations of 1.56% and 0.78% with 14.9 ± 0.01 and 11.17 ± 0.02 mm, respectively. That indicate higher anti-CPSA activity of CNE than RNE without any significant difference between them in the *in vitro* experiment.

As seen from Table 4, all films were transparent, with a slight yellowish tint according to the amounts observed for the b* value of the films except in the 1.56% chitosan with carvacrol nano-emulsion (Ch-CNE) film where there was a significant increase in b* value in comparison with chitosan free film (Ch-free). Chitosan film lightness was not affected by the addition of any concentrations of CNE or RNE. Additionally, yellow/blue (b*) coordinates and total colour difference (ΔE)

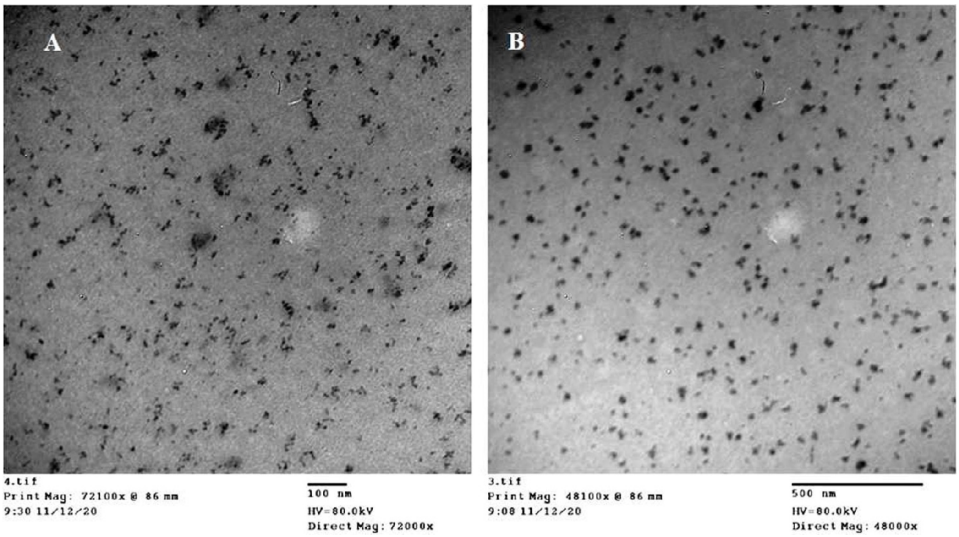

Fig. 2. Transmission electron microscopy for carvacrol nano-emulsion (A) and rosemary nano-emulsion (B) with average sizes 48.78 and 40.33 nm, respectively.

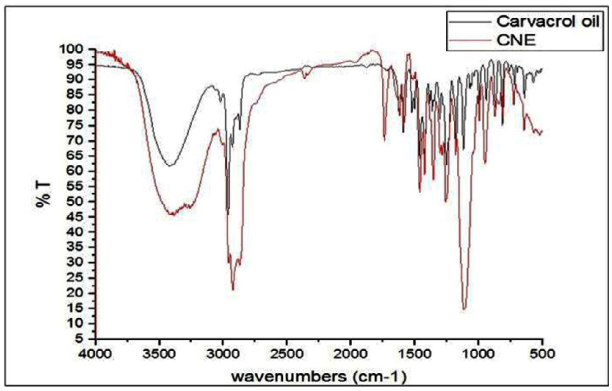

Fig. 3. Fourier-transform infrared spectroscopy (FTIR) of carvacrol essential oil and its nano-emulsion.

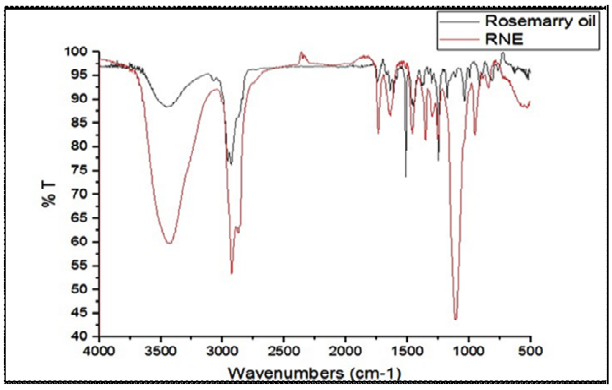

Fig. 4. Fourier-transform infrared spectroscopy (FTIR) of rosemary essential oil and its nano-emulsion.

Table 3. Antibacterial activity of nano-emulsions at different concentrations against coagulase-positive *Staph. aureus* (CPSA) by agar well diffusion method. Data are reported as mean values ± standard error (n=3)

Nano-emulsions and concentrations	Inhibition zone diameter (mm)	
	Carvacrol nano-emulsion	Rosemary nano-emulsion
Pure nano-emulsion	44.80±0.26	43.80±0.15
50%	40.40±0.23	39.70±0.12
25%	36.70±0.01	34.70±0.12
12.5%	29.20±0.12	28.20±0.13
6.25%	24.90±0.01	23.80±0.13
3.125%	20.30±0.12	19.30±0.10
1.56%	15.80±0.00	14.90±0.01
0.78%	12.20±0.14	11.20±0.02
0.39%	5.80±0.20	6.10±0.21

Table 4. Surface colour parameters and transparency of chitosan-based nano-emulsion films. Data are reported as mean values ± standard error (n=3)

Film types	L	a	b	ΔE	T_{660}
Ch-free	83.30±0.23	–0.84±0.25	5.67±0.62	12.57±0.52	95.64±0.80
Ch-CNE 0.78%	84.20±0.79	–1.55±0.14	9.24±1.05	16.86±1.20	90.60±0.75
Ch-CNE 1.56%	85.70±0.28	–2.09±0.54*	11.57±1.98*	19.56±1.01*	80.04±0.22*
Ch-RNE 0.78%	83.80±0.12	–1.03±0.21	7.45±0.56	14.67±0.57	93.74±0.90
Ch-RNE 1.56%	84.50±0.34	–1.71±0.34	8.67±0.64	17.22±0.58	91.87±0.70

L: lightness, a: red/green, b: yellow/blue, ΔE: total colour difference; T_{660}: transmittance at 660 nm, Ch-free: chitosan-free film, Ch-CNE: chitosan with carvacrol nano-emulsion film, Ch-RNE: chitosan with rosemary nano-emulsion film; * significant difference at P<0.01 vs control (Ch-free).

Table 5. Measurements of mechanical and physical properties of the chitosan-based nano-emulsion films. Data are reported as mean values ± standard error (n=3)

Film type	Thickness (mm)	Solubility (%)	Water vapour permeability (g/m^2.day)	Tensile strength (MPa)	Elongation (%)
Ch-free	0.060±0.001	12.23±0.02	2426±20	55.90±0.85	32.80±0.17
Ch-CNE 0.78%	0.062±0.001	10.56±0.03	2204±15	60.20±0.87	29.70±0.34
Ch-CNE 1.56%	0.063±0.002	9.75±0.02	1654±11*	65.40±0.42*	18.60±0.20*
Ch-RNE 0.78%	0.061±0.000	11.90±0.00	2301±20	53.40±0.30	35.50±0.25
Ch-RNE 1.56%	0.062±0.001	10.42±0.10	1982±15	50.70±0.70	37.60±0.47

Ch-free: chitosan-free film, Ch-CNE: chitosan with carvacrol nano-emulsion film, Ch-RNE: chitosan with rosemary nano-emulsion film; * significant difference at P<0.01 vs control (Ch-free).

were increased, whereas red/green (a*) values and transmittance at 660 nm were decreased with increasing nano-emulsion concentrations without a significant difference compared with Ch-free films but significantly different from 1.56% Ch-CNE films.

The thickness of the prepared films increased as the concentration of NEs increased from 0.060 to 0.063 mm (the highest) in the 1.56% Ch-CNE films without significant difference between the different lengths. The water vapour permeability (WVP) of Ch-free film was 2426 g/m^2/day and decreased significantly to 1654 g/m^2/day in Ch-CNE (1.56%) films by adding the NEs (Table 5). The Ch-CNE (1.56%) films had significantly higher pressure than control films (65.4 ± 0.42 MPa for TS and 18.6 ± 0.2% for E) (Table 5).

The data on Fig. 5 illustrate the significant decrease (P<0.01) in CPSA count at the 4^{th} day of storage at 4°C in samples packaged with Ch-CNE (1.56%) films. Complete eradication of the CPSA was detected at the 7^{th} day of storage compared with other films. The samples wrapped with Ch-CNE (1.56%) films showed significant good organoleptic properties without any unpleasant texture or appearance over the storage period, with the highest OAA (4 ± 0) and good pH (5.8) until the 12^{th} day (Fig. 6). However, these packaged samples had a light carvacrol odour for the first four days, which disappeared with the storage time

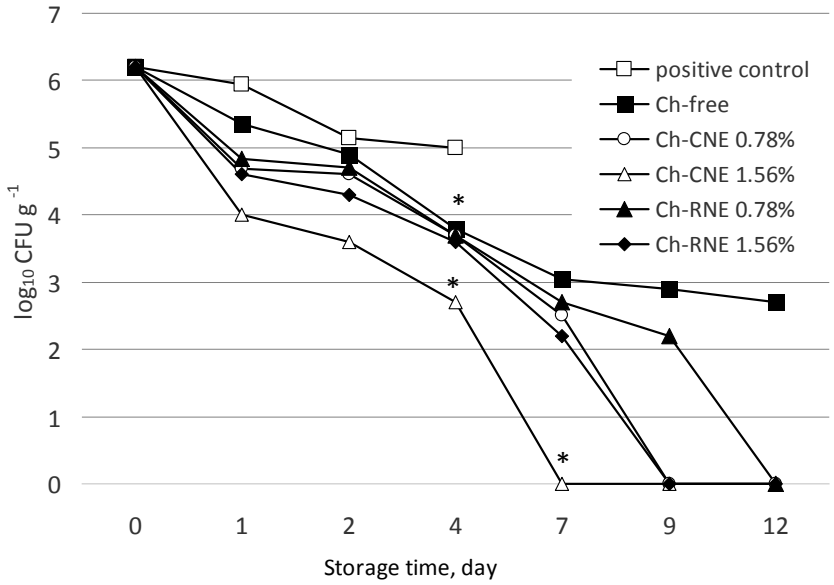

Fig. 5. Viability of coagulase-positive *S. aureus* (CPSA) inoculated in chicken meat packaged by chitosan-based nano-emulsion films during refrigerated storage (Ch-free: chitosan free; Ch-CNE: chitosan with carvacrol nano-emulsion; Ch-RNE: chitosan with rosemary nano-emulsion). * Significant difference at P<0.01 between inoculated chicken meat compared with controls. The positive control had completely deteriorated after the 4th day in refrigerated storage with pH 6.9 (not acceptable according to the Egyptian chicken meat standard).

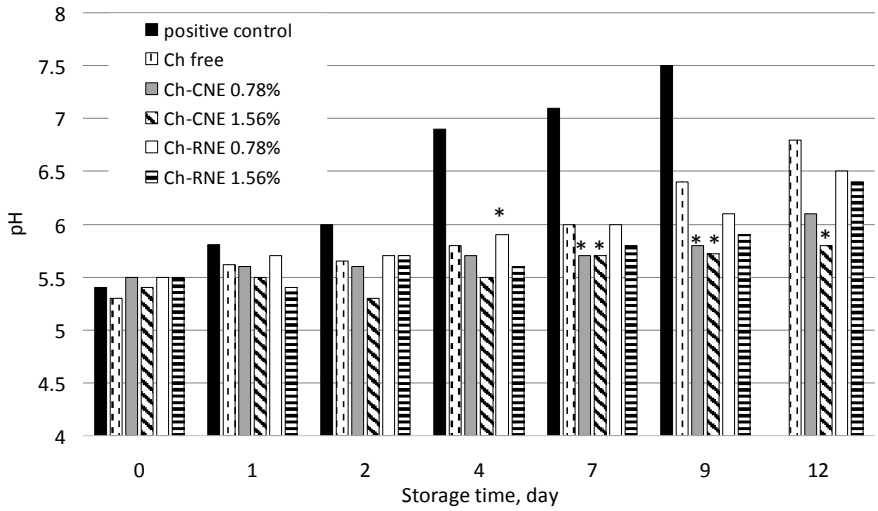

Fig. 6. The pH values of control and packaged chicken meat with chitosan-based nano-emulsion films during refrigerated storage (Ch-free: chitosan free; Ch-CNE: chitosan with carvacrol nano-emulsion; Ch-RNE: chitosan with rosemary nano-emulsion). * Significant difference at P<0.01.

Table 6. Sensory attributes of control and packaged chicken meat with chitosan-based nano-emulsion films during refrigerated storage.

Sensory attributes	Storage time, days	Negative control	Ch-free	Ch-CNE 0.78%	Ch-CNE 1.56%	Ch-RNE 0.78%	Ch-RNE 1.56%
Texture	1	4.3±0.5	4.4±0.5	4.3±0.2	4.4±0.3	4.3±0.5	4.4±0.5
	4	2.5±0.4	4±0.7	4.1±0.5	4.4±0.3	4±0.8	4.1±0.8
	7	2.2±0.2	3.5±0.5	3.5±0.5	4.1±0.5*	3.5±0.5	3.7±0.2
	9	1.1±0.2	3±0.2	3.2±0.4	3.8±0.5*	3.1±0.3	3.5±0.7*
	12	1±0.4	2.7±0.5	3±0.7	3.6±0.3*	2.7±0.2	3.3±0.3*
Odour	1	4.0±0.3	4.5±0.5	4.3±0.4	2.7±0.8*	4.4±0.5	3.2±0.5*
	4	2.3±0.5	4.1±0.5	4±0.3	2.9±0.6*	4.2±0.4	3.5±0.5*
	7	1.7±0.4	3.5±0.6	3.5±0.5*	3.4±0.7*	3.5±0.*7	4.0±0.7*
	9	1.0±0.0	3±0.4	3.1±0.5*	3.8±0.5**	3±0.5*	4.1±0.6**
	12	1.0±0.0	2.5±0.5	2.7±0.6	4.0±0.0**	2.5±0.5	3.5±0.2*
Appearance	1	4.8±0.7	4.8±0.3	4.8±0.4	4.7±0.4	4.7±0.5	4.7±0.5
	4	2.7±0.3	4.7±0.4	4.8±0.5	4.7±0.3	4.5±0.4	4.7±0.4
	7	2.3±0.4	3.8±0.5	4.7±0.9	4.5±0.5	4.5±0.4	4.2±0.5
	9	1.52±0.5	2.5±0.3	3.6±0.5*	4.1±0.4*	4.0±0.0*	4.1±0.5*
	12	1.05±0.6	1.5±0.7	3.2±0.4*	3.7±0.7*	3.2±0.7*	3.5±0.2*
Overall accepta-bility	1	4.8±0.7	4.8±0.5	4.8±0.3	4.7±0.5	4.8±0.3	4.8±0.7
	4	2.1±0.5	4.2±0.4	4.7±0.4	4.7±0.5	4.5±0.5	4.7±0.6
	7	1.3±0.02	3.1±0.5	4.5±0.4*	4.5±0.1*	3.8±0.6	4.5±0.5*
	9	1.03±0.03	2.4±0.6	4.1±0.3*	4.1±0.5**	3.5±0.4*	4±0.2**
	12	1.0±0.02	1.7±0.4	3.9±0.7*	4.0±0.0**	3.0±0.3*	3.9±0.7*

Ch-free: chitosan-free film, Ch-CNE: chitosan with carvacrol nano-emulsion film, Ch-RNE: chitosan with rosemary nano-emulsion film; * significant difference at P<0.01; ** P<0.0001 vs control (Ch-free).

until the completion of the trial (Table 6). The unwrapped group (positive control) showed the highest count of CPSA (5 $\log_{10}$ CFU/g), and complete deterioration was observed on the 4th day of refrigerated storage accompanied by a bad odour, change in organoleptic properties and OAA (2.1 ± 0.5) (Table 6), and an alkaline pH (6.9) (Fig. 6). Although chicken meat packaged with Ch-free films showed slight deterioration on the 7th day, they had an acceptable texture (3.5 ± 0.5), odour (3.3 ± 0.6), appearance (4.5 ± 0.5), and OAA (3.8 ± 0.5) with a reduction in CPSA count that was still detected at the end of the experiment (2.8 $\log_{10}$ CFU/g).

DISCUSSION

The prevalence of isolates in this study was nearly similar (41.8%) to result reported by Karmi (2013) whereas higher results (89%) were recorded by Abolghait *et al*. (2020). Other investigations from different countries (China, Turkey, Germany, and Brazil) found varied levels of CPSA in chicken meat – 8.1%, 30%, 71.5%, and 21.72%, respectively (Ahmed *et al*., 2016). The variable incidence may be due to the differences in handling and management practices of fresh and frozen chicken meat samples and their geographical location. In fact, the higher incidence of CPSA in fresh chicken meat may

be related to contamination during meat handling because of poor hygienic practices and cross-contamination during slaughtering or processing of chicken meat. The freezing temperature may also affect the growth and multiplication of the CPSA (Wu *et al.,* 2018; Parvin *et al.,* 2021).

PDI results indicated the stability of the prepared NEs as they were lower than 0.5, and the ratio of the surfactant used prevented coalescence at room temperature and for a long period, as mentioned in several previous studies (Alliod *et al*., 2018; Sharma *et al*., 2018; Hassanzadazar *et al*., 2019; Nirmala *et al*., 2020). The mean particle size largely depended on the surfactant-to-oil ratio (SOR), and decreased with an increase in SOR. In a study in this regard, Hassanzadazar *et al*. (2019) showed that obtaining smaller particle sizes than 100 nm was possible with a 30% increase of the surfactant and reduction of the EO concentration. Surfactants in NE preparation increase the stability and homogenisation of EO in water. Tween 80 is regarded as a safe surfactant for use in the pharmaceutical and food industries (D'Agostino *et al*., 2019).

FTIR is used to identify the functional groups and their means of attachment and provide a fingerprint of the molecule. IR spectroscopy is based on determining the energy difference (ΔE) between the excited and ground states of the molecule (Gurpreet *et al*., 2018). The difference in this peak indicates the interaction between the surfactant (Tween 80) and the EO to convert it to NE (Sugumar *et al*., 2015; Keawchaoon & Yoksan, 2011). The aromatic bond is essential as it determines the stability of the produced nano-emulsion by its saturation or if unsaturated, is also a characteristic of the aromatic substitution pattern (Zhuang *et al*., 2020). In this study, the aromatic ring of the prepared NEs indicates their stability and efficacy compared with that of the EOs. Additionally, C=O peaks were observed in all NEs at 1735 but missed in EOs. At this peak, a six-membered lactone ring was formed, which is considered the most stable ester and active functional group (Bidyarani *et al.*, 2020). The difference in peaks and the presence of more functional groups in NEs may be the main factor for its increased nanoproperties, stability, and antibacterial activity.

A very weak zone was detected by the lowest concentration (0.39%), so the concentrations of 1.56% and 0.78% were used in chitosan packaging films. The antibacterial effect of CNE and RNE against *S. aureus* was mentioned in several studies as Bidyarani *et al*. (2020) and Motta Felıcio *et al*. (2020) who proved the anti-*S. aureus* activity of CNE. Masoomi *et al*. (2016) and Hassanzadazar *et al*. (2019) determined the MIC of CNE and RNE at a concentration of 1% with mean inhibition zones of 30.4 ± 0.3 and 6.9 ± 0.1 mm, respectively.

The MIC (0.78% and 1.56%) of CNE and RNE was adjusted in the prepared chitosan-based films and tested for physical and mechanical properties. The results of films transparency were in good agreement with Rhim *et al*. (2006), Ojagh *et al*. (2010), Abdollahi *et al*. (2012) and Flores *et al*. (2021). It has been previously reported that the optical properties of films are affected by the developed internal and superficial microstructure because coalescence and creaming phenomena can occur during the film drying stage (Flores *et al*., 2021). A low transparency film may have a greater advantage because of a higher light barrier property and its antioxidant effect and because it is richer in active compounds (Elshamy *et al*., 2021). The 1.56% Ch-CNE films have a signifi-

cantly lower transparency (80.04 ± 0.22) than the others.

The thickness of the films is one of the most important parameters related to transparency, WVP, and mechanical properties. The results revealed that the film thickness was influenced by the solid content of the film-forming solution and the processing parameters. Therefore, our results agree with those of Abdollahi *et al*. (2012), Elshamy *et al*. (2021) and Flores *et al*. (2021). Water resistance is an important packaging material property for food protection, especially where water activity is high or when the film must be in contact with water permanently, such as with chicken meat products, as the water activities of these products are high and the packaging material must protect chicken meat products against exudation of fluids in either direction. As shown by the results presented in Table 5, Ch-free films had a low solubility value of 12.23% similar to that reported by Eldaly *et al*. (2018) and Dhankhar *et al*. (2021). When different concentrations of NEs were added to the film, there was a decrease in the percentage of water solubility. This fact is highly attributable to the cross-linking effects of CNE and RNE components to esters and/or amide groups (Ojagh *et al*., 2010) and that shown by FTIR. Additionally, the low solubility recorded may be due to the glycerol used as a plasticiser in the films as it has three hydroxyl groups that with the protonated amino groups in chitosan can interact with the water molecules to promote film solubility (Flores *et al*., 2021). A package with lower solubility is required during storage to avoid exudation, deterioration, dryness, and for lengthy preservation of the products (Khalaf *et al*., 2013).

WVP is an indicator of the film's resistance to transfer moisture or its ability to decrease moisture transfer between the food and the environment or even between heterogeneous components of food products to achieve longer shelf life (Fakhreddin *et al*., 2012; Elsherif *et al*., 2020). As mentioned previously, the addition of NEs, especially CNE, is believed to improve the barrier properties of polymers because of their disk-like morphology and their ability to create a tortuous path for molecule diffusion (Rhim *et al*., 2006). This is the case for the WVP decrease of chitosan/nanocomposites compared with pure chitosan. A significant decrease of WVP relates to the presence of ordered dispersed nanoparticle layers with relatively large aspect ratios in chitosan (ASTM, 1989). Further decreasing WVP in the presence of REO and CEO relates to their hydrophobic nature, which could affect the hydrophilic/hydrophobic property of the films. It may be that the hydrogen and covalent interactions between the chitosan network and NE ingredients limit the availability of hydrogen groups to form hydrophilic bonding with water, subsequently leading to a decrease in the affinity of chitosan film to water and decreasing WVP (Abdollahi *et al*., 2012; Higueras *et al*., 2014). Tensile strength (TS) is an indicator of the films' resistance to tension forces, whereas elongation at break (E) determines the films' stretching capacity. Our findings revealed that an increase in TS was accompanied by a decrease in E. Both TS and E are highly dependent on many factors such as the chemical structure of the film, different interactions among polymer networks, type of oils and their chemical composition, the chitosan natural resource, the degree of acidity of the filmogenic mixture, and other experimental factors like pH, relative humidity and emulsifiers (Sánchez-González *et al*., 2010). It seems

that NEs have shown a plasticising ability as an emulsifier. As for swelling, perhaps the presence of CNE changes the hydrogen-bonding network within the material and allows a better interaction between the nanofiller and the matrix (Abdollahi *et al*., 2012).

All other wrapped samples retained good organoleptic properties and OAA with a significant difference compared with unwrapped samples but nearly no difference among each other. Additionally, all NE-chitosan films had pH ranging from a good pH of 5.4 to a bad pH of 6.4 on the 7^{th} to 9^{th} day of storage except for Ch-CNE films, which had a pH of 5.8 on the 12^{th} day. The Ch-CNE (0.78%) and Ch-RNE (1.56%) completely reduced the CPSA in inoculated chicken samples on the 9^{th} day, whereas the Ch-RNE (0.78%) – on the 12^{th} day. These results were in agreement with data of Khalaf *et al*. (2013), Higueras *et al*. (2014) and Karimnezhad *et al*. (2017) who recorded that the antimicrobial activity of NEs of EOs loaded in chitosan films, especially at higher concentrations, indicated the increased release of the bioactive component of different phenolic compounds, which have excellent antioxidant and antibacterial activities. These phenolic compounds cause the inhibition of protective enzymes, increase the permeabilisation of bacterial cells, inhibit cytoplasmic membrane function, and disrupt membrane integrity, destroying microorganisms (Abdollahi *et al*., 2012; Napoli *et al*., 2020; Dhankhar *et al*., 2021). Furthermore, increased pH discloses the extent of meat spoilage because of protein decomposition by endogenous or microbial enzymes, which leads to the formation of free amino acids and subsequently, production of NH_3 and amines (Karabagias *et al*., 2011). Therefore, the shelf life of samples in the control group and Ch-free film would be 4 and 7 days, respectively, whereas for other Ch-NE films, it could be extended to 9 to 12 days except for 1.56% Ch-CNE films, which could be extended for more than 12 days in refrigerated storage. Additionally, the thickness and WVP of the prepared packaging could maintain the freshness of chicken samples, so the chitosan-based NEs maintained organoleptic properties (texture, odour, and appearance) and OAA better than the control or chitosan-free films. The reduction of exudates linked to NE incorporation in the films may be related to the enhanced water solubility of the films when NEs are added as previously reported in protein-based films (Pires *et al*., 2013). The liquid exuded from meat may have a negative impact on the sensory aspect as well as potentially enhance the proliferation of pathogens or spoilage microorganisms and act as microbial media (Otoni *et al*., 2016); thus, the reduction of exudates enhances the product quality and potentially its safety.

CONCLUSION

This study concluded that the percentages of CPSA isolated from the fresh chicken meat samples were higher than those in frozen ones. The isolated strains were confirmed genetically via PCR. The prepared CNE and RNE characterised using a zeta sizer demonstrated PDIs with the size nearly similar to that by TEM with a spherical shape. Additionally, the anti-CPSA activity evaluated using the agar well diffusion method showed MICs 1.56% and 0.78% for both NEs. On the basis of the results about antibacterial activity, and the good organoleptic properties of this packaged film during the storage period we conclude that Ch-CNE

(1.56%) film enhanced the functional properties of chitosan-based films and recommend it for potential applications in the chicken and other food industry.

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Correspondence:

W. M. Elsherif
Certified Food Laboratory,
Animal Health Research Institute (AHRI),
Agriculture Research Center (ARC), Egypt,
e-mail: me.elsherif@yahoo.com