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Improvement of the shelf life of grey mullet *(Mugil cephalus)* fish steaks using edible coatings containing chitosan, nanochitosan, and clove oil during refrigerated storage

Samar Aref[1*], Ramadan Habiba[1], Noha Morsy[2], Mohamed Abdel-Daim[3] and Fatma Zayet[1]

Abstract

The effects of edible coatings made of chitosan, or chitosan nanoparticles, and their combinations with clove oil on the physical, chemical, microbiological, and sensory properties of grey mullet (*Mugil cephalus*) steaks during refrigerated storage (4 °C) for 24 days were evaluated. The data indicated that metrics for all of these characteristics were improved significantly ($P \leq 0.05$) in coated fish steak samples compared to the control sample. The coated steaks texture values were higher than those of control sample through storage time with nanochitosan coating containing clove oil having best value 2.51 kg/cm^2 among tested samples at the end of storage period. However, pH values of coated samples were lower, a good indicator, than corresponding one of the control (6.46) with nanochitosan coatings with or without clove oil showed low mean values (6.11–6.13). Similar trend regarding chemical indices, i.e. total volatile basic-nitrogen, trimethylamine, peroxide, and thiobarbituric acid reactive substances values for samples coated with nanochitosan with or without clove oil giving better lower values than control or other treatments. Also, the coating delayed microbial (aerobic plate count, psychrotrophic bacteria, yeasts and molds, and total coliform bacteria) growth during the refrigeration storage period, and coated samples produced better results compared to the control sample. Sensory evaluations showed that the treatment enhanced the scores of fish steak samples compared to the control. The results showed that a nanochitosan coating with clove oil was the best treatment prolonged the shelf life of mullet steaks and efficiently maintained the quality attributes to an acceptable level during refrigerated storage for 24 days. Thus, these findings can be bases for producer to provide consumers with fresh fish steaks with good shelf life at refrigerated temperature for up to 24 days.

Keywords: Edible coating, Chitosan, Nanochitosan, Clove oil, Grey mullet fish, Storage

*Correspondence: Samar.moh.aref@gmail.com

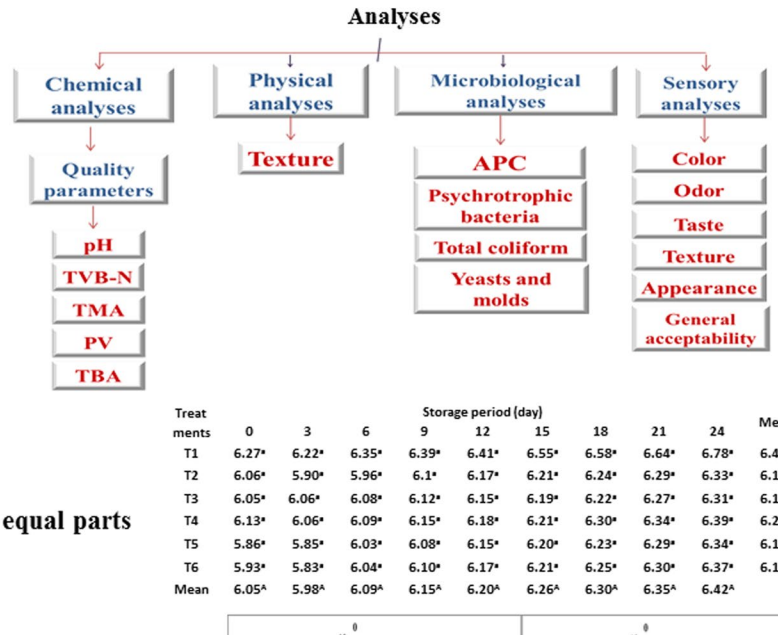
[1] Department of Food Technology, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt
Full list of author information is available at the end of the article

Graphical Abstract

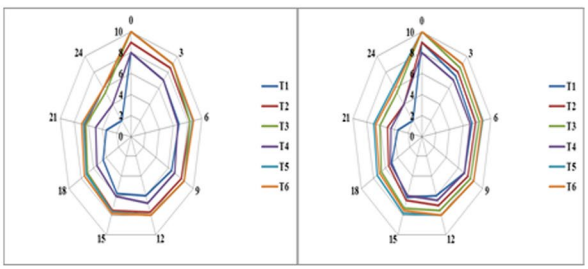
The fish slices were divided into six equal parts

T1	The control
T2	Chitosan 2%
T3	Chitosan 2% and clove oil – loaded samples.
T4	Clove oil 1.5%
T5	Nanochitosan particles 2%
T6	nanochitosan 2% and clove oil-loaded samples

Cold storage



Treatments	Storage period (day) 0	3	6	9	12	15	18	21	24	Mean
T1	6.27•	6.22•	6.35•	6.39•	6.41•	6.55•	6.58•	6.64•	6.78•	6.46A
T2	6.06•	5.90•	5.96•	6.1•	6.17•	6.21•	6.24•	6.29•	6.33•	6.14A
T3	6.05•	6.06•	6.08•	6.12•	6.15•	6.19•	6.22•	6.27•	6.31•	6.16A
T4	6.13•	6.06•	6.09•	6.15•	6.18•	6.21•	6.30•	6.34•	6.39•	6.20A
T5	5.86•	5.85•	6.03•	6.08•	6.15•	6.20•	6.23•	6.29•	6.34•	6.11A
T6	5.93•	5.83•	6.04•	6.10•	6.17•	6.21•	6.25•	6.30•	6.37•	6.13A
Mean	6.05A	5.98A	6.09A	6.15A	6.20A	6.26A	6.30A	6.35A	6.42A	



Changes in sensory properties of coated fish steaks

Introduction

Fresh fish is extremely perishable because of its biological composition. Deterioration of fish muscle caused by changes associated with biological processes such as lipid oxidation, decline in protein functionality, reactions generated by the activities of the fish's autolytic enzymes, and the metabolic activity of microorganisms. Lipid oxidation plays a vital role in the quality deterioration of fish during storage because it changes the flavor of fish, while protein oxidation alters the texture of the fish (Shahidi & Hossain 2022). These actions cause fish and fish products to have a limited shelf life (Arashisar et al. 2004). It is therefore desirable to improve natural protective coatings by adding antibacterial and antioxidant characteristics to extend the shelf life of fish and fish products.

Over the last two decades, the use of edible coatings to extend storage periods, particularly for highly perishable foods, by delaying or inhibiting microbiological and/or oxidative deterioration, has increased rapidly (Gómez-Guillén et al. 2009). Edible coatings are tiny layers made of natural polymers that are applied to food surfaces using various methods including spraying, immersing, and brushing (Dhall 2013), or using electrical deposition (Poverenov et al. 2014).

The main benefits of coating with edible coating include that the coating can be consumed with the food product, prevents moisture loss during frozen storage, maintains the color of fresh meat, improves flavor and texture, minimizes lipid oxidation, decreases spoilage, and decreases environmental contamination (Arkoun et al. 2018). Ali and Ahmed (2018) have been carried out into the use of edible coating as a carrier for food additives, such as anti-browning and antimicrobial agents, dyes, flavor donors, nutrients, and seasonings.

Chitosan (poly-b-(1–4)-D-glucosamine) is a biopolymer formed from the deacetylation of chitin obtained

from the exoskeletons of crustaceans and mollusks (Jasour et al. 2015). Chitosan has been shown to have antioxidant properties (Rajalakshmi et al. 2013). It can scavenge free radicals and bind metal ions by donating hydrogen or a single pair of electrons (Lin et al. 2009). The amino and hydroxyl functional groups of chitosan associated with metal ions initiate numerous processes including adsorption, ion exchange, and chelation**.** Chitosan is effective at killing gram-positive and gram-negative bacteria (Kong et al. 2010). Chitosan's antibacterial properties are affected by a variety of factors such as the type of chitosan and its polymerization rate, pH, and molecular weight. Nanochitosan (CSNPs) is a natural substance with desirable physicochemical characteristics. The applications of CSNPs as food preservatives, drug carriers, active packaging material, and encapsulating agents for bioactive components have been subjects of increasing interest for the last few years (Divya & Jisha 2018). CSNPs are more active in preventing bacterial growth than chitosan itself, and it can damage the cell membrane of bacteria such as *Salmonella choleraesuis*, *Escherichia coli, Vibrio parahaemolyticus*, and *Staphylococcus aureus*.

Edible coatings combined with numerous essential oils were revealed to have antimicrobial properties against foodborne pathogens (Fernández-Pan et al. 2012). Clove oil (*Syzygium aromaticum*, Lin) is a natural essential oil with antimicrobial and antioxidant activities (Gülçin et al. 2012; Oskoueian et al. 2013). Hosseini et al. (2009) have shown that clove essential oil, which is primarily composed of eugenol, is an efficient inhibitor of pathogenic microorganisms such as *S. aureus, L. monocytogenes*, *Campylobacter jejuni*, *E.coli* and *Salmonella enteritidis***.**

The objectives of the current study were to evaluate the efficacy of using edible coating with chitosan, and nanochitosan, with or without clove oil on the chemical, microbiological, and sensory quality qualities of fresh mullet steaks during refrigerated storage as to improve shelf life of the steaks.

Materials and methods

Practical grade chitosan from crab shells (Brookfield viscosity > 200 cP, 1% in 1% acetic acid, degree of deacetylation ≥ 75%, and molecular weight 190–375 kDa) was obtained from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Clove seeds (*Syzygium aromaticum*) were purchased from local markets. Nanochitosan particles and nanochitosan loaded with clove oil were obtained from Nano Gate Co., Mokattam, Cairo. Other chemicals and microbial media used were of analytical grade, and were purchased from El-Saudia for Chemicals, Ismailia, Egypt.

Clove oil preparation and extraction

Twenty five grams of clove seeds was transferred into a beaker and 400 mL of methanol was added. At room temperature, the combination was coated and shaken with a mechanical shaker for 24 h. The extract was then filtered using Double Rings Filter Paper. The filtrate was obtained, and the residue was extracted twice more. The extracts were then combined. The solvent in the extract was then evaporated using a rotary evaporator under low pressure at 45 °C (Khamsah et al. 2006) to obtain clove oils.

Preparation of chitosan solutions

Chitosan coatings were prepared using the methodology described by Günlü and Koyun (2013). Chitosan (2% v/v) was blended with glacial acetic acid (1% v/v) for an hour at 40 °C using a magnetic stirrer. Then,

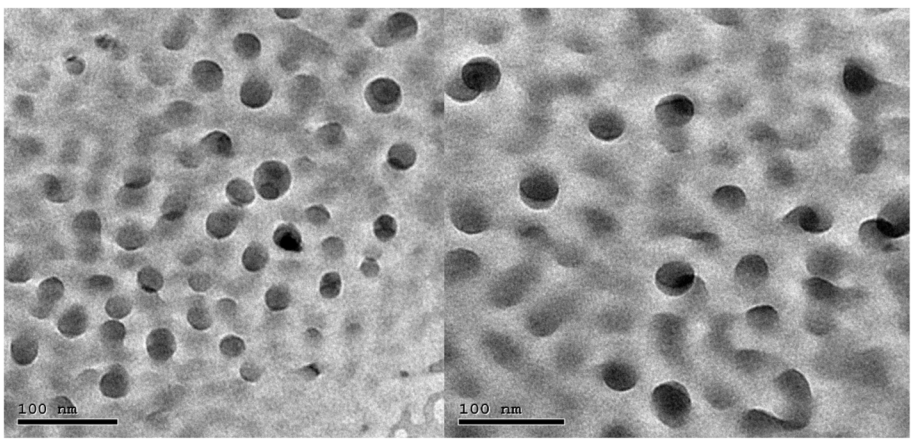


Fig. 1 TEM image of 2% chitosan nanoparticles with an average particle size of 25 ± 5 nm

glycerol (2%) was gently added to the liquid as a plasticizer, and stirred for 10 min using a heated magnetic stirrer. The resulting chitosan coating mixture was filtered using Whatman No. 102 filter paper to eliminate indissoluble parts.

To make a chitosan mixture combination with clove oil, glycerol (0.5 ml /g chitosan) and Tween 60 (0.1%w/v, to assist in the dissolving of clove oil) were added to the chitosan solution and stirred for 30 min. Then, clove oil (1.5 g) was added up to 100 ml of the chitosan combination with glycerol and Tween 60, and stirred for 6 h at room temperature (final clove oil concentration = 1.5% w/v). All stirring was conducted using a magnetic stirrer at room temperature.

Tests on chitosan nanoparticles

Transmission electron microscopy (TEM) was conducted using a JEOL JEM-2100 high resolution TEM with a 200 kV accelerating voltage was used to estimate the chitosan particle size (Fig. 1).

Fish experiments

Fresh aquaculture grey mullet (*Mugil cephalus*) fish (about 25 kg) obtained from a local aquaculture farm were transported immediately to the laboratory under refrigeration. The fish were beheaded, gutted, washed, and sliced to a thickness of 2 cm. The fish slices were divided into six equal parts for the experiments. The following treatment groups were prepared: control without coatings (Control) (T1); Chitosan 2% (T2); Chitosan 2% and clove oil-loaded samples (T3). Clove oil 1.5% was added onto the surface of the fish slices (T4). Nanochitosan particles 2% (T5); and nanochitosan 2% and clove oil-loaded samples (T6). The fish slices were kept in a refrigerator at 4 °C and the chemical, microbial, and sensorial changes of the treatments were evaluated on days 0, 3, 6, 9, 12, 15, 18, 21, and 24.

For coatings, the different fish steak samples were immersed in different solutions for 5 min and then dried at room temperature for 30 min. Control samples were performed in the same manner except that the slices

Table 1 Changes in the texture (kg/cm^2) of fish steak samples during storage at 4 °C

Treatments	Storage period (days)									Mean
	0	3	6	9	12	15	18	21	24	
T1	2.52^{eg}	2.46^{ag}	2.63^{ae}	2.40^{ah}	2.10^{ah}	1.92^{bh}	1.68^{dh}	1.42^{gh}	1.05^{h}	2.02^{BC}
T2	2.59^{af}	2.46^{ag}	2.15^{ah}	1.82^{ch}	1.74^{dh}	1.65^{eh}	1.43^{fg}	1.24^{h}	1.10^{h}	1.79^{C}
T3	2.96^{ac}	2.53^{ag}	2.35^{ah}	2.11^{ah}	2.01^{ah}	1.84^{ch}	1.76^{dh}	1.70dg	1.61^{eh}	2.09^{BC}
T4	3.06^{ab}	2.96^{ac}	2.49^{ag}	2.30^{ah}	2.24^{ah}	2.10^{ah}	1.94^{bh}	1.72^{dh}	1.48^{eh}	2.25^{AB}
T5	2.56^{ag}	2.46^{ag}	2.35^{ah}	2.15^{ah}	1.82^{ch}	1.64^{eh}	1.42^{gh}	1.26^{h}	1.20^{ah}	1.87^{C}
T6	3.54^{ah}	3.11^{a}	3.03^{ab}	2.82^{ad}	2.54^{ag}	2.30^{ah}	1.96^{ah}	1.74^{dh}	1.58^{eh}	2.51^{A}
Mean	2.87^{A}	2.66^{AB}	2.50^{AB}	2.26^{BC}	2.07^{CD}	1.90^{CDE}	1.69^{DEF}	1.51^{EF}	1.33^{F}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters have significant differences among treatments at a significance level of $P \leq 0.05$

Table 2 Changes in pH of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	6.27^{a}	6.22^{a}	6.35^{a}	6.39^{a}	6.41^{a}	6.55^{a}	6.58^{a}	6.64^{a}	6.78^{a}	6.46^{A}
T2	6.06^{a}	5.90^{a}	5.96^{a}	6.1^{a}	6.17^{a}	6.21^{a}	6.24^{a}	6.29^{a}	6.33^{a}	6.14^{A}
T3	6.05^{a}	6.06^{a}	6.08^{a}	6.12^{a}	6.15^{a}	6.19^{a}	6.22^{a}	6.27^{a}	6.31^{a}	6.16^{A}
T4	6.13^{a}	6.06^{a}	6.09^{a}	6.15^{a}	6.18^{a}	6.21^{a}	6.30^{a}	6.34^{a}	6.39^{a}	6.20^{A}
T5	5.86^{a}	5.85^{a}	6.03^{a}	6.08^{a}	6.15^{a}	6.20^{a}	6.23^{a}	6.29^{a}	6.34^{a}	6.11^{A}
T6	5.93^{a}	5.83^{a}	6.04^{a}	6.10^{a}	6.17^{a}	6.21^{a}	6.25^{a}	6.30^{a}	6.37^{a}	6.13^{A}
Mean	6.05^{A}	5.98^{A}	6.09^{A}	6.15^{A}	6.20^{A}	6.26^{A}	6.30^{A}	6.35^{A}	6.42^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters have significant differences among treatments at a significance level of $P \leq 0.05$

were not covered with any coating. During storage, the samples were randomly taken every 3 days for analyses.

Physical and chemical parameters

The texture of the fish steak samples was assessed using a Y2 laboratory penetrometer, and the results were recorded as kg/cm^2. The pH readings were evaluated in homogenous solutions of fish steak and distilled water (1:9, w:v), using a standardized pH meter (Jenway 3010; UK). Total volatile basic-nitrogen (TVB-N) was determined using steam distillation of TCA-fish extracts using the modified method of Malle and Tao (1987). Trimethylamine (TMA) content was assessed according to the method published by Malle and Poumeyrol (1989). Peroxide value (PV) content was evaluated in the fish samples using the method of Mattissek et al. (1992). The value of thiobarbituric acid reactive substances (TBARS) in mg malondialdehyde equivalent (MDA eq)/kg was evaluated utilizing a spectrophotometric technique (Tarladgis et al. 1960).

Microbiological analysis

Ten grams of each blended sample were aseptically taken out of the Petri dish, and 90 ml of sterile buffered peptone water was subsequently added. The samples were then homogenized for 2 min. The aerobic plate count (APC) and psychotropic counts (TPC) were determined using the pour plate method. Serial dilutions were prepared, and 1 ml of each was placed onto plate count agar media (Merck, Darmstadt, Germany). The plates were incubated for two days at 35 °C for APC and 10 days at 7 °C for TPC (Arashisar et al. 2004). Yeast and molds were determined using plate count agar containing 100 µg/ml cidostane. The poured plates were incubated at 25 °C for 48 h. For the total coliform count, violet red bile (VRB) agar was used as a medium. Plates were incubated at 35 °C for 18 – 24 h. The results are expressed as log CFU per gram of sample.

Sensory quality

Sensory evaluation was performed as described by Ojagh et al. (2010). Fish slices samples preserved at 4 °C were given to the panelists, who had to judge the samples with respect to five quality parameters: color, odor, texture, appearance, taste and general acceptability on a 1–10 point scale. The panelists that conducted the tests were staff members of the Food Technology Department, Suez Canal University.

Statistical analysis

The data were analyzed using Analysis of Variance (ANOVA) tests, performed using SPSS software (version 16.0 for Windows, SPSS Inc., Chicago). Bartlett's multiple range tests were used to identify significance among treatment means at $P \leq 0.05$.

Results and discussion

Quality parameters of treated fish steak samples

Texture

The texture of fish is the main feature used to indicate it's the freshness (Chèret et al. 2006). Changes in the texture of the tested fish steaks during cold storage are shown in Table 1. The texture values ranged from 2.52 to 3.54 kg/cm^2 at the beginning of storage. Treatment with 2% of nanochitosan and clove oil improved the texture of fish steak samples.

According to Alishahi & Ader (2012), nanochitosan coatings have many mechanisms of action, including the production of conjugates and polymerization by endogenous enzymes. Samples treated with clove oil had a higher texture value (3.06 kg/cm^2) than control samples. As statistical analysis indicated, refrigerated storage

Table 3 Changes in TVB-N (mg/100 g) of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	11.15^{mo}	15.12^{go}	20.26^{go}	25.40^{em}	30.32^{ci}	35.40^{be}	39.70^{bd}	46.50^{b}	59.70^{a}	31.50^{A}
T2	10.11^{mo}	13.91^{lo}	15.17^{lo}	18.83^{io}	20.22^{go}	25.16^{em}	30.42^{ci}	32.01^{ch}	35.21^{bf}	22.33^{B}
T3	10.85^{mo}	15.21^{lo}	19.18^{ho}	22.40^{fn}	26.80^{dl}	28.92^{cj}	32.43^{cg}	35.01^{bf}	37.51^{em}	23.83^{B}
T4	9.92^{no}	11.12^{mo}	15.72^{ko}	18.20^{io}	24.20^{em}	28.40^{ck}	30.50^{ci}	34.70^{bf}	40.40^{bc}	23.68^{B}
T5	8.83^{o}	9.12^{o}	11.14^{mo}	15.20^{lo}	17.22^{jo}	20.50^{go}	22.50^{en}	25.01^{em}	30.15^{em}	17.74^{C}
T6	8.91^{mo}	10.28^{mo}	13.15^{mo}	15.12^{lo}	16.21^{jo}	18.71^{io}	20.50^{go}	22.40^{fn}	25.20^{em}	16.66^{C}
Mean	9.96^{H}	12.46^{GH}	15.77^{FG}	19.10^{EF}	22.49^{DE}	26.18^{CD}	29.34^{BC}	32.60^{AB}	35.72^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters indicate significant differences among treatments at a significance level of $P \leq 0.05$

Table 4 Changes in TMA (mg/100 g) of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	0.460^{j}	0.801^{j}	1.45^{gj}	5.05^{ce}	5.10^{ce}	6.48^{bd}	7.14^{bc}	8.24^{ab}	9.82^{a}	4.94^{A}
T2	0.330^{j}	0.723^{j}	0.892^{j}	1.11^{hj}	1.62^{gj}	1.77^{gj}	2.11^{gj}	2.67^{fj}	3.11^{ei}	1.59^{C}
T3	0.320^{j}	0.470^{j}	0.687^{j}	0.872^{j}	0.950^{ij}	1.50^{gj}	1.78^{gj}	2.11^{gj}	2.50^{gj}	1.23^{CD}
T4	0.340^{j}	8.20^{ab}	1.42^{gj}	1.72^{gj}	2.20^{gj}	2.73^{fj}	3.23^{eh}	3.52^{eg}	4.61^{df}	3.10^{B}
T5	0.287^{j}	0.441^{j}	0.522^{j}	0.651^{j}	0.801^{j}	0.885^{j}	0.930^{ij}	1.20^{hj}	1.57ij	0.80^{D}
T6	0.178^{j}	0.232^{j}	0.263^{j}	0.478^{j}	0.721^{j}	0.832^{j}	0.921^{j}	0.941^{ij}	1.32^{gj}	0.76^{B}
Mean	0.31^{F}	1.81^{CD}	0.87^{EF}	1.64^{DE}	1.89^{CD}	2.36^{BCD}	2.68^{BC}	3.11^{AB}	3.97^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters indicate significant differences among treatments at a significance level of $P \leq 0.05$

produced a significant effect on texture during the storage period, with fish samples coated with edible nanochitosan coatings containing clove oil (T6) having the best values (2.51 kg/cm^2) among the tested samples at the end of the storage period.

pH value

pH is an important indicator of the freshness of fish products (Brewer et al. 2006). The variations in the pH values of the treated fish samples are shown in Table 2. At time zero, the pH value of the control sample was 6.27 and it reached 6.78 by the end of refrigerated storage. With regard to the pooled mean pH values, the control samples had a significantly ($P \leq 0.05$) the higher mean pH value (6.46) than the other treatments. The samples covered with nanochitosan (T5 and T6) had the lowest mean pH values (6.11 and 6.13). Treatment containing a combination of 2% chitosan and clove oil had a low pH value (6.31), compared with the control sample. Mohan et al. (2012) found that sardines coated with chitosan had a significantly ($P \leq 0.05$) lower pH value than uncoated fish.

The pH constantly increased with storage time, with statistically significant differences ($P \leq 0.05$) between the treatments during the storage period. Nirmal and Benjakul (2011) found that the pH values increased gradually during the storage period, because of the accumulation of basic compounds generated from autolytic processes by endogenous enzymes. Pawar et al. (2013) found a slightly increased pH value of *Catla catla* from 6.50 to 6.79 when preserved at refrigerator temperatures (−2 to −4 °C).

Total volatile basic-nitrogen

Total volatile nitrogen is always utilized to assess fish quality because it is directly related to microorganism growth and the production of basic components, like methylamine ammonia, diethylamine, and trimethylamine, as a consequence of bacterial metabolism (Amin 2012). Changes in the TVB-N values of the

Table 5 Changes in PV (meq O_2 /kg lipid) of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	0.923 k	1.24^{ik}	2.43^{fk}	2.97^{dk}	3.52^{dk}	5.70^{cd}	8.10^{c}	11.12^{b}	15.16^{a}	5.68^{A}
T2	0.656 k	1.15^{jk}	1.78^{fk}	2.24^{fk}	2.87^{ek}	3.42^{dk}	4.32^{dg}	4.79^{df}	5.20^{de}	3.00^{B}
T3	0.642 k	0.780 k	0.998 k	1.15^{jk}	2.40^{fk}	2.81^{ek}	3.51^{dk}	3.75^{dj}	4.25^{dh}	2.25^{BC}
T4	0.525 k	0.720 k	1.01 k	1.45^{ik}	2.15^{fk}	3.41^{dk}	3.94^{di}	4.72^{df}	5.24^{de}	2.58^{BC}
T5	0.342 k	0.506 k	0.781 k	1.05^{jk}	1.67^{hk}	2.58^{ek}	3.78^{dj}	3.89^{di}	4.50^{dk}	2.12^{CD}
T6	0.132 k	0.256 k	0.456 k	0.528 k	0.829 k	1.52^{hk}	1.93^{gk}	2.54^{ek}	3.50^{dk}	1.29^{D}
Mean	0.53^{E}	0.78^{E}	1.32^{DE}	1.56^{DE}	2.23^{CD}	3.24^{C}	4.28^{B}	5.13^{B}	6.30^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters have significant differences among treatments at a significance level of $P \leq 0.05$

Table 6 Changes in TBARS (mg MDA/kg) of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	0.454^{fh}	0.678^{fh}	1.52^{eg}	2.32^{de}	3.47^{cd}	3.82^{bc}	4.52^{ac}	4.79^{ab}	5.21^{a}	2.98^{A}
T2	0.245^{h}	0.352^{fh}	0.372^{fh}	0.424^{fh}	0.473^{fh}	0.601^{fh}	0.642^{fh}	0.801^{fh}	0.924^{fh}	0.54^{B}
T3	0.278^{gh}	0.361^{fh}	0.397^{fh}	0.421^{fh}	0.578^{fh}	0.594^{fh}	0.621^{fh}	0.723^{fh}	0.896^{fh}	0.54^{B}
T4	0.321^{gh}	0.421^{fh}	0.521^{fh}	0.582^{fh}	0.678^{fh}	0.741^{fh}	0.787^{fh}	0.998^{fh}	1.61^{de}	0.74^{B}
T5	0.256^{h}	0.321^{gh}	0.381^{fh}	0.432^{fh}	0.524^{fh}	0.632^{fh}	0.652^{fh}	0.781^{fh}	0.806^{fh}	0.53^{B}
T6	0.224^{fh}	0.272^{gh}	0.321^{gh}	0.382^{fh}	0.471^{fh}	0.521^{fh}	0.542^{fh}	0.652^{fh}	0.742^{fh}	0.45^{B}
Mean	0.29^{E}	0.40^{E}	0.58^{DE}	0.76^{CDE}	1.03^{BCD}	1.15^{BC}	1.29^{AB}	1.46^{AB}	1.69^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters have significant differences among treatments at a significance level of $P \leq 0.05$

samples during cold storage are shown in Table 3. At time zero, the TVB-N values in the samples ranged from 8.83 to 11.15 mg/kg. The TVB-N values were significantly ($P \leq 0.05$) lower in all treated samples than in the control sample. During storage, all samples showed a steady increase ($P \leq 0.05$), with the rate of increase being faster in the control sample. The TVB-N value for the control sample increased from 11.15 to 59.70 mg/kg, and exceeded the maximum level allowed for seafood to be considered safe (35 mg/kg) after the twelfth day of storage.

The samples which contained a combination of 2% nanochitosan and clove oil (T6) had the lowest pooled mean TVB-N value (16.66 mg/100 g). Zarei et al. (2015) demonstrated that treating silver carp with nanochitosan coatings slowed the rise in TVB-N concentration compared with other treatments. Previously, nanochitosan coating has been found to be more effective than chitosan coating in inhibiting the increase in TVB-N content in silver carp fillets in cold storage (Ramezani et al. 2015).

Trimethylamine

Trimethylamine (TMA) content in muscle is the most used indicator for fish spoilage (Shahidi & Hossain 2022). Changes in the TMA values of the studied samples during storage are given in Table 4**.** At time zero, the TMA of the control sample was 0.460 reaching 9.82 mg/100 g by the end of storage. The TMA levels were significantly lower ($P \leq 0.05$) in all coated samples than in the control. Fish samples coated with a combination of nanochitosan and clove oil (T6) had the lowest TMA pooled mean (0.76 mg/100 g) as compared to the control sample (4.94 mg/100 g). This finding could be related to nanochitosan's antibacterial properties. Samples containing chitosan coating had low TMA values compared to the control. This reduction in TMA production when applying chitosan coating of fish has also been reported by Günlü and Koyun (2013), and Tsiligianni et al. (2012). A rejection level of 5 – 10 mg of TMA/100 g of flesh has been established for fish products (Ocaño-Higuera et al. 2011).

Table 7 Changes in aerobic plate count (APC) (log cfu/g) of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	3.84^{ej}	4.49^{dj}	5.90^{ag}	6.70	7.35^{ae}	7.86^{ad}	8.33^{ac}	8.79^{ab}	8.90^{a}	6.91^{A}
T2	2.32^{gj}	2.86^{fj}	3.13^{fj}	3.54^{fj}	3.75^{fj}	4.67^{dj}	5.34^{ai}	5.82^{ah}	6.32^{af}	4.19^{B}
T3	2.26^{hj}	2.53^{fj}	2.30^{gj}	3.51^{fj}	3.82^{ej}	4.40^{dj}	8.82^{a}	5.65^{ah}	6.21^{af}	4.39^{B}
T4	1.84^{ij}	2.22^{hj}	2.85^{fj}	3.21^{fj}	4.12^{ej}	4.62^{dj}	5.63^{ah}	6.23^{af}	6.43^{af}	4.18^{A}
T5	2.44^{fj}	2.76^{fj}	3.35^{fj}	3.61^{fj}	4.10^{ej}	4.49^{dj}	4.88^{cj}	5.33^{ai}	5.62^{fj}	4.06^{B}
T6	1.30^{fj}	1.72^{j}	2.63^{fj}	3.56^{fj}	3.97^{ej}	4.47^{fj}	4.83^{cj}	5.20^{bj}	5.49^{ah}	3.50^{B}
Mean	2.33^{E}	2.76^{DE}	3.36^{CDE}	4.03^{BCD}	4.52^{BC}	4.80^{B}	6.30^{A}	6.25^{A}	6.49^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters have significant differences among treatments at a significance level of $P \leq 0.05$

Peroxide value

Peroxide value (PV) is a popular method for determining the presence of primary oxidation compounds (peroxides) in oils and fats (Zhang et al. 2010). Table 5 shows the changes in the PV of fish steak samples during storage at 4 °C. In this study, the mean PV value of mullet steak samples was in the range 1.29 to 5.68 meq/kg of fish lipid. Initial PV values for all treated fish finger samples were significantly ($P \leq 0.05$) lower than those for the control. The PV in all samples rose during storage, with the control sample having the greatest level (15.16 meq/kg) after 24 days of refrigerated storage from a value of 0.923 meq/kg of lipid at time zero.

Fish steaks subjected to treatment containing nanochitosan and clove oil (T6) had the lowest mean PV (1.29 meq/kg). Jeon et al. (2002) reported that nanochitosan coating could act a barrier between the meat and its surroundings, to retard the permeation of oxygen. The phenolic components in essential oils delay the propagation of radicals and the oxidation of fish oils by scavenging free radicals from the first stages of oxidation, initial and propagation, as reported by Maqsood and Benjakul (2010). A level of 5 meq/kg is the maximum allowable PV for fish oils (Piedrahíta Márquez et al., 2019), and all coated samples were below this level, for up to 21 days of storage.

Thiobarbituric acid reactive substances

The thiobarbituric acid reactive substances (TBARS) value is among the indicators used to determine the fish quality, and is calculated as malondialdehyde equivalent (MDA eq) (Sheard et al. 2000). The TBARS values of fish samples are shown in Table 6. At time zero, the TBARS of the control was 0.454 reaching 5.21 mg MDA eq/kg by the end of storage. The control sample had an acceptable level of MDA eq for 21 days of storage. As shown in Table 6, there were no significant differences in TBARS

Table 8 Changes in psychrotrophic bacteria (log cfu/g) of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	2.72^{mp}	4.46^{fo}	5.38^{dj}	6.48^{af}	6.83^{ae}	7.41^{ad}	7.52^{ac}	8.21^{ab}	8.56^{a}	6.39^{A}
T2	2.51^{op}	2.92^{lp}	2.42^{op}	3.81^{hp}	4.51^{fo}	4.82^{em}	5.30^{dk}	6.11^{bg}	6.51^{af}	4.32^{B}
T3	2.50^{op}	2.85^{lp}	3.17^{lp}	3.42^{jp}	3.79^{hp}	4.41^{fo}	4.82^{em}	5.42^{cj}	6.30^{bg}	4.07^{BC}
T4	2.68^{np}	3.20^{kp}	3.43^{jp}	3.81^{hp}	4.30^{fo}	4.72^{en}	5.60^{ci}	6.40^{bf}	6.73^{ae}	4.54^{B}
T5	1.71^{p}	2.63^{np}	3.01^{lp}	3.53^{jp}	3.84^{hp}	4.42^{fo}	4.93^{el}	5.41^{cj}	5.97^{fn}	3.93^{BC}
T6	1.65^{p}	2.42^{op}	2.73^{mp}	3.41^{jp}	3.72^{hp}	3.93^{hp}	4.21^{go}	4.83^{el}	5.81^{ch}	3.64^{C}
Mean	2.29^{H}	3.08^{G}	3.35^{FG}	4.07^{EF}	4.52^{DE}	4.95^{CD}	5.39^{BC}	6.08^{AB}	6.64^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters have significant differences among treatments at a significance level of $P \leq 0.05$

Table 9 Changes in coliform bacteria (log cfu/g) of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	2.20^{ln}	3.86^{em}	5.19^{cj}	5.81^{bg}	6.21^{ae}	6.71^{ad}	7.81^{ab}	8.41^{a}	8.61^{a}	6.09^{A}
T2	2.64^{ln}	2.85^{jn}	3.69^{fn}	3.93^{em}	4.32^{dl}	4.74^{cl}	5.30^{ci}	5.83^{bf}	6.71^{ad}	4.44^{B}
T3	2.35^{ln}	2.92^{in}	3.54^{fn}	3.72^{fn}	3.94^{em}	4.42^{dl}	5.10^{ck}	5.60^{bg}	5.89^{bf}	4.15^{BC}
T4	2.74^{kn}	3.03^{hn}	3.40^{gn}	3.82^{em}	4.63^{cl}	4.83^{cl}	5.40^{bh}	5.84^{bf}	6.91^{ac}	4.51^{B}
T5	1.40^{n}	1.67^{mn}	2.60^{ln}	3.10^{hn}	3.73^{fn}	3.92^{em}	4.62^{cl}	4.80^{cl}	5.83^{em}	3.51^{CD}
T6	1.04^{ln}	1.90^{mn}	2.65^{ln}	2.76^{kn}	3.61^{fn}	3.84^{em}	4.41^{dl}	4.74^{cl}	5.71^{bg}	3.40^{D}
Mean	2.06^{H}	2.70^{GH}	3.51^{FG}	3.85^{EF}	4.40^{DE}	4.74^{CD}	5.44^{BC}	5.86^{AB}	6.61^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters have significant differences among treatments at a significance level of $P \leq 0.05$

Table 10 Changes in yeasts and molds (log cfu/g) of fish steak samples during storage at 4 °C

Treatments	Storage period (day)									Mean
	0	3	6	9	12	15	18	21	24	
T1	3.86^{ho}	4.13^{gm}	5.20^{di}	5.71^{cg}	6.81^{bd}	7.26^{ac}	7.80^{ab}	8.82^{a}	8.90^{a}	6.49^{A}
T2	2.44^{mr}	2.34^{nr}	3.20^{jr}	3.50^{ip}	3.89^{hn}	4.43^{fk}	5.76^{cg}	5.92^{cf}	6.41^{be}	4.21^{BC}
T3	2.07^{ho}	2.32^{nr}	2.74^{kr}	3.41^{jr}	3.73^{hp}	3.94^{hn}	4.73^{ej}	5.41^{dh}	5.81^{cg}	3.79^{CD}
T4	2.47^{mr}	2.82^{kr}	3.47^{jq}	3.78^{hp}	4.43^{fk}	4.70^{ej}	5.41^{dh}	5.83^{cg}	6.62^{bd}	4.39^{B}
T5	1.77^{qr}	2.14^{or}	2.64^{li}	2.83^{kr}	3.52^{ip}	3.75^{hp}	4.72^{ej}	5.81^{cg}	6.01^{fj}	3.68^{D}
T6	1.53^{r}	2.32^{nr}	2.74^{kr}	3.42^{gr}	3.60^{ip}	4.32^{fl}	4.73^{ej}	5.74^{cg}	6.23^{be}	3.84^{CD}
Mean	2.35^{F}	2.67^{F}	3.33^{E}	3.77^{DE}	4.33^{CD}	4.73^{C}	5.52^{B}	6.25^{A}	6.66^{A}	

T1 Control, T2 Chitosan 2%, T3 Chitosan 2% and clove oil, T4 Clove oil, T5 Nanochitosan 2%, T6 Nanochitosan 2% and clove oil

Means within the same column having different capital letters are significantly different at a significance level of $P \leq 0.05$ for treatments, and means within the same row having different capital letters are significantly different for time of storage. Means within the same column having different small letters have significant differences among treatments at a significance level of $P \leq 0.05$

mean values between coated fish steaks. Similar results were found by Ramezani et al. (2015), who showed that the TBARS concentration of silver carp did not differ significantly among chitosan and nanochitosan coatings during cold storage.

The combination of nanochitosan and clove oil treatment had the lowest TBARS value. Zhang et al. (2008) demonstrated that the usage of Ch-tripolyphosphate nanoparticles maintained antioxidant activity in vitro, utilizing a free radical scavenging activity test and a reducing power test. This phenomenon might occur because of the nanochitosan's tiny particle size and large surface area per unit volume, which increased the chitosan's ability to trap OH radicals.

Microbiological changes in fish steaks during refrigerated storage

Aerobic plate counts

Microbial activity is a limiting factor in the quality of the fish, APC are used as an acceptability index for fish products because of the importance of bacteria in spoilage. Table 7 shows the APC in fish steak samples during cold storage. At time zero, the APC of the control sample was 3.84, reaching 8.90 (log cfu/g) by the end of the refrigerated storage period. The rise in APC in the control sample may be due to a rise in simple nitrogenous molecules (amino acids and nucleotides) and fatty acids created by the degradation of fat and protein by native fish enzymes, which resulted in

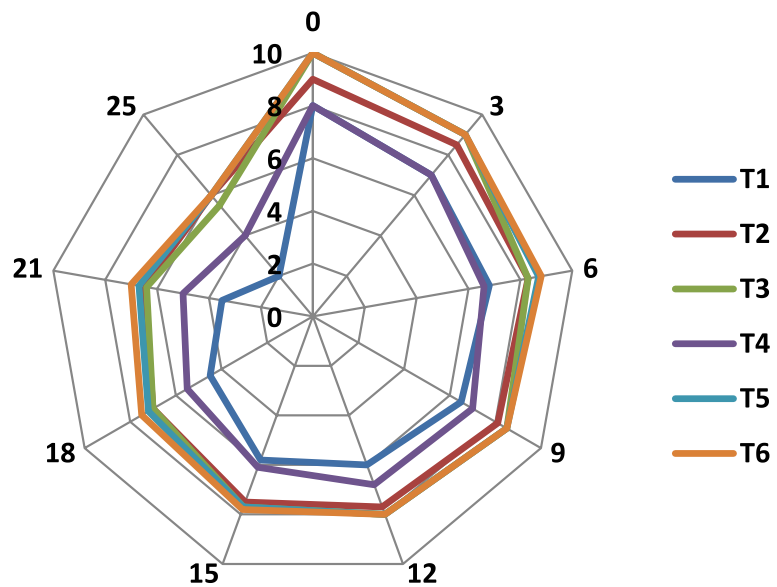


Fig. 2 Changes in color of fish steak samples during storage at 4 °C

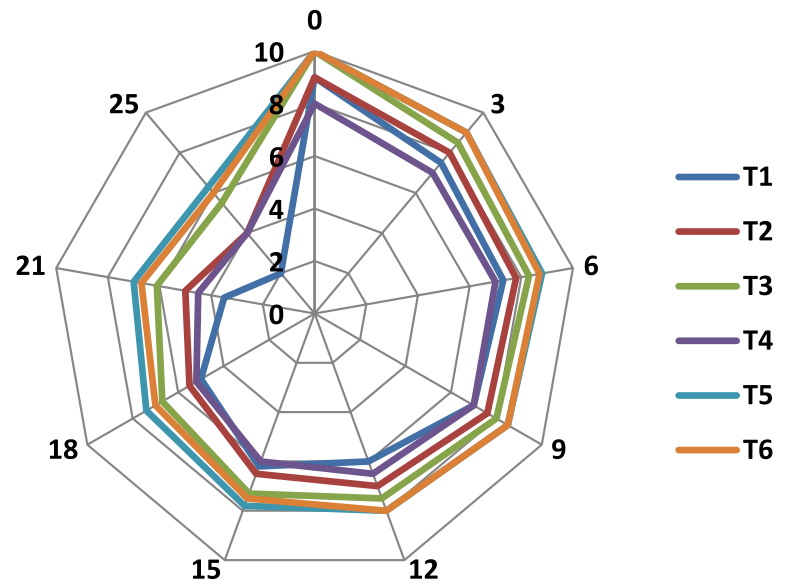


Fig. 3 Changes in odor of fish steak samples during storage at 4 °C

appropriate conditions for the growth of bacteria. The mean APC values of treated mullet steaks samples (T2-T6) were around 3.50–4.39 log cfu/g. These values did not reach the maximum permissible limits (7 log CFU/g) specified by the International Commission on Microbiological Specifications for Foods for fresh fish (ICMSF 2002). The APC decreased after the addition of chitosan, clove oil, and nanochitosan, due to their synergistic influence.

The inhibitory impact of nanochitosan coating on microbes may to be related to the oxygen parrying ability of nanochitosan coatings, which inhibit the entrance of the O_2 necessary for microbial respiration (Abdel-Wahab et al. 2020).

Psychrotrophic bacteria

Psychrotrophic bacteria like *Alteromonas, Shewanella*, *Flavobacterium,* and *Pseudomonas* have been identified as the predominant bacteria in fish. As shown in Table 8, the initial level of psychotropic bacteria in the various treatments ranged from 1.65 to 2.72 log CFU/g and rose throughout the storage period. After the twelfth day of

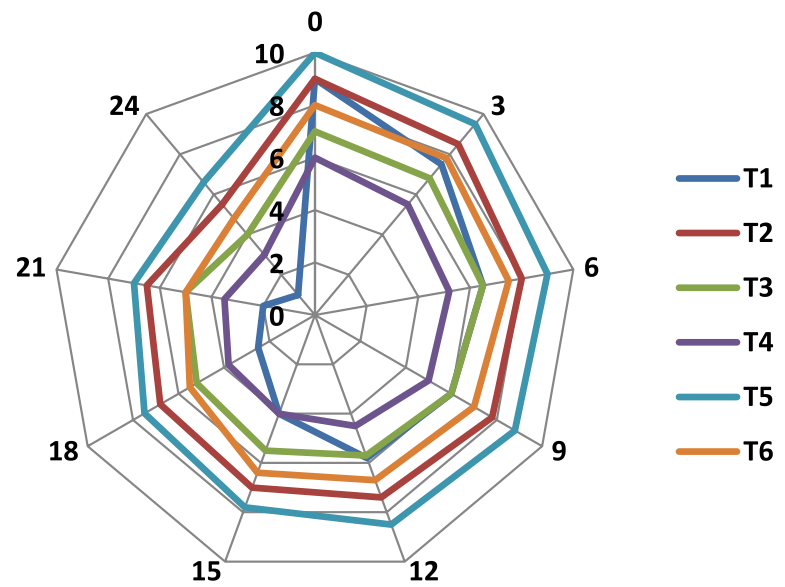


Fig. 4 Changes in taste of fish steak samples during storage at 4 °C

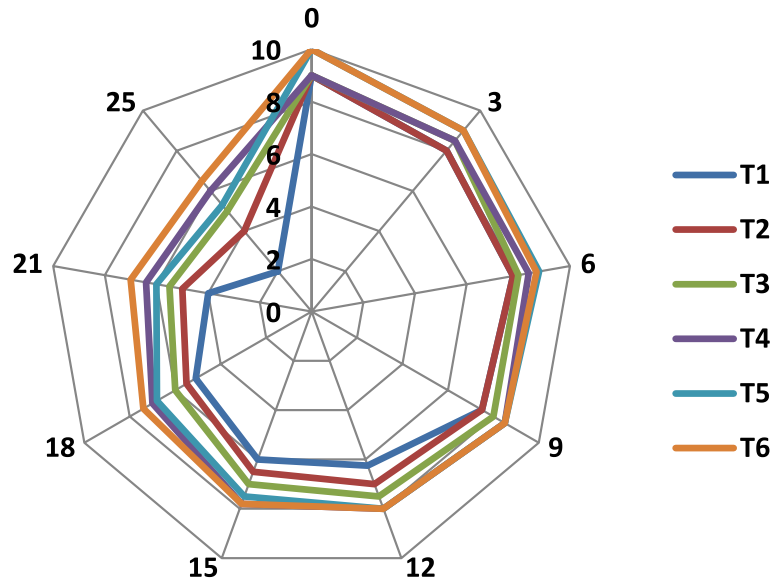


Fig. 5 Changes in texture of fish steak samples during storage at 4 °C

refrigerated storage, the control samples surpassed the allowable limit level (7.41 log CFU/g). Ojagh et al. (2010) also revealed that utilizing chitosan coatings combined with essential oils could keep psychrotrophic bacteria at acceptable limits for up to 12 days of cold storage.

Coliform bacteria

Table 9 shows the levels of coliform bacteria in fish steak samples during cold storage. The coliform bacteria count mean in fish steak samples ranged from 3.40 to 6.09 log cfu/g. A significant ($P \leq 0.05$) increase in coliform bacteria was detected for the control treatment compared to the other treatments. Treatments containing chitosan, or combining nanochitosan and clove oil (T5 and T6), had the lowest number of coliforms because of their antibacterial properties.

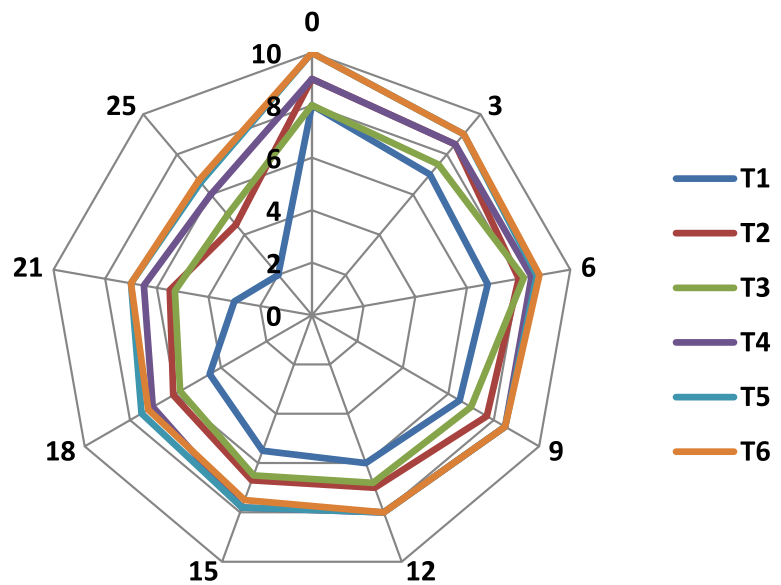


Fig. 6 Changes in appearance of fish steak samples during storage at 4 °C

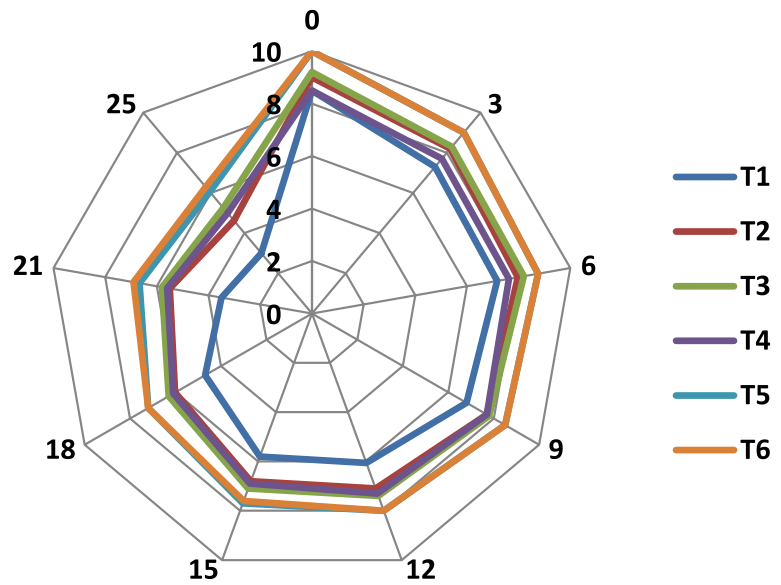


Fig. 7 Changes in general acceptability of fish steak samples during storage at 4 °C

Yeasts and molds

Table 10 shows the count of yeasts and molds in fish steak samples during refrigerated storage. At time zero, the count of the control was initially 3.86 log cfu/g, and reached 8.90 log cfu/g at the end of the refrigerated storage time. Treatment containing nanochitosan coating (T5) had the lowest mean yeast and mold counts (3.68 log cfu/g). Chitosan's fungicidal and bactericidal activity is believed to be achieved by electrostatic interactions among the protonated amino group in chitosan and the negative groups on cell surfaces, which retard the microbial growth.

Sensory evaluation of fish steaks

Color

The changes of color of fish steaks are given in Fig. 2. All sensorial scores for the control group over the storage time were significantly lower than those of the treated samples. The color score for the control sample was acceptable for only 12 days of storage. Statistical analysis indicated that treatments and refrigerated storage produced a significant effect on color changes during storage period.

Odor

Changes of odor of fish steaks are shown in Fig. 3. On the initial 0-day of storage, the odor attribute of clove oil treatment had the lowest odor score (8), probably due to the distinct flavor of clove oil, while the nanochitosan treatments had the highest odor score (10). Songsaeng (2014) found that the odor score decreased with the concentration of clove oil. The treatments and storage time produced statistically significant ($P \leq 0.05$) effects on the odor scores of fish steaks with nanochitosan having the highest mean odor score. According to Soultos et al. (2008) and Roller et al. (2002), the addition of nanochitosan to sausage improved the acceptability of odor and flavor.

Taste

Changes of taste of fish steaks are shown in Fig. 4. On the initial 0-day of storage, the taste attributes of clove oil samples were the poorest, probably due to the distinct flavor of clove oil, while the control samples had pleasant taste. At the end of storage, the control sample had the lowest taste score (1) and treatment containing nanochitosan 2% had a relatively high taste value (6.5). The treatments and storage periods had significant effects on the taste of the fish samples.

Texture

The sensory changes in the texture of fish steaks are presented in Fig. 5. Clove oil treatment produced a higher mean texture value (8.10) than those of the control and other samples. At the end of storage, the control sample had the lowest texture score (2) and treatment containing a combination of chitosan 2% and clove oil had a high texture value (6.5). Throughout the storage time, the mean texture values declined consistently, with a significant difference ($P \leq 0.05$) from an initial 9.3 to 4.8 at the end of the storage period.

Appearance

Figure 6 shows the changes in the appearance of fish steak samples during cold storage. The treatments and

storage periods had significant effects on the appearance of the fish samples. All samples had acceptable sensory characteristics at the beginning of the storage period, which declined as the storage time increased. Statistical analysis indicated, treatments and refrigerated storage had a significant effect on the appearance of the fish during the storage period.

General acceptability

Samples were regarded as safe for human consumption until the sensory value reached 4 (Ojagh et al. 2010). Figure 7 shows changes in the general acceptability of fish steak samples during cold storage**.** Control sample scores declined rapidly with increasing storage time. However, treatments improved the acceptability score, with the highest mean score for the samples coated with nanochitosan (8.05). Li et al. (2012) found a significant decline in general acceptance after eight days of storage of uncoated large yellow croaker, which corresponded well with a concomitant increase in bacterial counts.

The use of a nanochitosan and clove oil-based coating for fish steaks resulted in the preservation of sensory quality by preventing oxidation. Nanochitosan has been shown to be an effective antioxidant and chelating agent, allowing it to react with oxidation enzymes and prevent their impact (Tayel 2016).

Conclusions

The shelf life of fish steaks refrigerated storage can be prolonged by using natural edible coatings like chitosan, nanochitosan. Also, a combination of nanochitosan and clove oil treatment was more effective in improving the chemical, microbiological and sensory characteristics of the fresh fish steaks than the other treatments studied during refrigerated storage for 24 days. These findings can be bases for producers to provide consumer with fresh fish steaks with good shelf life at refrigerated temperature.

Abbreviations

CSNPs: Nanochitosan; TEM: Transmission electron microscopy; TVB-N: Total volatile basic-nitrogen; TMA: Trimethylamine; PV: Peroxide value; TBARS: Thiobarbituric acid reactive substances; MDA eq: Malondialdehyde equivalent; APC: Aerobic plate count; TPC: Psychotropic counts; VRB: Violet red bile; ANOVA: Analysis of Variance.

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Authors' contributions

Samar Aref: data collection, sample analysis, and writing the manuscript. Ramadan Habiba: Research conceptualization and design, acquisition of funds, research supervision, reviewing and final editing of the manuscript. Noha Morsy: reviewing the manuscript and Research design. Fatma Zayet: helped in sample collection. Mohamed abdeldiam: helped in sample collection. The authors read and approved the final version of the manuscript.

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Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Food Technology, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt. [2]Department of Food Technology (Home Economics), Faculty of Agriculture, Suez Canal University, Ismailia, Egypt. [3]Department of Pharmacology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt.

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