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ORIGINAL PAPER

Assessment of nanochitosan packaging containing silver NPs on improving the shelf life of caviar (*Acipenser persicus*) and evaluation of nanoparticles migration

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Abstract

Nano packaging plays a major role in reducing microbial load and preserving proper quality. The aim of this study was to evaluate antimicrobial characteristics of nanochitosan-silver packaging against both Gram positive and negative microorganism. The total count test was used to measure the antimicrobial effects. Additionally, the minimal inhibitory concentration and minimum fungicidal concentration of nano chitosan were both measured. Finally, the antimicrobial effects of the silver and chitosan nanoparticles were evaluated. Caviar samples were inoculated to *Coagulase-positive Staphylococcus aureus*, *Escherichia coli* using 0/5 McFarland standard. Also, *Fusarium solani* was inoculated to caviar samples using 10^5 CFU/mL. Samples were checked out in the 1st, 15th, 30th, 60th, and 70th days, respectively. To determine the size and distribution of nanoparticles) NPs), scanning and transmission electron microscopy (SEM&TEM), dynamic light scattering (DLS), and atomic force microscopy (AFM) analyses were used. The images from SEM and TEM were indicative of spherical silver nanoparticles (AgNPs), and the average size of the AgNPs was lower than 100 nm. The average size of chitosan particles was 60–100 nm as revealed by DLS and Zeta potential analyses, and the chitosan nanoparticles were completely homogenous. The results showed that silver and chitosan NPs had significant antibacterial effects compared to blank low-density polyethylene samples ($P < 0.05$), and reduced the bacterial and fungal loads more than one logarithmic cycle. Migration of nanoparticles was measured using the atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectrometry (ICP-MS). AAS results showed no migration of nanoparticles although, the ICP-MS showed 0/165 ppm from nanopackaging.

Keywords Migration · Nanochitosan · Nanosilver · Antimicrobial · LDPE · Caviar

Introduction

Caviar is a nutritional food containing high amounts of protein (22–28%) and lipid (15–78%), as well as different types of vitamins [1–3]. Recently, numerous studies are conducted on the preservation of seafoods, such as caviar. However, methods such as modified atmospheric packaging (MAP) and high hydrostatic packaging cannot decrease microorganisms significantly, prevent fat oxidation, or diminish enzymatic activity. Therefore, to address the aforementioned problems, the application of packaging techniques that contain various nanoparticles have gained more attention [4–6]. Mixture of nanoparticles with Packaging matrixes imply antimicrobial effects that can help increasing the shelf life of food products [7–11]. Nanoparticles smaller than 50 nm, have greater antimicrobial activities, which is a direct result of the increased ratio of the surface area to overall volume [12–16].

As a natural biodegradable carbohydrate polymer, Chitosan (poly-β-1-4-*n*-acetyl-d-glucosamine) originated from the acetylation of chitin [17–21]. Numerous studies have reported the antimicrobial role that chitosan plays against a variety of food pathogens [21, 22]. A few factors can affect nano chitosan antimicrobial activity, including molecular weight, pH, specific chitosan type, as well as the degree of polymerization. Some studies investigated the antimicrobial effects of Chitosan. For example, Hu et al. (2015)

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investigated the result of Chitosan-cinnamon on pork meat in 15 days at refrigerator temperature [23]. Maghami et al. (2019) evaluated the effects of chitosan nanoparticles and the essential oils of fennel along with MAP on *Huso huso* fish fillet shelf life after being in refrigeration temperatures for 18 days [17]. Hassanzadeh et al. (2018) evaluated the effect of 2% chitosan coating along with 0.10% grape seed extract (GSE) on microbial (*Mesophils* and *Psychrophils* counts), chemical (thiobarbituric acid and peroxide value (PV)) and sensorial properties on rainbow fillet at 4 °C during 15 days [24]. Other studies have investigated the antimicrobial effect of silver NPs. The antimicrobial activity mechanism of silver nanoparticles is as follows: silver ions released from the Nanosilver package, which prevent or reduce microbial growth. In other words, silver reacts with the thiol groups of microorganisms enzymes, resulting in cell death [25–28]. In 2019, Anvar et al. investigated differing percentages of silver NPs on the reduction of Beluga caviar microbial [29]. Paidari et al. (2021) conducted a study on evaluation of antibacterial activity of Silver and Clay nanocomposites against *E. coli*, *S. aureus* and *V. parahaemolyticus* inoculated to shrimp samples [30]. Hosseini et al. (2017) and Barani et al. (2018) evaluated the effects of silver Nanofilms on *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*) that inoculated in *Penaeus semisulcatus* and *Sander lucioperca* samples respectively, then by utilizing the disk diffusion methodology they measured the inhibition zone diameter [31, 32].

Due to their high thermal resistance and extensive spectrum effects on different batteries, the silver nanoparticles mainly use in the antimicrobial packaging. However, its maximum amount of migration (0/01 mg/kg) should be assessed [33]. According to the commission regulation (EC) NO.10/2014, the maximum migration limit of non-authorized substances through a functional barrier is 0/010 mg/kg. Besides, the size, concentrate, and molecular weights of food samples, as well as the distribution of nanoparticles, are critical essential criteria for evaluation of the migration rate in food samples [34]. Since the migration ratio of the NPs is a significant concern, there are numerous studies on the measurement of Ag^+ NPs For instance, Pezzato et al. (2015) evaluated the migration ratio of silver in cheese samples for 24 days using the atomic absorption spectroscopy (AAS) and inductively coupled plasma mass spectrometry (ICP-MS) methods [35]. Besides, Li et al. (2018) evaluated both the physicochemical and microbial properties of cottage cheese, which were packed using poly lactic acid (PLA), PLA/TiO_2, as well as PLA/TiO_{2+}Ag, and were stored at 5 + 1 °C for 25 days [36]. They also analyzed the movement of Ti and Ag nanoparticles from the films to Hexane as a simulant solution using the inductively coupled plasma atomic emission spectroscopy (ICP-AES). Moreover, Polat et al. (2018) took measurements of silver nanoparticles migrating from polypropylene into isooctane, ethanol, and acetic acid [37].

This paper aimed to investigate the potential antimicrobial effects of Nano chitosan containing 7% nanosilver films on caviar samples as well as to measurement of their migration rates using AAS and ICP-MS techniques during the storage period.

Materials and methods

Caviar (*Acipenser persicus*) was purchased from the culture food industry company. Chitosan samples and 7% silver nanofilms were obtained from Sigma (Denmark) and Iran polymer institute, respectively. *Fusarium Solani* (*F. solani*) (PTCC 5284), *S. aureus* (PTCC 1431), as well as *E. coli* PTCC (1399) were obtained through the Persian Culture Collection Institute (PTCC, Iran). Sabouraud dextrose agar (SDA), tryptic soybean broth (TSB), plate count agar (PCA), MacConkey agar (MCA), *Escherichia coli* broth (ECB), and Baird-Parker agar (BPA) were purchased from Merck chemical Co.

Preparation of nano chitosan suspension

In this study, we prepared the nano chitosan by the methodology developed by Jafari et al. with modification [38]. 0/3 g chitosan was poured into 100 mL of distilled water containing 1 cc acetic acid. After 3 h of stirring and adjusting the temperature to 40 °C, a brown solution was prepared. Then, Glycerol was added to the solution (0/75 cc per 1 g of Chitosan) as a softener. The prepared solution was purified using the Watman paper (grade3) under vacuum condition. Following this, we added 2% tween 80 to the chitosan solution, and then the solution was stirred for a period of 30 min. Lastly, the solution was sonicated at 80 and 40 kHz frequency.

Sterilization procedure

Since the dominant microorganism existing in caviar are yeasts, as mentioned in a review conducted by Safari and Razavilar (2003) yeasts increase the microbial load dramatically, several methods are developed to decrease microbial count, which can be used in combination with salt [38]. As a result, to perform the fungal test, the caviar sample was sterilized using different methods. In the first method (A) according to Ovissipour et al. (2018), due to organoleptic changes, the optimum temperature to pasteurization of fish roe in the hot water bath within a glass of jar should not exceed 70 °C, so according to this theory, 25 g of caviar was inserted into a jar glass and immersed at 65 °C water for 70 min [39]. Additionally, in a study conducted by Moini et al. (2009), they noted that the gamma radiation up to 3 kGy resulted in the highest quality of the fish fillets, plus it decreased microbial

contamination [40]. Hence, Gamma-ray 3 kGy was used to sterilize 50 g of caviar (sample (B) for 28 min). Finally, it is important to note that various studies investigated using ultraviolet radiation to increase the shelf life of food, such as chicken meats and rainbow trout fillets.[28,29] Therefore, caviar samples were exposed to ultraviolent-C (UV-C 254 nm) light after gamma radiation for 1 h (C) and 3 h (D).

Evaluation of antibacterial and antifungal effects of nano chitosan suspension and nanosilver film

We performed fungal tests in accordance with the CLSI[1]M38 standard. Using the microdilution method, we predicted the minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC), as described in the following: initially, 100 µL RPMI was added to each 69 well plates, then 100 µL of chitosan solution was inserted, and the serial dilution was prepared. Finally, 100 µL of fungal suspension stock was poured into each cell. The prepared solutions were put into incubator about 28 °C for 48 h. To determine MFC, 100 µL of MIC and former MIC cells were cultured into SDA at 28 °C for 72 h.

To determine the MIC and MFC, the microdilution method was used. Initially, 12 test tubes, each containing 1 cc Mueller Hinton broth (MHB), were used to prepare a serial dilution. 1 cc of the nano-chitosan solution was poured into the first test tube, and finally, 0/5 McFarland was added. Eventually, the test tube was kept at an incubator at 37 °C for 24 h [41].

Caviar samples were transferred to the laboratory under cold conditions, and *E. coli* (PTCC1399) *Coagulase S. aureus* (PTCC1431) and *Fusarium solani* (PTCC 5284) were inoculated to caviar samples. The total count test was performed according to the ISO 4833-1:2013. Besides, we performed identification and enumeration of *E. coli* and *S. aureus* according to the ISO (7251, 4832, 6888-1 and 6888-3), respectively. Total count test was performed for all samples in each day. Per every 5 g of caviar, 550 µL *Fusarium solani* was added. Also, *S. aureus* and *E. coli* were inoculated to samples using the 0/5 McFarland concentration. Using the inoculation method, 312/5 µL and 156/25 µL nano chitosan were added to the samples to evaluate the MIC of bacterial load and MIC of fungal load, respectively. Then, caviar samples were packed using the 7% nanosilver packaging and LDPE packaging as a blank. Nano silver packaging was sterilized using 70% alcohol and UV light for 6 h. Finally, to prepare serial dilution, 1 g of each sample was inserted into the tubes and serial dilution was prepared. Cultures were incubated according to Barani et al. (2018) [32].

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Analyses of SEM and TEM

To evaluate the surface characteristics of nanofilms, the SEM was used. Firstly, the samples were sliced into 2×2 cm^2 sections. Next, the films were all golden coated with a thickness of 15 nm (sputter coater, KYKY, china). Following a period of ten minutes, the films were then relocated to the scanning electron microscope (KYKY-EM3200). As which point, we obtained a SEM image at a voltage of 26 kV and magnified of 40KX. TEM analysis was performed using ZIESS-EM900 (Germany), and the TEM showed that the internal structure of the film and the sample preparation were very sensitive; this process was controlled by an operator, because the sample had to be turned into a thin sheet [31].

Atomic force microscopy (AFM)

Silver NPs surfacy morphology was surveyed, with 2D and 3D forms prepared, using AFM analysis [13].

Dynamic light scattering (DLS)

To measure the size of NPs in aqua solution (Nano-chitosan) Dynamic Light Scattering device was used (HORIBA-SZ100, Japan) and the concentration of the sample was the most crucial factor to determine the particle size. Therefore, it was essential to prepare the samples and achieve the appropriate concentration [42].

Migration of NPs

To measure the migration of silver NPs to caviar samples, Atomic Absorption Spectroscopy was used (VARIYAN-200, Australia). To achieve higher precision, induced conductive Plasma-Mass Spectroscopy (ICP-MS) was used. To digest caviar samples, 5 g of caviar were digested in a test tube using nitric acid (HNO_3) (5 mL) and sulfuric acid (H_2SO_4) (5 mL). After passing a while, the test tube was inserted into the digestive device and heated for a while (60 °C, 30 min). After cooling down, 10 mL HNO_3 was poured out into the test tube and heated again (120–150 °C). After cooling down, 1 mL hydrogen peroxide (H_2O_2) was added to the test tube. Finally, the solution becomes transparent, and absorbance was gained [31].

Statistical analyses

Data were Study data analyzed by conducting an ANOVA (analysis of variance) in Excel 2010 and SAS software version 9.4. In using LSD (least significant difference), we were able to conduct multiple comparisons of the treatments with

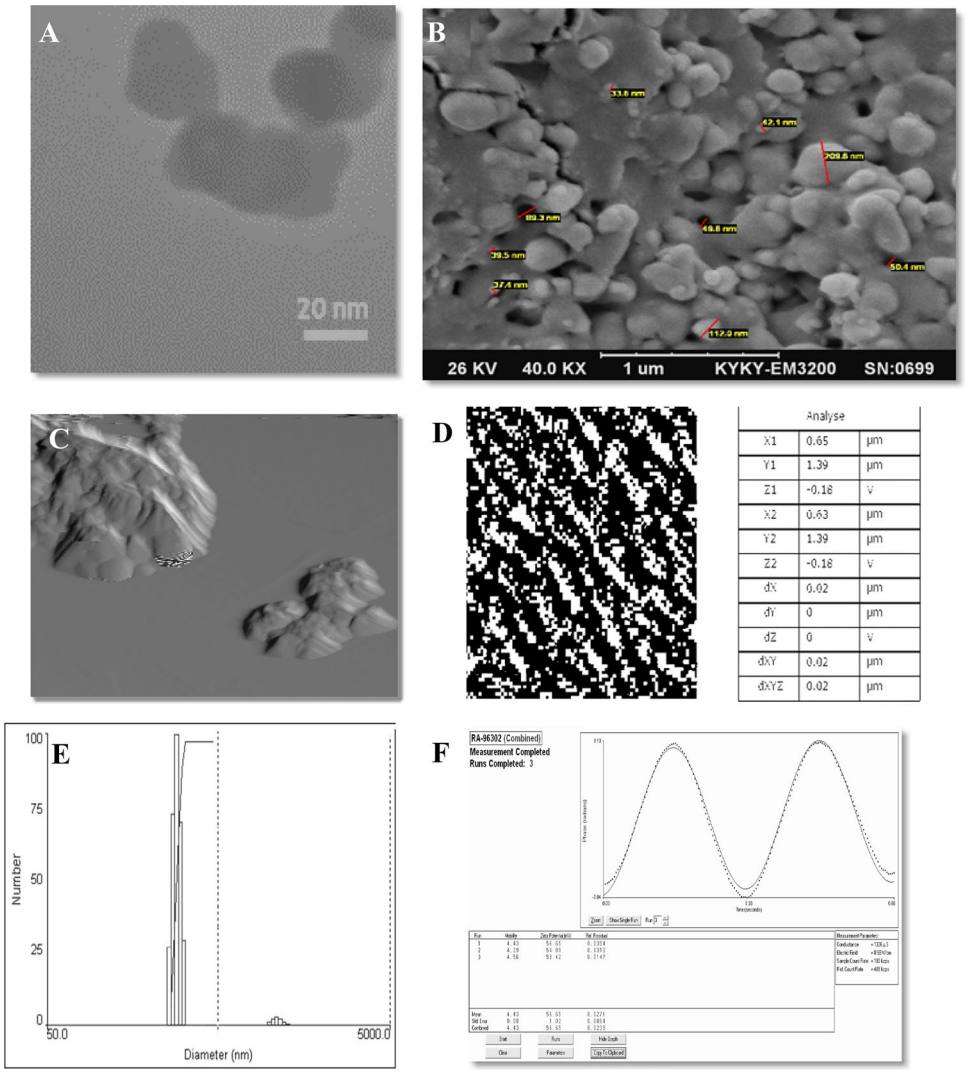

Fig. 1 **A** TEM images of nanosilver coating. **B** Depicts size, shape, and distribution of silver NPs in LDPE film by scanning electron microscope. **C**, **D** AFM analysis of silver Nano powders. **E** Result of DLS analysis for nano chitosan. **F** Result of zeta potential for nano chitosan

substantial differences in an ANOVA test. All test were carried out in triplicate.

Results

SEM and TEM analyses

The SEM and TEM analyses of Ag-LDPE films are shown in Fig. 1A, B respectively. According to the figure, the shape of AgNPs was spherical, and the average size of AgNPs was lower than 100 nm.

AFM analysis

Figure 1C and D show the size and structure of AgNPs using the 3D software. Atomic force microscopy (AFM) figure analysis noted that the silver particle powders were approximately 20 nm in size.

Dynamic light scattering analysis

Analysis of DLS (Fig. 1E (used for nano chitosan solution revealed the average size of NPs, in which the size of the Chitosan NPs was mainly between 60 and 100 nm. Also, zeta potential analysis showed the unique distribution and stability of the chitosan solution (Fig. 1F).

ICP-MS and AAS results

Since there is no clear attitude toward the application of NPs and its potential hazards, evaluating its potential release to food material from nanosilver is necessary. To evaluate the potential migration of silver NPs from 7% nanosilver film to caviar samples, the ICP-MS, and AAS analyses were performed at the 30th and 60th days of the experiment. It worth noting that, according to the previous studies, the ICP-MS showed higher precision than AAS However, the precision of these two methods is comprised of the current study.

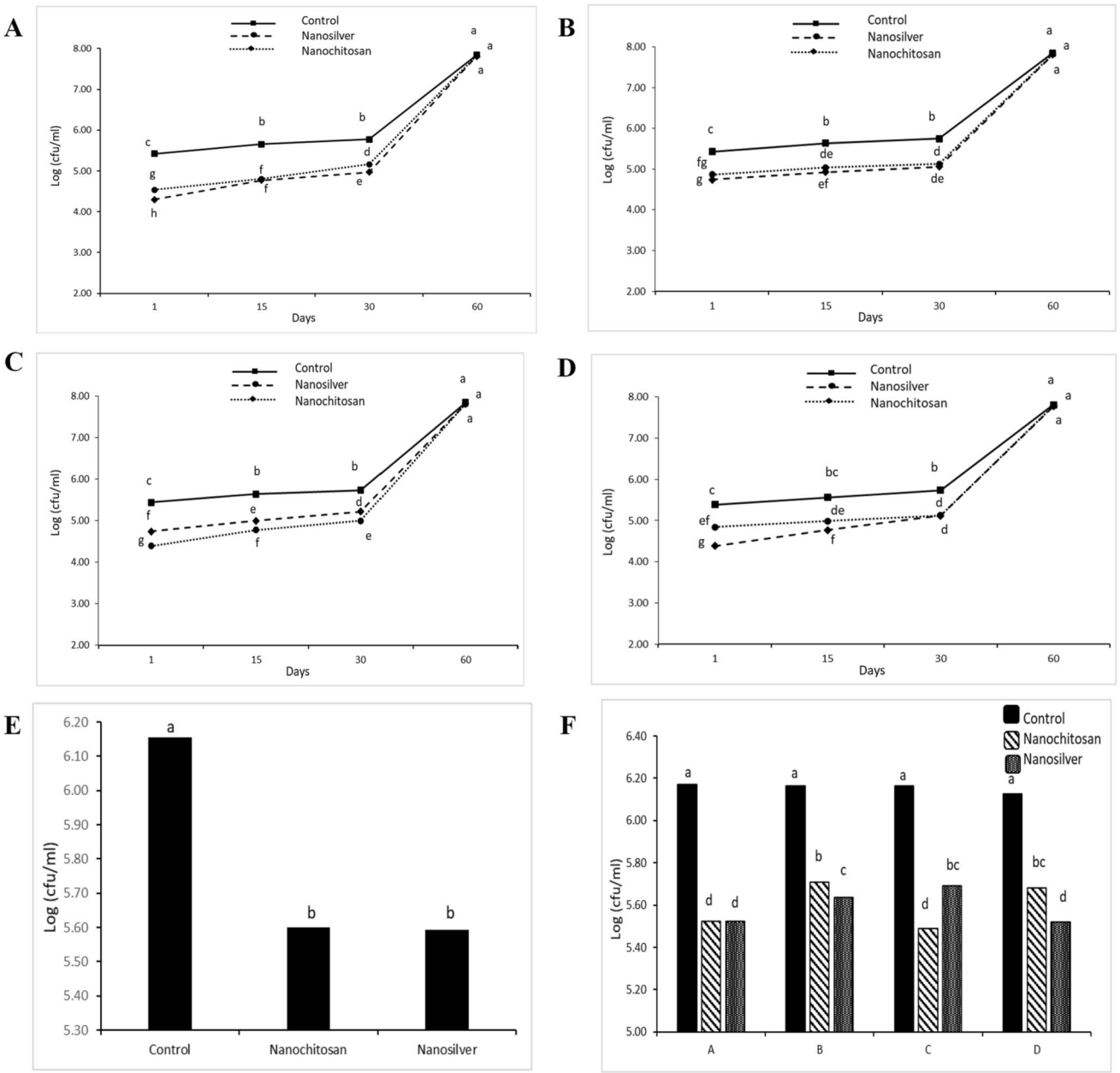

Fig. 2 **A**–**D** Mean comparison of nano treatments during the days in A, B, C, D sterile methods in *F. solani*. **E** Log (CFU/ml) in each Nano treatment in *F. solani.* **F** Mean comparison of CFU in Nano treatment and sterile method in *F. solani*

According to the results, although. AAS results showed no migration from nano packaging, the ICP-MS results showed that 0/165 ppm of silver was released to caviar samples at the 30th and 60th days of the experiment.

Antibacterial and antifungal analyses

The trend in Fig. 2A–D demonstrates, the number of colonies on the 1st, 15th, and 30th days in the blanks increased considerably. According to nano chitosan and nanosilver treatments, there was no significant difference between nano chitosan and nanosilver ($P < 0.05$) (Fig. 2E). However, we noted that between nano chitosan and nano silver, there was a statistically significant difference ($P < 0.0001$). As shown in Fig. 2F, in general, the combined use of sterile methods, nano chitosan, and nanosilver more reduced the microbial colonies compared to blanks (LDEP) ($P < 0.0001$). While only in the sterilized sample A, there was no significant differences between nano treatments, although there was a difference among sterilized samples, including B, C, and D ($P < 0.0001$). In the samples B and D, the effect of nanosilver fungicidal was greater than nanochitosan.

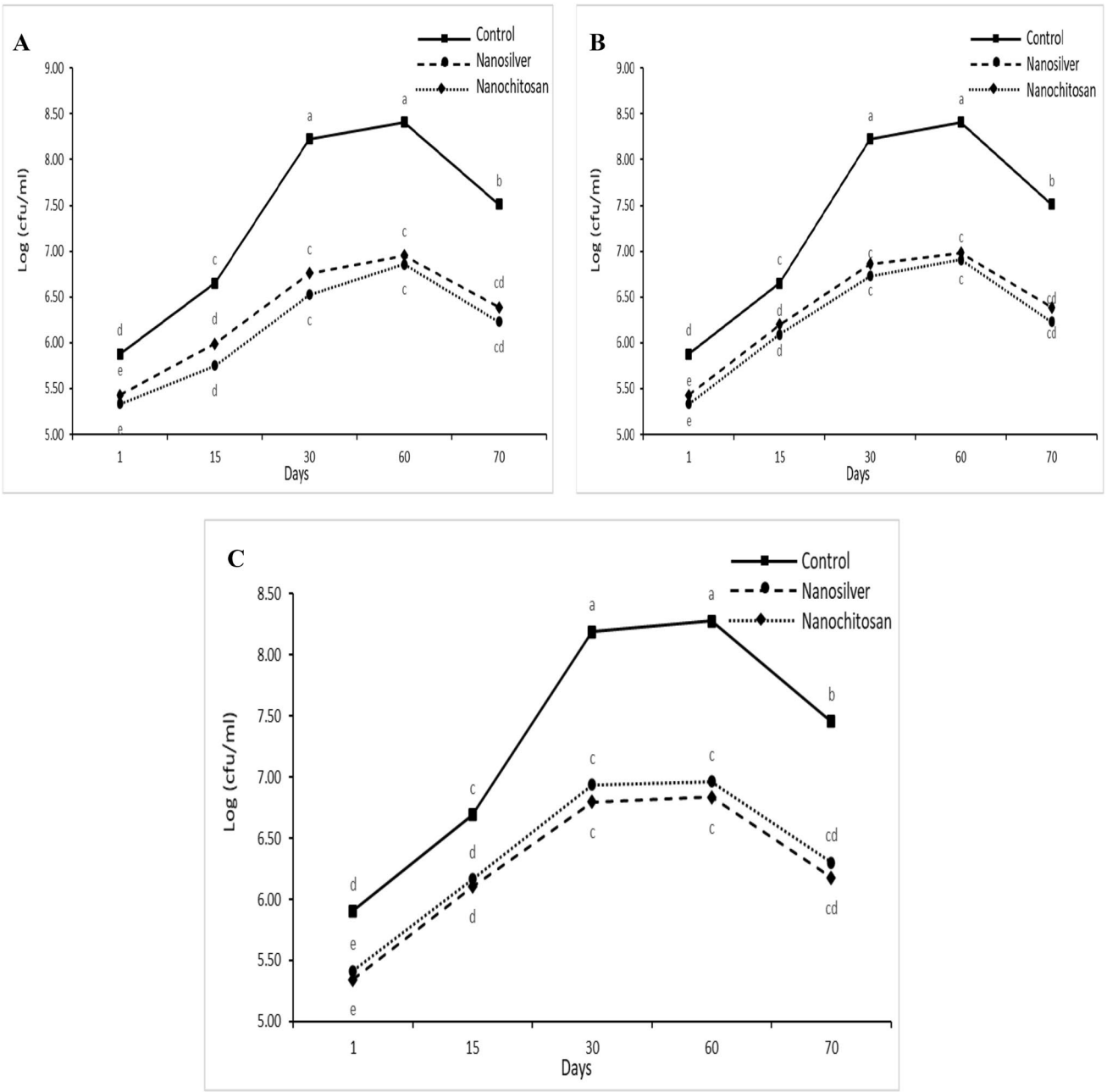

Fig. 3 **A** Mean comparison of nano treatments during the days in total count. **B** Mean comparison of nano treatments during the days in *staphylococcus areuse*. **C** Mean comparison of nano treatments during the days in *E. coli*

According to Fig. 3A–C, the number of colonies on 1st, 15th, 30th, and 60th day in blanks increased significantly. Compared to the nano treatments and for all periods, the number of colonies decreased, mainly due to bacteria death. For instance, on the 30th and 60th days, nano treatments caused more reduction than one log of staph and *E. coli* in comparison with the control. Generally, we did not observe any statistically significant differences attributed to the number of colonies in nano treatments, all day long and between 30 and 60th days, went up slightly in blanks and nano treatments.

Discussion

Packaging technology is improving due to the high demands of consumers for longer shelflife periods. Besides, because of the antimicrobial effects of NPs, more attention is paying

to them. Hence, further studies should be conducted to investigate its potential hazards or migration of NPs [13].

Hu et al. (2015) showed that chitosan-cinnamon could significantly decrease the microbial load of pork meat 15 days after the experiment [23]. In their study, Maghami et al. (2019) noted an increase in the shelf life of *Huso huso* fish after utilizing nano chitosan and essential oil of fennel, in addition to MAP packaging [17]. Hassanzadeh et al. (2018) have demonstrated that coating with Chitosan singly and combined with grape seed extract had significant effect on reducing the microbial population (*Mesophilic* and *Psychrotrophic*) compared to control and GSE, between control and CH-GSE coated was depicted less than 1.20 log cycle difference also, PV value and TBA were highest in control samples. The control samples decreased the sensorial quality of rainbow trout fillets after nine days storage whereas, coating with Chitosan and CH-CSE showed minimized spoilage signs [24]. In 2019, Anvar et al. reported that the average size of silver NPs was 20–45 nm, and *E. coli, S. aureus*, A. *flavus* were observed in the blank samples [29]. Also, Hosseini et al. (2017) conducted a study on silver packaging. They reported that the average size of used NPs was 30–48 nm, which was approximately the same as that of ours. The inhibition zone had the largest size on the first day of the experiment [31]. In 2013, Barani et al. conducted a study where they demonstrated that 63 nm silver particles significantly decreased *S. aureus* and *E. coli* load during shelf life. On the other hand, they used the sol–gel and melt mixing method to evaluate the antimicrobial effects. Their results revealed that the melt mixing methodology had a superior antimicrobial effect compared to the sol–gel producing methodology [32]. In line with the current study, Lotfi et al. (2019) reported that nanosilver increased chicken meat shelf life, as well as limited bacterial growth, including both *E. coli* and *S. aureus*. Also, a film that contained 5% Ag and 5% TiO_2 inhibited the growth of colonies more effectively, but contrary to the results of the current study, this effect in *S. aureus* was higher than *E. coli*. Moreover, according to our study, Ag nanoparticles were spherical in shape, with an average size of 20 nm [12]. Consistent with our study, Ghasemi-Varnamkhasti (2018) reported that combined use of gamma-rays and polyethylene films containing silver nanoparticles was effective in maintaining the quality of bottom mushroom during shelf life [43].

In an experiment conducted by Pezzuto et al. (2015) migration of silver NPs was 250 times higher than EFSA standards. In 7 days, the total migration of silver NPs was 0/103 mg/kg; however, in the current study, 0/165 ppm was released to caviar samples after 60 days [35]. In line with the current study, in 2018 Li et al. noted that the total bacterial colonies in PLA./TiO_2 and PLA./TiO_2 + Ag significantly lower than PLA and LDPE in all days $P < 0/05$ and the amount of Ag, Ti nanoparticles migration was less than the threshold of 10 mg/kg [36]. Also, consistent with our results, Polat et al. (2018) reported that the amount of silver migration from nano packaging was lower than the European standard [37]. According to the result, the application of small sized nanoparticles as antimicrobial packaging as well as nano chitosan can significantly reduce the microbial load of both bacteria and yeasts during specific storage time. In other words, silver particles reduce the bacterial load of more than one logarithmic cycle. Additionally, the study results demonstrated that between the Gram-negative and Gram-positive bacteria, there was no significant difference. The migration rate of silver from the packaging cover to the caviar sample was measured using the ICP-MS and AAS on 30th and 60th days, and it was revealed that the AAS device did not record any migration, while the ICP-MS device, which is a more accurate device, recorded a migration rate of 0.165PPM.

Conclusion

According to the results of the current study, application of nano chitosan packaging containing various percentage of silver nanoparticles can lead to a significant decrease in microbial load of caviar samples in prolonged shelf life periods. Based on chemical results, application of nanoparticles having lower size could intensify the antimicrobial effects. Application of wider spectrum on nanoparticles as well as different methods of synthesis of nano chitiosan particles is suggested for further studies.

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Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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