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Research Article

Influence of Ag/LDPE nanocomposite films on the microbial growth of Beluga (*Huso huso*) fillets during the refrigerated storage period

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ABSTRACT

In the present study, 7% silver-low-density thin polyethylene films were successfully prepared through the melt mixing method. These films were applied to reduce the harmful antibacterial properties of *Huso huso* fillets at refrigeration temperatures, thus extending their shelf life. The total viable bacteria count for *H. huso* fillets wrapped in a 7% Ag/LDPE nanocomposite was 3.04±2.43 (log CFU/g) in 10^{-2}-diluted fish samples, which is significantly lower than those of the control and the LDPE group after two days of cold storage. The lowest psychrophilic bacteria count (log CFU/g) was recorded for the 10^{-2}-diluted fish samples encased in Ag/LDPE-NC in which *Staphylococcus aureus* count (SAC) reached 1.3±1.40 log CFU/g on days 2 and 4 at 10^{-2} dilution, respectively. It was extraordinarily going up and reached 6.97±3.91 log CFU/g for group Ag/LDPE showing high effectiveness of Ag/LDPE on retarding the SAC sampled from the caviar fish fillets up to the fourth day. It is concluded that application of 7% Ag/LDPE film produced by the melt mixing method can remarkably retard growth of bacteria on fresh *H. huso* fillets. Thereupon, the total viable bacterial count and psychrophilic bacteria growth and *S. aureus* activity were significantly mitigated up to four days of preservation at the refrigerator at which the values were below acceptable limits for fresh fish.

Keywords: Nanocomposite, Ag/LDPE, *Huso huso*, Fillet, Storage

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Introduction

Today, various types of packaging have been introduced throughout the world. In general, food packaging extends shelf life of food, medicines, and many other perishable materials before they are made available for sale or use (Tsironi and Taoukis, 2018). New packaging technology prolonged storage, appearance, shape, and the texture of foods through storage and transportation (Mital *et al.*, 2015). Food packaging is modified by several materials, which enables the quality of the products to be well-maintained (Shahbazi and Shavisi, 2019).

Among these materials, LDPE is a polymer that is extensively used in food packaging due to its low price, strong physical capacity, water-blocking characteristic, and transparency (Azlin-Hasim *et al.*, 2016), although some reports evidenced that it caused side effects concerning the physiological attributes of fish (Banaee *et al.*, 2019). Ag/LDPE, a well-known chemical antibacterial nanocomposite, significantly prevents growth of microorganisms, mainly bacteria (Hosseini *et al.*, 2017, Anvar *et al.*, 2019, Khanipour *et al.*, 2020). A recent finding indicates that Ag/LDPE has a low holding tonicity at rest but strong antibacterial characteristics with the intensification of content (Ebrahimi *et al.*, 2019). In addition to inhibition of microbial growth, Ag/LDPE nanoparticles (NPs) can also induce minor alterations in the fractions of the bacteria concerning the content. This finding suggests that these polymers which have antibacterial characteristics can be applied in the cold chain of food industries. The antibacterial properties of Ag/NPs are thought to be the result of adhesion between silver ions and the thiol group (sulfhydryl-SH) of bacterial cell walls (Liau *et al.*, 1997). Furthermore, the small size of Ag/N particles (Rai *et al.*, 2009, Wijnhoven *et al.*, 2009) provides plenty of contact area, resulting in significant bacteria penetration and physical alterations to the cell wall through the formation of holes on cells' surfaces, thereby causing the bacterial cells to die.

Microbial contamination mostly occurs on the surfaces of food products. Therefore, different methods can be used to increase the shelf life of these products. To this end, antibacterial agents are introduced onto the surface of food products either by spraying or immersion. Nanocomposite coatings containing metal nanoparticles are also applied to protect the packaging layer (Azlin-Hasim *et al.*, 2015). Films and coatings are thin layers of edible polymer materials that can be used as antimicrobial compounds, antioxidants, and other additives (e.g., flavors and stains) that generally preserve the nutritional value and organoleptic characteristics of food and increase their shelf life and safety (Hosseini and Gómez-Guillén, 2018). Plastics comprise a wide range of materials that have been used increasingly often for food packaging in recent past years (Andrady and Neal, 2009). Cushen *et al.* (2014) reported that the results of silver release from exposure to the

composition with food are far less significant than the results for other compounds under severe conditions.

Fish and other seafood are highly perishable, meaning that they need to be packaged carefully so that they remain fresh (Mital *et al.*, 2015). On the other hand, fish and seafood products with high levels of nutrients and protein, are deeply susceptible to bacterial growth while they are preserved at the refrigerating temperature or above it (Tsironi and Taoukis, 2018). This is due to oxidation of unsaturated fatty acids and high concentrations of hematin and metal ions in the muscles of fish (Jeon *et al.*, 2002). According to a recent report (IFO, 2018), Iran extended the Acipenseridae fish culture to 2618 metric tons by 2018. *H. huso* is the largest of caviar fishes, and its principal habitats are in Caspian Sea and Black Sea (Vecsei *et al.*, 2002). This species has a flavorful fillet that is expensive in comparison to other fish fillets. Caviar fish are susceptible to spoilage by microbes due to their high nutritional value and abundance of fat tissues in their bodies. Thus, the way these fish are packaged affects their shelf life.

Based on the above introduction, the objective of this study was to assess the effects of an Ag/LDPE-NC (nanocomposite) film at a concentration of 7% Ag produced through melt mixing on the shelf life extension of *H. huso* fillets stored at refrigeration temperature.

Materials and methods

Silver nanoparticle and LDPE preparation

Ag NPs were purchased from Sigma-Aldrich (Germany), and the method of Song and Kim (2009) was applied to them. Film grade LDPE with a melt flow index (MFI) of 25 g/10 min (190°C/2.16 kg), which was deemed suitable for nanocomposite usage, was purchased from Aria Sasol Polymer Petrochemical (Iran). Nanosilver (at a concentration of 7%) was mixed with polyethylene glycol monostearate (PGE) (Sigma-Aldrich, Germany) for 15 min to stabilize the dispersion of Ag NPs into LDPE medium. The mixed solution was introduced to LDPE media at 140°C during the melting procedure. Heating and cooling method of Jokar *et al.* (2012) was carried out.

Treatment design

Fish were divided into 9 separate groups, depending on the different treatments. In group 1(G1) fish were stored in normal bags (LDPE) and total viable count (TVC) was performed. In groups 2 (G2) and 3 (G3), the fish were respectively encased in 7% Ag/LDPE-NC films and placed in the plates without any coating and evaluated for TVC test. The fish coatings of groups 4 (G4), 5 (G5), and 6 (G6) were respectively similar to groups 1-3, but their fish were sampled to assay psychrophilic bacterial count (PBC). Accordingly, the coverage of groups 7 (G7), 8 (G8), and 9 (G9) were similar to groups afore-mentioned, but their fish were examined to enumerate *S. aureus.* Each group was evaluated in triplicate.

Staphylococcus aureus inoculation

Lyophilized *S. aureus* (PTCC, 1133) samples were prepared from microbiology lab of the laboratory complex of IAU, Science and Research Branch, Tehran, Iran. The lyophilized bacteria were retrieved by mixing into a sterile tube containing Brain Heart Infusion (BHI) fluid and placed in a 37°C incubator for 24 h. One loop-full of final culture was inoculated on Mueller-Hinton agar (MHA) at the same conditions. After 24 h of incubation, the bacteria were grown on media to prepare a 0.5 McFarland standard to about 3×10^8 CFU/mL read at 625 nm, following the method of Sohbatzadeh *et al.* (2010).

Fish samples preparation

H. huso fillets were freshly prepared from the fish farmed in fields located in Ahmedabad Mostofi and transferred to the laboratory under sterile conditions and ice. The fish were then removed from the pockets and washed several times with sterilized distilled water and then with 70% alcohol. Then the fish were cut into 10 g pieces which were placed under UV sterilized conditions for 20 minutes (Gómez-Estaca *et al.*, 2007). The pieces were inoculated with one cc of 0.5 McFarland standard of *S. aureus* and then were put into special bags (Remya *et al.*, 2016) of Pulsifier machine in order the inoculum to permeate the pieces (Fung *et al.*, 1998). After removing the excess water from fish samples, the coating was closed using a lid closing machine, and the samples were packed and kept in a refrigerator at 4°C.

Sampling and bacteriological tests

The fish were sampled two, four, six, and eight days after being stored in refrigerator. On each testing day, 30 minutes before the test, samples were removed from the refrigerator, placed into a stomacher bag (to which 90 mL sterilized physiological serum containing 0.85% NaCl solution had been added), and well-homogenized. Afterwards, 1 mL of the mixed sample was diluted in 99 mL of a sterilized physiological serum to obtain an even serial dilution of up to 10^{-10} (Kavoosi *et al.*, 2013). When counting the CFU, a value below 30 CFU/g was considered very low, and a value above 300 CFU/g was considered very high.

Total viable count

A volume of 100 µL was removed from each diluted tube and inoculated in a petri dish prefilled with a 10 mL plate count agar (PCA, Merk, Darmstadt, Germany) then the plates were counted at 37°C after 48 h (Bereda *et al.*, 2018). Colony-forming units (CFUs) per gram of fish were subsequently measured.

Psychrophilic bacterial count

A volume of 100 µL from each diluted tube was removed and inoculated on the surface of a sterile plate prefilled with 10 cc plate count agar (Sigma Aldrich, Germany) and stored in a refrigerator (7°C) for 7-8 days. Creamy rod-shaped colonies were numerated (Ojagh *et al.*, 2010, Alves *et al.*, 2018).

Staphylococcus aureus count

From each tube, 100 µL of the diluted substance was removed and transferred to the surface of a medium in a petri dish containing 10 cc Baird Parker Agar (Sigma Aldrich, Germany) and incubated at 37°C for 24-36 h, following the method of Lali Sarab *et al.* (2019).

Statistical analyses

The data for TVC, psychrophilic bacteria, and *S. aureus* isolated from fillets of *H. huso* were analyzed using a repeated-measure ANOVA within the general linear model technique of SPSS V. 18 (SPSS Inc., IL, and USA). A factorial assignment was applied by use of the fixed factors, containing the bacteria species, sample dilution, and time of sampling. The marginal mean of the number of bacteria from the fillets was considered significantly different at $p<0.05$. When significant *p*-values were obtained, the differences between the individual estimated marginal means were compared using the post-hoc Tukey's HSD test ($p<0.05$). All counts were performed in triplicates. Also the Pillai's trace was tested by performing the multivariate test of MANOVA.

Results

Figure 1 shows the films produced in this study. According to the multivariate test (Table 1), the high values of Pillai's trace indicated the notable effectiveness of days, groups of films, and serial dilution of bacteria (CFU/g) during the eight days of the experiment.

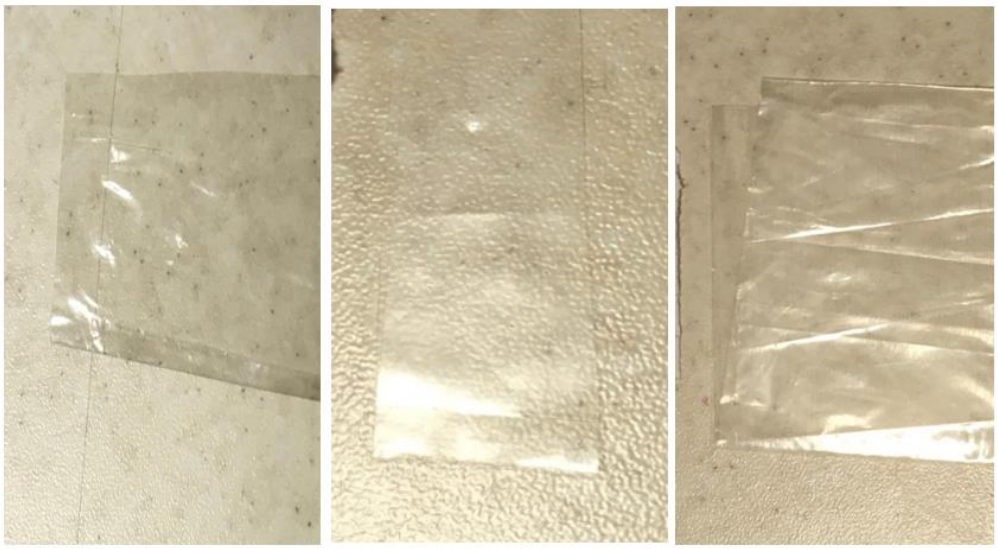

Figure 1: Ag/LDPE-NC (nanocomposite) films produced in this study at a concentration of 7%, 3% and 1% Ag from left to right.

Table 1: The multivariate test of MANOVA showed the effect of independent variables on the quantity of bacteria.

Effect	treatment	Value	F	Sig.	Partial Eta Squared
Days*G.*S. D. Pillai's Trace	1-3	2.97	416.30	0.000	0.99
Days*G.*S. D. Pillai's Trace	4-6	2.97	371.89	0.000	0.99
Days*G.*S. D. Pillai's Trace	7-9	2.97	416.30	0.000	0.99

G. =Group, S. D. =Serial Dilution

Mauchly's sphericity test revealed that sphericity was violated (W=0.072, $\chi 2$=541.11, p=0.000; W=0.120, $\chi 2$=111.93, p=0.000; and W=0.391, $\chi 2$=49.54, p=0.000) for the three groupings of treatments mentioned in Table 1. Variances of the differences between each pair of days were equal (p<0.05). Therefore, the value of Greenhouse-Geisser was adjusted so that the values for the crossing variables (Table 2) were significant (p<0.05). The number of days elapsed (2, 4, 6, and 8) significantly influenced the reduction of bacteria (CFU/g) isolated from minced fillets of caviar fish for the effect of: "Days of the experiment*Groups of films*Serial dilution."

Table 2: The test of within-subjects "effects for each grouping treatments".

Effect	Treatments	df	F	Sig.	Partial Eta Squared
Days*G.*S.D. Greenhouse-Geisser	1-3	22.04	521.79	0.000	0.994
Days*G.*S.D. Greenhouse-Geisser	4-6	22.05	605.98	0.000	0.994
Days*G.*S.D. Greenhouse-Geisser	7-9	28.97	447.70	0.000	0.993

G. = Group, S. D. = Serial Dilution

Interactions among attributes, including days of the experiment were considered, with different time elapsed and groups of plastic films encased the fish fillets employed as grouping (1-3, 4-6, and 7-9) and their effects on shelf life extension of caviar fish fillets (CFU/g) were of significance [F(1, 54)=221826.67, p=0.000, η^2=1.00; F(1, 16)=104581.58, p=0.000, η^2=99.99 and F(1, 16)=121564.56, p=0.000, η^2=99.89] in groups, 1-3, 4-6, and 7-9, respectively. These values indicated that almost 100% of previously unexplained variances for the mentioned bacteria could be explained by the interaction between serial dilution and the groups of plastic films used in the experiment. According to Table 3 and Figure 2, the estimated marginal means (EMM) of TVC was 3.04±2.43 (log CFU/g) in 10^{-2}-diluted fish samples encasing in Ag/LDPE coatings (G2), which is significantly lower than those of G3 and G1 after two days of cold storage. This difference was increased by 4 logs on the fourth day and decreased by 1 log on the sixth and eighth days of the preservation in refrigerator. Accordingly, TVC of G2 enumerated on the 2nd day was not significantly different (p<0.05) from its value after four days (3.45±2.34 log CFU/g) at refrigerated storage, although it was slightly increased. The TVC of 10^{-}

^{3}-diluted fish samples obtained from G2 was significantly decreased by 1.5 logs compared to that of the lower diluted one (Table 3). The pattern for TVC was created from a serial dilution of 10^{-2} repeated at dilutions of 10^{-4} and continued until the last dilution (10^{-10}) of fish samples.

Table 3: Estimated marginal means of total count of bacteria (log CFU/g) through the effect of days of preservation× sample dilutions × groups of fish coatings.

Sample Dilution ($n=10^{-n}$)	Days	Coating groups			
		1	2	3	SE
2	2	4.84^{bA}	3.04^{aA}	3.46^{bA}	2.43
	4	7.90^{bB}	3.45^{aA}	7.89^{bB}	2.34
	6	7.91^{bB}	6.46^{aB}	7.89^{bB}	3.94
	8	7.96^{bB}	6.62^{bB}	7.89^{bB}	7.12
3	2	4.37^{cA}	1.49^{aA}	3.11^{bA}	2.43
	4	7.86^{bB}	3.34^{aB}	7.79^{bB}	2.34
	6	7.90^{bB}	6.37^{aC}	7.66^{bB}	3.94
	8	7.95^{bB}	6.59^{aC}	7.62^{bB}	7.12
4	2	4.10^{cA}	0.00^{aA}	2.99^{bA}	2.43
	4	7.78^{bB}	3.10^{aB}	7.69^{bB}	2.34
	6	7.90^{bB}	6.32^{aC}	7.83^{bB}	3.94
	8	6.87^{aC}	6.47^{aC}	7.77^{bB}	7.12
5	2	6.72^{cB}	0.00^{aA}	2.44^{bA}	2.43
	4	7.61^{bC}	3.12^{aB}	7.51^{bC}	2.34
	6	7.84^{bC}	6.17^{aC}	7.72^{bC}	3.94
	8	4.86^{bA}	3.21^{aB}	4.76^{bB}	7.12
6	2	2.34^{bB}	0.00^{aA}	2.07^{bA}	2.43
	4	5.12^{cB}	2.14^{cB}	7.12^{bA}	2.34
	6	6.50^{cB}	6.08^{cB}	6.26^{bA}	3.94
	8	4.29^{cB}	3.02^{cB}	4.24^{bA}	7.12
7	2	1.84^{bA}	0.00^{aA}	0.00^{aA}	2.43
	4	3.96^{bB}	2.44^{aB}	3.96^{bC}	2.34
	6	6.10^{bC}	4.99^{aC}	5.90^{bD}	3.94
	8	3.91^{bB}	2.96^{aB}	3.08^{aB}	7.12
8	2	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	4	3.68^{bC}	0.00^{aA}	3.95^{bC}	2.34
	6	4.54^{bB}	0.00^{aA}	4.36^{bC}	3.94
	8	3.54^{cB}	0.00^{aA}	3.05^{bB}	7.12
9	2	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	4	3.47^{bD}	0.00^{aA}	3.47^{bC}	2.34
	6	2.50^{bC}	0.00^{aA}	2.07^{bB}	3.94
	8	2.04^{bB}	0.00^{aA}	1.86^{bB}	7.12
10	2	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	4	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	6	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	8	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00

Groups 1, 2 and 3 = fish respectively encased in plastic, Ag / LDPE nanocomposite films and control for total count assay; N.D. = not detected. Means with different superscripted small letters differ significantly ($p<0.05$) in the same row and different superscripted capital letters differ significantly ($p<0.05$) in the same column.

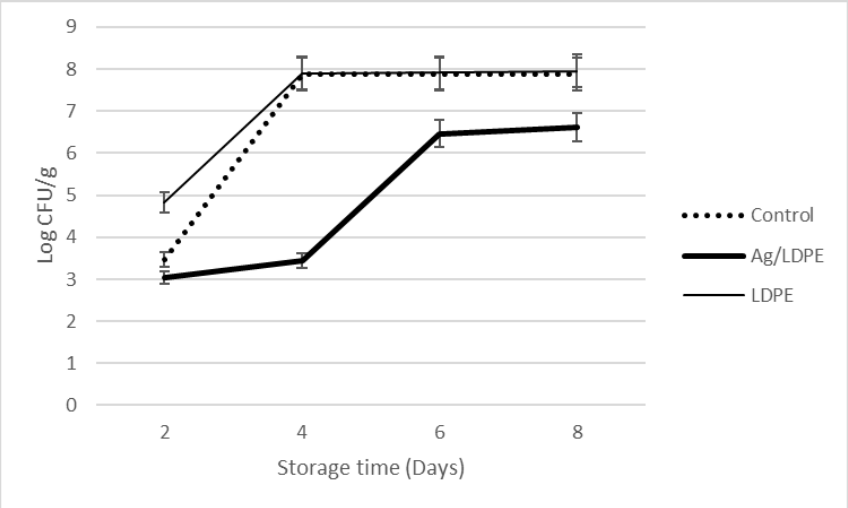

Figure 2: Trend of total viable counts (CFU/mL) of the bacteria through days for each fish fillet group at 10^{-2} dilution.

Results of the EMM of PBC amounted for fish samples are present in Table 4 and Figure 3. Table 4 indicates that the lowest EMM of PBC (0.0 log CFU/g) was recorded for the 10^{-2}-diluted fish samples encased in Ag/LDPE-NC at the 2nd day of preservation, increased by 3-7 logs up to sixth days and decreased by 1 log on the eighth day of cold preservation. The PBC of fish kept in G6 was 2.93±2.43 log CFU/g significantly lower (p<0.05) than that of the fish coated with LDPE alone (3.83±2.43 log CFU/g). This difference recurred on the fourth day but decreased afterward. The effect of Ag/LDPE NC exhibited greater antibacterial activity on reducing PBC from *H. huso* fillets when compared with TVC (Tables 3-4 and Figs. 2-3). Another finding of the present study highlighted high effectiveness of Ag/LDPE-NC film (group 5) on inhibiting growth of psychrophilic bacteria (3.07±5.17 log CFU/g) when compared with groups 4 and 6 (control), whose values for PBC were 7.84±5.17 and 5.86±5.17 log CFU/g after 96 h. The data (Table 4) showed that PBC increased as dilution decreased, and as time increased.

The MMEs of SAC are listed in Table 5 and Figure 4. Group 8 was composed of significantly less SAC than the other two groups. Moreover, its values for this group in which the fish were kept in an Ag/LDPE NC film were 1.3±1.40 and log CFU/g on days 2 and 4 at 10^{-2}, respectively. This increased significantly and reached 6.97±3.91 log CFU/g for group 8, showing the appropriate effects of Ag/LDPE on retarding the SAC sampled from caviar fish fillets in up to four days. The MME of SAC for control and group 7 (the fish were encased in LDPE) was remarkably increased (p<0.05) by 0.4 and 1.5 log CFU/g when compared with group 8. This difference between groups remarkably decreased after the fourth day. On dilution of 10^{-3}, however, SAC for the group of Ag/ LDPE was 0.00 log CFU/g significantly less (p<0.05) than those of the groups, LDPE and control

(2.81±3.70 and 2.53±3.70 log CFU/g, respectively). This pattern was recurred up to a dilution of 10^{-5} and subsequently, the MMEs of SAC generally reached zero in all groups.

Table 4: Estimated marginal means of psychrophilic bacteria (log CFU/g) through the effect of days of preservation × sample dilutions × groups of fish coatings.

Sample Dilution ($n=10^{-n}$)	Days	Coating groups			
		4	5	6	SE
2	2	3.83^{cA}	0.00^{aA}	2.93^{bA}	2.43
	4	7.84^{cB}	3.07^{aA}	5.86^{bA}	5.17
	6	7.89^{bB}	6.97^{aA}	7.86^{bA}	6.38
	8	7.91^{bB}	6.58^{aA}	7.85^{bA}	6.11
3	2	3.80^{bA}	0.00^{aA}	3.23^{bA}	2.43
	4	7.81^{bB}	2.35^{aB}	7.80^{bB}	5.17
	6	7.88^{bB}	6.92^{aC}	7.82^{bB}	6.38
	8	7.94^{bB}	6.54^{aC}	7.84^{bB}	6.11
4	2	3.68^{bA}	0.00^{aA}	3.70^{bA}	2.43
	4	7.57^{bB}	1.84^{aB}	7.77^{bB}	5.17
	6	7.68^{bB}	6.87^{aC}	7.80^{bB}	6.38
	8	7.86^{bB}	6.46^{aC}	7.71^{bB}	6.11
5	2	3.72^{bA}	0.00^{aA}	3.72^{bA}	2.43
	4	7.12^{bB}	0.00^{aA}	6.94^{bB}	5.17
	6	7.39^{bB}	4.38^{aB}	7.72^{bB}	6.38
	8	7.80^{bB}	4.95^{aB}	7.70^{bB}	6.11
6	2	2.46^{bA}	0.00^{aA}	3.34^{bA}	2.43
	4	7.15^{bB}	0.00^{aA}	2.91^{bB}	5.17
	6	7.12^{bB}	4.00^{aB}	6.78^{bC}	6.38
	8	7.82^{bC}	4.93^{aC}	7.36^{bC}	6.11
7	2	2.43^{bA}	0.00^{aA}	3.00^{bA}	2.43
	4	4.46^{cB}	0.00^{aA}	2.44^{bA}	5.17
	6	6.45^{cC}	0.00^{aA}	4.46^{bB}	6.38
	8	7.10^{bC}	4.30^{aB}	7.07^{bC}	6.11
8	2	2.34^{bA}	0.00^{aA}	3.04^{bB}	2.43
	4	4.32^{cB}	0.00^{aA}	2.17^{bA}	5.17
	6	5.43^{cC}	0.00^{aA}	4.45^{bC}	6.38
	8	6.81^{bD}	0.00^{aA}	7.02^{bD}	6.11
9	2	2.25^{bA}	0.00^{aA}	0.00^{aA}	2.43
	4	4.32^{bB}	0.00^{aA}	0.00^{aA}	5.17
	6	5.41^{cC}	0.00^{aA}	4.41^{bB}	6.38
	8	5.46^{bC}	0.00^{aA}	5.46^{bB}	6.11
10	2	2.04^{bA}	0.00^{aA}	0.00^{aA}	2.43
	4	3.07^{bB}	0.00^{aA}	0.00^{aA}	5.17
	6	5.37^{cC}	0.00^{aA}	4.37^{bB}	6.38
	8	5.44^{bC}	0.00^{aA}	5.11^{bB}	6.11

Groups 4, 5, and 6 = fish respectively encased in plastic, Ag / LDPE nanocomposite films and control for psychrophilic; N.D. = not detected. Means with different superscripted small letters differ significantly ($p<0.05$) in the same row and different superscripted capital letters differ significantly ($p<0.05$) in the same column.

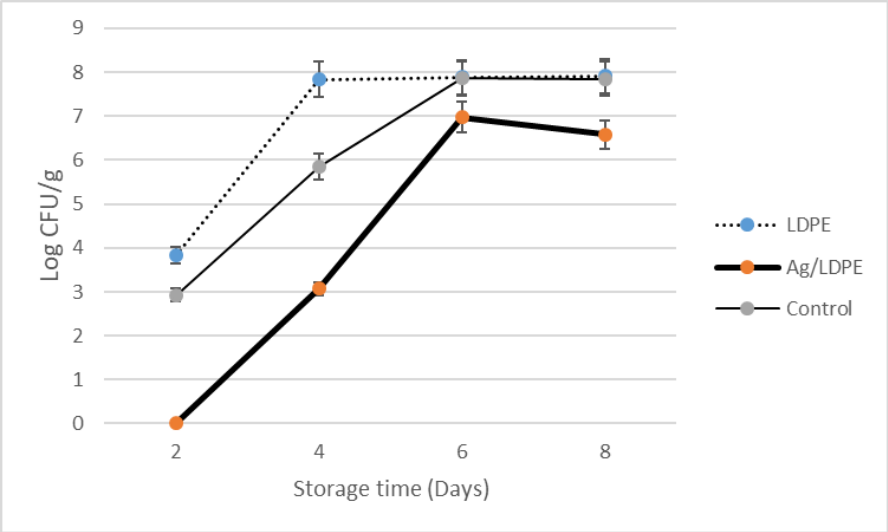

Figure 3: Trend of psychrophilic bacteria (CFU/mL) through the days for each fish fillet group at 10^{-2} dilution.

Table 5: Estimated marginal means of *Staphylococcus aureus* bacteria log (CFU/g) through the effect of days of preservation × sample dilutions × groups of fish coatings.

Sample dilution ($n=10^{-n}$)	Days	Coating groups			SE
		7	8	9	
2	2	2.89^{bA}	1.30^{aB}	1.71^{aA}	1.40
	4	2.81^{bA}	0.00^{aA}	2.53^{bB}	3.70
	6	7.89^{bB}	6.97^{aC}	7.86^{bC}	3.91
	8	7.91^{bB}	6.58^{aC}	7.85^{bC}	3.97
3	2	1.44^{bA}	0.00^{aA}	1.46^{bA}	1.40
	4	2.90^{cB}	0.00^{aA}	1.97^{bA}	3.70
	6	7.88^{bC}	0.00^{aA}	7.82^{bB}	3.91
	8	7.94^{bC}	6.54^{aB}	7.84^{bB}	3.97
4	2	1.39^{bA}	0.00^{aA}	1.45^{bA}	1.40
	4	1.28^{bA}	0.00^{aA}	1.46^{bA}	3.70
	6	7.68^{bB}	0.00^{aA}	7.80^{bB}	3.91
	8	7.86^{bB}	6.46^{aB}	7.71^{bB}	3.97
5	2	1.36^{bA}	0.00^{aA}	1.20^{bA}	1.40
	4	1.07^{bA}	0.00^{aA}	1.46^{bA}	3.70
	6	7.39^{bB}	4.38^{aB}	7.72^{bB}	3.91
	8	7.80^{bB}	4.95^{aB}	7.70^{bB}	3.97
6	2	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	4	0.47^{cB}	0.00^{aA}	1.43^{bB}	3.70
	6	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	8	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
7	2	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	4	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	6	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	8	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
8	2	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	4	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00
	6	0.00^{aA}	0.00^{aA}	0.00^{aA}	0.00

Table 5 (continued):

Sample Dilution ($n=10^{-n}$)	Days	Coating groups			SE
		7	8	9	
	8	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00
9	2	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00
	4	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00
	6	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00
	8	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00
10	2	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00
	4	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00
	6	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00
	8	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00

Groups 7, 8 and 9 = fish respectively encased in plastic, Ag / LDPE nanocomposite films and control for *Staphylococcus aureus*; N.D. = not detected. Means with different superscripted small letters differ significantly (p<0.05) in the same row and different superscripted capital letters differ significantly (p<0.05) in the same column.

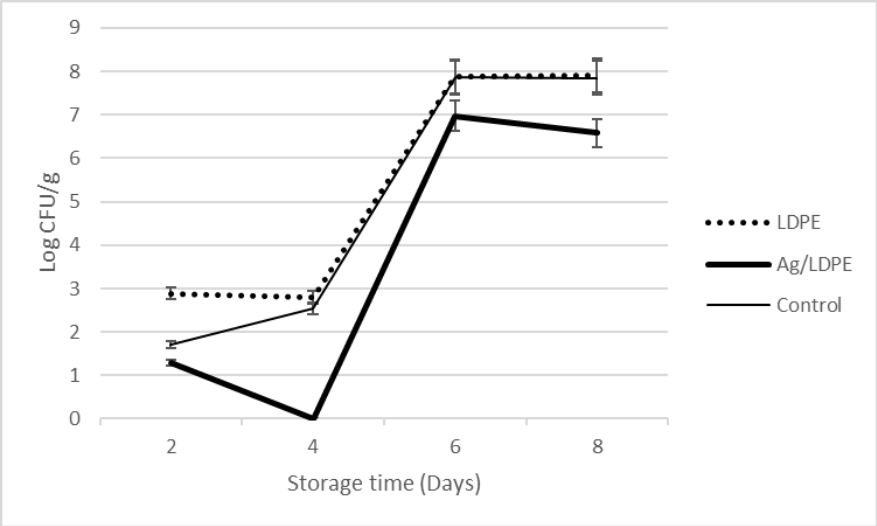

Figure 4: The trend of *Staphylococcus aureus* (CFU/mL) through the days for each fish fillet group at 10^{-2} dilution.

Discussion

The objective of this study was to evaluate the effect of 7% Ag/LDPE coating on microbial growth of Beluga (*H. huso*) fillets during refrigerated storage period. From bacteriological point of view, acceptable limit for TVC of fresh fish and refrigerated fish fillets is 5.7 (Pyz-Łukasik and Paszkiewicz, 2018) and 4.87 log CFU/g (Hamzeh and Rezaei, 2012, ISIRI, 2000). Herein, the initial TVC value before sterilization and inoculation was 4.04 log CFU/g. It exceeded 0.8 logs CFU/g LDPE-fish samples (Fig. 2) at a dilution of 10^{-2} on the second day, unlike those of the control group and Ag/LDPE, which were less than the above mentioned limits. Any values of TVC above these limits raise concerns about hygienic status and level of spoilage of the product, and the situation of spoilage it could have undergone. The initial TVC load of fresh fish fillets could be related to the contamination of fish during processing or fish habitats (Alparslan *et*

al., 2014). The results in Table 3 showed that growth inhibition of total bacteria initiated on the second day while Ag/LDPE coating was used. The maximum time to preserve Ag/LDPE coated *H. huso* fillets under the explained limits was 96 hours at refrigerated temperature as opposed to the other fish fillets coated with LDPE or control fish, which maintained up to 48 h at the same condition (Fig. 2). Though the precise mechanism of silver nanoparticles' antimicrobial activities has not been entirely clarified, various antibacterial actions, including disruption of cell wall and perforation of cytoplasmic membrane, denaturation of ribosomes, interruption of adenosine triphosphate (ATP) production, and the interference of deoxyribonucleic acid (DNA) replication have been proposed in a previous study (Yin *et al.*, 2020).

Similarly, chitosan LDPE incorporated with cinnamon (2% w/v+ 1.5% v/v) revealed that the bacteriostatic effect for TVC in trout fillets was up to 96 h (Ojagh *et al.*, 2010). In samples taken from flounder (*Paralichthys orbignyanus*) fillets covered by agar film incorporated with clove EO after 48 h TVC reached 3.7 log CFU/g considerably 0.5 log CFU/g more than that of Ag/LDPE-fillet samples (Table 3) at the same condition (da Rocha *et al.*, 2018). In another work, the gelatin enriched with 1% laurel leaf essential oil (EO) had bacterial growth-reduction effect on trout fillets after 10 days (Alparslan *et al.*, 2014). The decline of TVC reached 4.2 log CFU/g at a dilution of 10^{-5}, which was fairly similar to the results of this study (Table 3) showing a 3.45 log CFU/g reduction under the same conditions.

Proteolytic psychrophilic bacteria are the most prominent microorganisms involved in aerobic spoilage of refrigerated fresh fish (Uçak, 2019). Psychrophiles usually grow faster than psychrotrophic bacteria because their optimum temperature range is within the normal refrigeration temperatures (Mhango *et al.*, 2010). The maximum acceptable level for psychrophilic bacteria for chilled fish is 6 log CFU/g (Mol *et al.*, 2007). The results (Table 4) showed that Ag NC film (G5) had a significant effect on retarding the value of PBC in comparison to effects of encasing fish in LDPE (G4) and placing fish in plates without coatings (G6) respectively. Consequently, its capability showed that it could eliminate psychrotrophic bacteria from *H. huso* fillet up to the second day and retard it (3.07 log CFU/g) on the fourth day (Fig. 3) in agreement with bacterial examination on Cod (*Gadus morhua*). Fillets exhibited that LDPE-packaging material containing nano-ZnO features inhibitory activity against mesophilic and psychrotrophic bacterial growth up to the 3rd day (Mizielińska *et al.*, 2018a). In other words, psychrophiles reached 7.8 and 3.8 log CFU/g in salmon (Alves *et al.*, 2018) and *Sander lucioperca* fillets (Barani *et al.*, 2018), encased in chitosan and Ag/TiO2 LDPE films, respectively, under the same conditions. In a study, psychrophiles decreased by 1.5 log CFU/g in the cooled mullet fillets coated with LDPE

enriched with 5% thyme after four days when compared with a control condition (Yasin and Abou-Taleb, 2007). Meanwhile, the present study indicated decreases of 3 and 2.8 log CFU/g for fillets wrapped in 7% Ag NC films in comparison to control conditions (G6).

Furthermore, a mixture of chitosan extract and rosemary nanocomposites administered to *H. huso* reduced the growth of inoculated *Listeria monocytogenes* from 4.14 log CFU/g to 2.23 log CFU/g (less than 2 logs CFU/g mitigation) after 16 days of storage at 4°C (Jafari *et al.*, 2017). Mizielińska *et al.* (2018b) showed that LDPE cellulose/2% ZnO NPs could decrease PBC (1×10^4 CFU/g) in refrigerated *Gadus morhua* fillets after 72 h. The explanation mentioned above highlights the ability of 7% Ag NCs to reduce psychrophilic bacteria in *H. huso* fillets. The efficacy of 7% Ag NC on SAC (Table 5) of *H. huso* fillets exhibited retarding and elimination properties, respectively, on the second and fourth days at refrigerated temperature and dilution of 10^{-2}. As such, the PBC and TVC were gradually increased up to four days, but *S. aureus* was eliminated from *H. huso* fillets at the same time. Another research showed that different types of nanocomposites, such as alginate/clay, exhibit no antimicrobial activity against *S. aureus* (Alboofetileh *et al.*, 2014). Similarly, Menezes *et al.* (2019) examined Ag/NC films enriched with gelatin and found that such films have inadequate efficacy to act as a bactericidal or bacteriostatic compound unless tannic acid is added. Their conclusions were based on the concept that the thick peptidoglycan layer of Gram-positive bacteria prohibits infiltration of Ag/NPs (Mariselvam *et al.*, 2014). Hosseini *et al.* (2016) evaluated the effect of fish gelatin coating containing *Origanum vulgare L* essential oil on shelf life extension of rainbow trout (*Oncorhynchus mykiss*) fillet stored under refrigerated storage (4±1ºC); and it was also revealed that this was an effective method for maintaining the storage quality of rainbow trout fillet stored at refrigerated temperature. Our study is another investigation in line with the survey of Hosseini *et al.* (2016) evaluating the effects of chemical elements on the shelf life extension of fish fillet stored under refrigerated storage. Kanmani and Rhim (2014) showed that the efficiency of Ag/NC films on *S. aureus* reduction was significantly associated with the concentration of the enriched matter added to NP and Ag/NP concentration, which is in agreement with our previous research (Barani *et al.*, 2018). Therein, the optimum efficacy of Ag/TiO_2 LDPE on *S. aureus* reductions in fish fillets was observed when either the minimum concentrations of Ag and TiO_2 were 5% and 2.5%, respectively, or when the method of Ag/NC production was melt mixing. This was in line with the study of Cho *et al.* (2005) who observed that the minimum mitigating concentrations of Ag-NPs to protect against *S. aureus* and *E. coli* were 5 ppm, and 10 ppm, respectively. This finding is in line with our study showing the effectiveness of

7% Ag/LDPE films on the elimination of *S. aureus* from caviar fish fillet.

Our previous study on pikeperch (*Sander lucioperca*) fillets (Barani *et al.*, 2018) confirmed that the 5% Ag/TiO2-NC fabricated by melt mixing exhibit more antibacterial activity against *S. aureus* than nanocomposites fabricated via a sol-gel method. On the fourth day of preservation, the Ag/LDPE nanocomposite film manufactured through melt mixing properly eliminated *S. aureus* from *H. huso* fillets (Fig. 4) preserved at refrigerator temperature. The standard acceptable SAC level for fresh fish in EU countries and Japan is $1\times10^3/cm^2$ (Topić Popović *et al*., 2010). The findings presented in this study showed that fresh *H. huso* fillets encased in 7% Ag NC films could retard the SAC and keep it below the standard value for up to four days at refrigerated condition. EMM of the bacteria in fish samples coated with LDPE (Tables 4-6) was significantly higher than that of the control group up to four days. This could be due to a delay occurred for passing the cold weather through the LDPE film and reaching the fish body. This finding indicates that the most suitable LDPE film for fish packaging is the perforated one.

The comprehensive study of the Ag/LDPE composite film exhibited here offers practical visions into active food packaging material which has a high potential to preserve the fresh fish quality and extend shelf life of the product. It is concluded that application of 7% Ag/LDPE film produced by the melt mixing method can remarkably retard growth of bacteria on fresh *H. huso* fillets. Accordingly, total viable bacterial count, psychrophilic bacteria growth, and *S. aureus* activity were significantly mitigated up to four days of preservation at refrigerator at which the values were below the acceptable limits for fresh fish set by national standards and the International Commission on Microbiological Specification for Foods. Thus, the 7% Ag/LDPE film is a safe material, as shown in our parallel study (Kargar *et al*., 2021), that can be used as coating in order to extend the shelf life of chill stored *H. huso* fresh-fillets.

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