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Bi-Layer packaging Approach with Rosemary Encapsulated on Nanochitosan for Increasing the Shelf Life of Sturgeon Fillets

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ABSTRACT: Recently, the integration of nanoparticles with natural polymers has emerged as a promising approach for enhancing the mechanical and antimicrobial properties of active and smart packaging. In this study, a bilayer film was developed that harnesses the unique cationic properties of chitosan in addition to the antioxidant and antimicrobial effects of rosemary. This film was utilized for packaging sturgeon fish fillets and for storage under refrigerated conditions. The bilayer film was formulated with an optimal composition of 20% chitosan nanoparticles encapsulating rosemary extract (RChNPs). The microbial, chemical, and sensory qualities of the packaged fish were evaluated on the first, third, seventh, and fourteenth days. Over time, the results from the microbial and chemical analyses indicated a significant reduction in microbial growth, total volatile nitrogen, and aldehyde compounds. Additionally, sensory evaluations revealed that the rate of quality decline was markedly slower than that of the control samples. Consequently, the developed bilayer film significantly extended the shelf life of fish at refrigeration temperatures. Considering the critical importance of food safety and the need to prevent spoilage and food waste, antimicrobial-based packaging represents a valuable advancement in enhancing the shelf life of fresh protein sources, including fish.

Keywords: *Bilayer, Nanochitosan, Rosemary, Shelf Life, Sturgeon Fish*

Introduction

Packaging under appropriate physicochemical conditions leads to an increased shelf life of food products. The primary goals of food packaging are to delay spoilage, preserve the positive effects of processing, extend storage life, and maintain or enhance the quality and safety of food. Additionally, packaging serves a protective role against external chemical, physical, and biological factors (Priyadarshi and Rhim, 2020). The term "antimicrobial packaging" encompasses any packaging method used to control microbial growth in food products. The antimicrobial agents employed in packaging must prolong the lag phase of microorganisms and reduce their growth phase to extend the shelf life of the product while preserving its quality and

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safety. (Fadiji *et al*., 2023). Nanotechnology can address the weaknesses of the packaging industry by incorporating specific materials into packaging to create containers that can prevent the invasion of pathogens and microorganisms that threaten food safety (Ahari and Lahijani, 2021). "Chitosan," a polysaccharide derived from the deacetylation of chitin, possesses inherent antimicrobial properties. The antimicrobial mechanism of this polymer includes the destruction of microbial cell membranes and leakage of cellular components into the environment. Additionally, chitosan can penetrate cell nuclei, leading to the disruption of mRNA synthesis. Another mechanism mentioned in the literature regarding the antimicrobial properties of this polymer is its chelating ability (ion bonding with metals) (Asl *et al*., 2021, Abdeltwab *et al*., 2019). The creation of nanoscale dimensions improves mechanical properties, enhances antimicrobial activity, and reduces biochemical changes. Encapsulation of natural phenolic compounds increases the stability of bioactive components and protects them from interference with food components (Ahari *et al*., 2021). "Rosemary" is recognized for its strong antioxidant and antimicrobial properties; the antioxidant and antimicrobial activities of rosemary extract (RE) are primarily due to carnosic acid and carnosol (Lee *et al*., 2019). Many food products are perishable, necessitating measures to prevent spoilage during processing, preparation, storage, and distribution. Furthermore, the trade of food products and their transportation to remote areas increases the importance of creating suitable conditions to prevent spoilage (Ekhtiarzadeh *et al*., 2011). Aquatic products, such as shrimp and fish, despite their high nutritional value, spoil faster than other meats. Generally, fish and shrimp spoilage can be classified into two categories: bacterial and chemical (autolytic) spoilage. Microbial and chemical spoilage leads to a reduction in the quality of proteins and unsaturated fatty acids in fish(Khoshbou Lahijani *et al*., 2019). The primary reasons for this perishability and lack of long-term stability are related to their intrinsic properties (intense enzymatic activity in fish and shrimp) and the neglect of proper handling and storage of aquatic products after harvesting. Freshness is one of the primary attributes of fish and shrimp in both markets and consumers. These products cannot be stored for long periods (dos Santos Silva and e Barros, 2020) , Various strategies have been proposed to prevent or delay the spoilage of fish and its products, with active packaging being one of these methods. Active packaging, which is based on antimicrobial activity, reduces food waste and consequently extends shelf life (Appendini and Hotchkiss, 2002). Maintaining the quality and freshness of food products, as well as protecting them from oxidation and microbial spoilage, plays a significant role in food packaging. Advances in active packaging technology through the addition of active compounds have improved both antimicrobial and antioxidant properties. In this study, a two-layer film containing rosemary extract encapsulated in chitosan nanoparticles was investigated for the coating of fish fillets.

Materials and Methods

- ***Preparation and Production of Two-Layer Film and Its Characteristics***

After synthesizing chitosan nanoparticles (ChiNPs) using the sol-gel method and extracting rosemary extract with ethanol and water, the rosemary extract was encapsulated within the chitosan nanoparticles (Esmaeili *et al*., 2023; Khoshboui Lahijani *et al*., 2024).

The first layer (outer layer) was created by adding chitosan nanoparticles at weight percentages of 2.5%, 5%, 10%, 20%, and 40% to a polycaprolactone (PCL) solution. This mixture was then dried using the casting method in petri dishes for 24 h. The second layer (adjacent to the fish) was prepared by creating a gelatin solution and adding rosemary extract encapsulated in chitosan nanoparticles (RChiNPs). The prepared solution was subsequently cast over the first layer (Homayounpour *et al*., 2021; Nguyen and Lee, 2012). As shown in Fig 1, all compositions for making the two-layer film were examined, ultimately selecting a combination of polycaprolactone (20%) and gelatin (20%) as the suitable composition for investigating the enhancement of refrigerated shelf life for fish fillets. This selection was based on the higher biodegradability observed at greater nanoparticle percentages, superior mechanical properties, and lower water vapor transmission rate (WVTR) (Figure 1).

- ***Preparation and Processing of Sturgeon Fillets Fish***

The Sturgeon fish, obtained live from the Plour fish farm, was placed in a cool box layered with crushed ice at a ratio of 3:1 and quickly transported to the laboratory. The fish was cut into 10-gram pieces, and these pieces were packaged in a two-layer film containing RChNPs and regular freezer bags (used as a control) and stored in the refrigerator (Fig. 2) (Khoshbouy Lahidjani *et al*., 2020).

- ***Microbiological Testing***

In order to prepare a suspension for microbiological testing, a 10-gram sample was placed in 90 mL of 0.85% NaCl solution and homogenized using a stomacher.

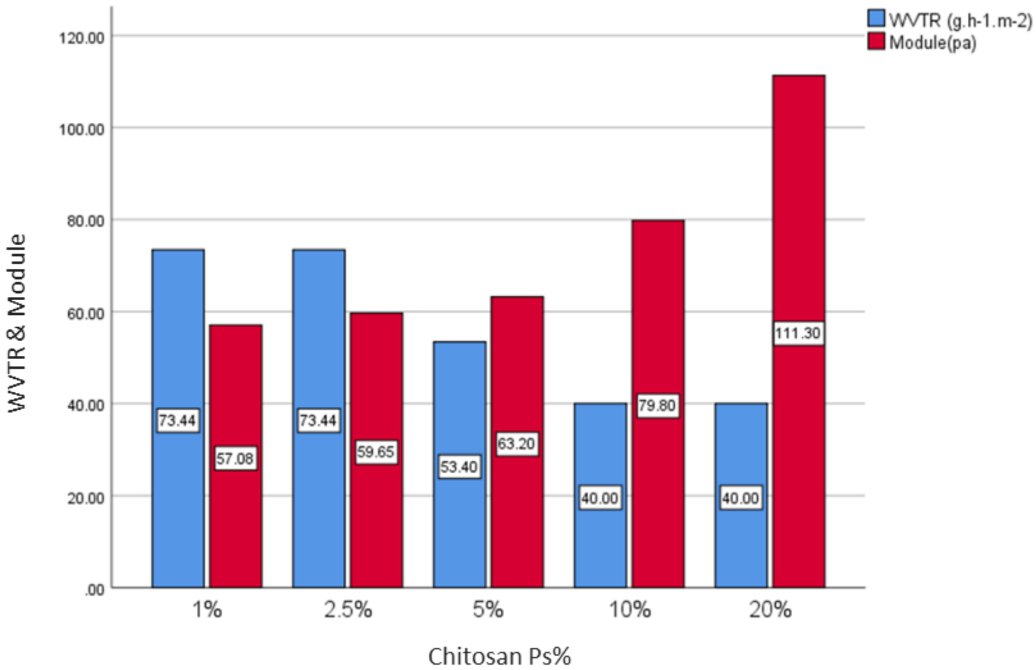

Fig. 1. Results of the investigation of the elastic modulus and water vapor transmission rate in various compositions of the two-layer film, indicating that the two-layer film with a formulation of 20% nanoparticles in each layer was selected as the most suitable formulation(Khoshboui Lahijani et al., 2024).

Fig. 2. Packaging of fish fillets in bilayer film integrated with RChNPs.

- ***Total Viable Count (TVC)***

For the total bacterial count, the surface plate method was used. The homogenized samples were used for microbial culture, and the cultured plates were incubated at 37°C for 48 h before counting. The counts were recorded as log10 cfu/g, and the tests were performed in The tests were performed in triplicate order (Abdeldaiem *et al*., 2018).

- ***Psychrophilic Bacterial Count***

For psychrophilic bacterial counts, plate count agar and the surface plate method were used. Homogenized samples were cultured and the plates were incubated at 10°C for 7 days. The tests were performed in triplicate order, and the counts were recorded as log10 cfu/g after seven days (Ozogul *et al*., 2017).

- ***Chemical Tests***
- ***Measurement of Total Volatile Basic Nitrogen (TVN)***

Volatile nitrogen compounds were measured through direct distillation of 10 g of fish after adding magnesium oxide, based on the amount of nitrogen in milligrams per 100 g of sample. This test was conducted on days 1, 3, 7, and 14 for the fish packaged in the two-layer film containing RChNPs and in regular freezer bags (used as a control), with three repetitions for each condition (Alkuraieef *et al*., 2021).

- ***Measurement of Secondary Oxidation Products Using the Thiobarbituric Acid (TBA)***

Ten grams of the homogenized fish sample were dissolved in 25 mL of butanol. Five milliliters of this solution was placed in a test tube containing 5 mL of thiobarbituric acid reactive reagent and mixed. The mixture was heated in a boiling water bath for 90 min. Before measurement, it was cooled in an ice bath for 10 min, and its absorbance (As) was read at a wavelength of 532 nm. The absorbance of the control sample (Ab) was also measured, and the absorption was converted to the thiobarbituric acid index, which is expressed as milligrams of malondialdehyde per kilogram of sample, using equation 1.

Equation: 1 $TBA = 50 \times (As - Ab) / 200$

- ***Sensory Evaluation Using the 5-Point Hedonic Scale:***

The sensory evaluation of different treatments of raw fish fillets was conducted with twenty expert sensory assessors using a hedonic test to assess liking and pleasantness on a five-point scale, focusing on texture, color, and odor parameters. The sensory characteristics assessed included the following:

Color Change: 5 = no color change; 1 = severe color change

Odor Change: 5 = very pleasant; 1 = very unacceptable or foul

Texture Change: 5 = hard; 1 = very soft

The average scores were defined as overall acceptance: 5 = very desirable; 1 = very unacceptable

Sensory evaluation tests were conducted at intervals of 1, 3, 7, and 14 days (Hassanzadeh *et al*., 2018).

- ***Statistical Analysis***

The statistical population consisted of two groups of Sturgeon fish fillets: those packaged in a two-layer film containing RChNPs and those in the control film. Data were collected through chemical, microbiological, and sensory tests on fish fillets that were packaged and stored under refrigeration for 1, 3, 7, and 14 days. Graphs were plotted and group analyses were performed using SPSS software and one-way ANOVA. Mean comparisons were conducted using Duncan's method with a 95% confidence level.

Results and Discussion

- ***Microbiological Tests***
- ***Total Viable Count***

As shown in Table 1 and Fig. 3, the presence of the two-layer film generally led to a reduction in the increase in total microorganisms compared to the control sample, which can be attributed to the antimicrobial properties of rosemary (Brandt *et al*., 2023) However, the microbial load increased until the third day of storage, which may be due to the pH-dependent release properties of the chitosan nanoparticles. Fig. 4 illustrates the results of the study on pH-dependent release in rosemary extract-loaded nanoparticles through dialysis bags, indicating a gradual release after 72 hours (Khoshboui Lahijani *et al*., 2024) Furthermore, considering the low acidity of fish meat and the increased alkalinity during the release of amino compounds over time, we generally deal with an alkaline environment (Kim *et al*., 2023). According to research by Farahani and colleagues, which aimed at reducing the rate of release of anticancer compounds from chitosan nanocarriers, it was shown that the release of anticancer compounds in neutral and alkaline environments is longer and more stable than that in acidic environments. This is due to the NH_2 functional group in acidic environments converting to NH_3^+, which increases the protonation of chitosan, leading to repulsion of chitosan branches and an explosive, immediate release of anticancer compounds (Gooneh-Farahani *et al*., 2021). According to research by Kalouri and colleagues examined the antimicrobial properties of rosemary essential oil, both alone and encapsulated in chitosan, and showed that rosemary essential oil encapsulated in chitosan has significant effects against gram-negative bacteria and fungi, indicating its potential to become a new generation of plant-based antibiotics (BOLOURI *et al*., 2023).

- ***Psychrophilic Bacterial Count***

According to Table 2 and Fig. 5, the presence of the two-layer film generally controlled the increase in psychrophilic bacteria up to the seventh day compared to

Table 1. Measurement of total microbial count (CFU/ml) during the storage period of fish fillet samples

Day	Control Sample	Bi-layer Fim
Day1	2.70E+05[a]	1.70E+05[a]
Day3	NC	5.6E+06[a]
Day7	NC	9.5E+07[a]
Day14	NC	7.2E+08[b]

* Lowercase letters indicate a significant difference between treatments.

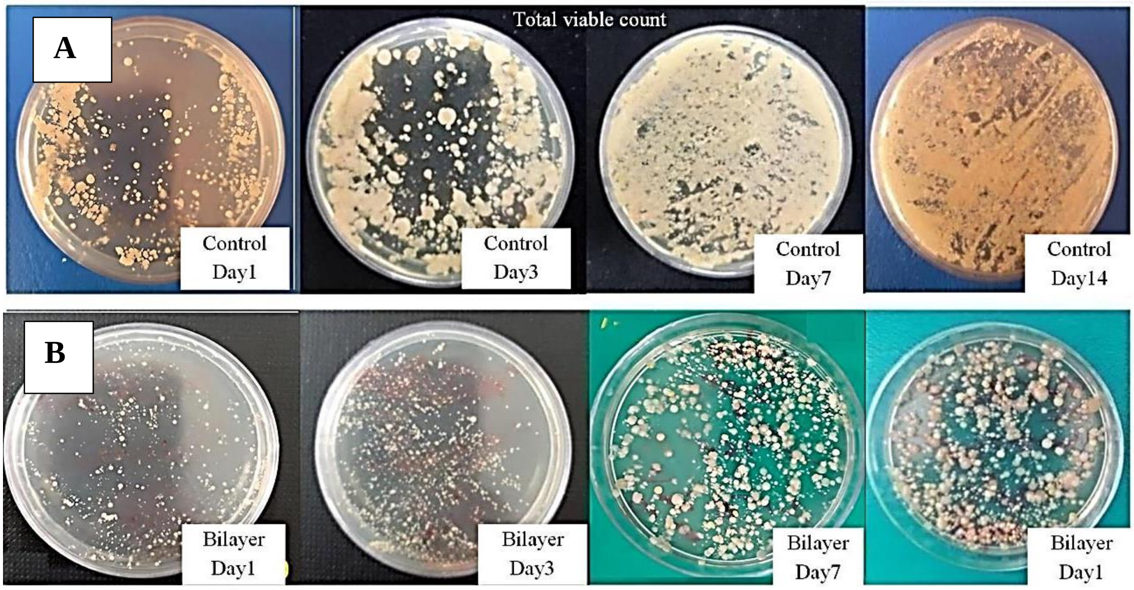

Fig. 3. A: Illustration of TVC for the control sample during the storage period of fish fillet samples B: Illustration of TVC for the Bi-layer sample during the storage period of fish fillet samples.

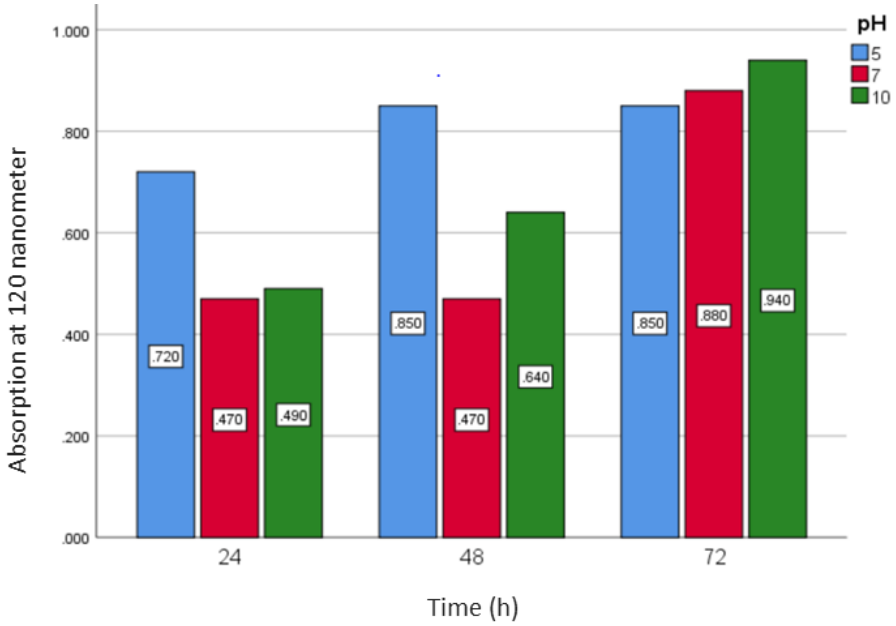

Fig. 4. UV absorbance graph depicting the release of rosemary microencapsulated in chitosan nanoparticles under acidic, neutral, and alkaline conditions after 24, 48, and 72 hours. Higher absorbance values indicate greater release(Khoshboui Lahijani *et al.*, 2024).

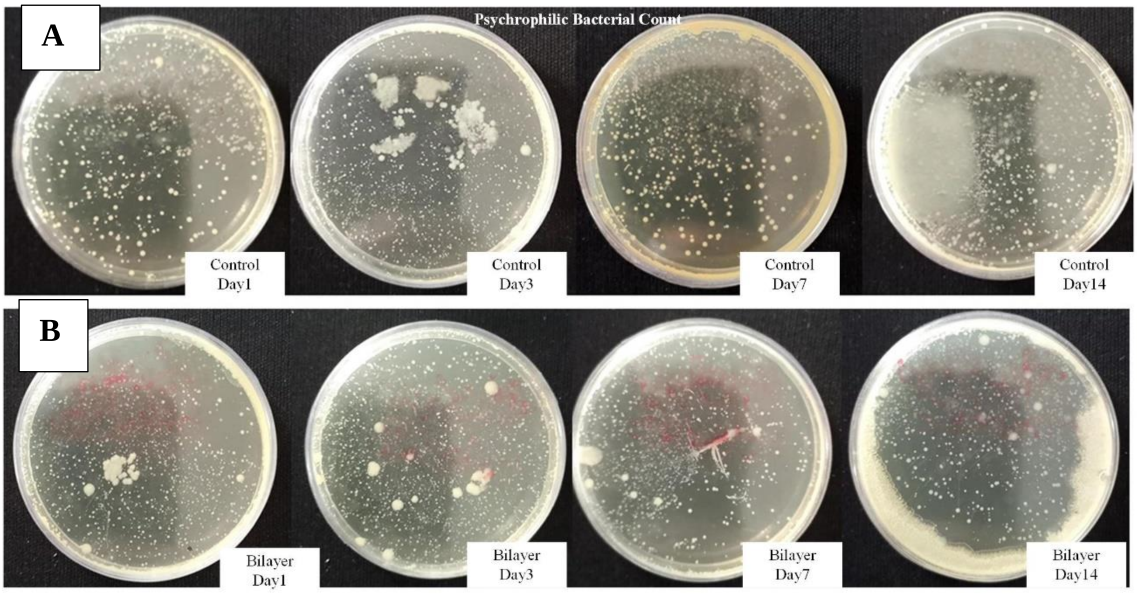

Fig. 5. A: Illustration of Psychrophilic Bacterial Count for the control sample during the storage period of fish fillet samples B: Illustration of Psychrophilic Bacterial Count for the Bi-layer sample during the storage period of fish fillet samples.

Table 2. Measurement of psychotropic bacterial counts (CFU/ml) during the storage period of fish fillet samples

Day	Control Sample	Bi-layer Fim
Day1	1.35E+05[a]	1.19E+05[a]
Day3	1.11E+06[a]	3.70E+05[a]
Day7	3.96E+06[a]	7.86E+05[a]
Day14	1.56E+08[b]	1.70E+06[a]

* Lowercase letters indicate a significant difference between treatments.

the control sample, indicating the antimicrobial properties of rosemary in the psychrophilic range as well. According to research conducted by Farokhzad *et al*. who examined the effects of chitosan and rosemary essential oil on the quality characteristics of chicken burgers, the microbial growth rate decreased with the addition of both rosemary essential oil and chitosan compared to the control sample. This reduction in microbial growth is attributed to the antimicrobial properties of these two substances (Farokhzad *et al*., 2023). Essential oils disrupt the cytoplasmic membranes of microorganisms, leading to cell death (Zhang *et al*., 2018). The antimicrobial properties of chitosan are attributed to its polycationic nature, which disrupts the cell membrane. However, this may also be due to chelation behavior, water-binding properties, and inhibition of mRNA synthesis. According to research conducted by Külcü *et al*. who examined the modeling of the shelf life of ground chicken treated with rosemary extract, the addition of rosemary extract resulted in a 1.5-logarithmic cycle reduction by the last day of storage (Külcü and Kalkan, 2022).

- ***Chemical Tests***
- ***Measurement of Total Volatile Nitrogen***

As shown in Fig. 6, the two-layer film resulted in a slower increase in volatile basic nitrogen compared to the control sample, aligning with the findings of Jafari *et al*. (2017), who studied the effects of chitosan and rosemary extract coatings on the quality of fish fillets inoculated with *Listeria monocytogenes* during refrigerated storage. This could be due to reduced bacterial populations or the decreased deamination capacity of bacteria for non-protein nitrogen compounds. In any case, as storage time increases, the amount of volatile basic nitrogen increases, but in the treated sample, the rate is lower than in the control (Jafari *et al*., 2017; Yu *et al*., 2017).

- ***Measurement of Secondary Oxidation Products Using TBA***

As shown in Fig. 7, the amount of TBA increased with increasing storage time, although this increase occurred at a much slower rate in the samples packaged in the two-layer film than in the control. This aligns with the results obtained by Nawaz *et al*. (2020), who studied the effects of rosemary extract coating combined with chitosan on the quality of stored mulberries. Additionally, based on the findings of Can *et al*. (2018), who focused on gelatin recovery for producing biodegradable films, chitosan helps to keep metals away from fish lipids through chelation, playing an antioxidant role in stabilizing the fat content in food. The coating also acts as a barrier, reducing the rate of oxygen diffusion from the surrounding air to the fillet surface, thus preventing lipid oxidation. Furthermore, the addition of rosemary extract to the chitosan coating showed a synergistic effect. The antioxidant activity of most plant extracts is attributed to their ability to break the free radical chain by donating hydrogen atoms (Can *et al*., 2018; Nawaz *et al*., 2020).

- ***Sensory Evaluation Using a 5-Point Hedonic Scale***

According to the results shown in Table 3, the samples packaged in the two-layer film had the highest shelf life compared to

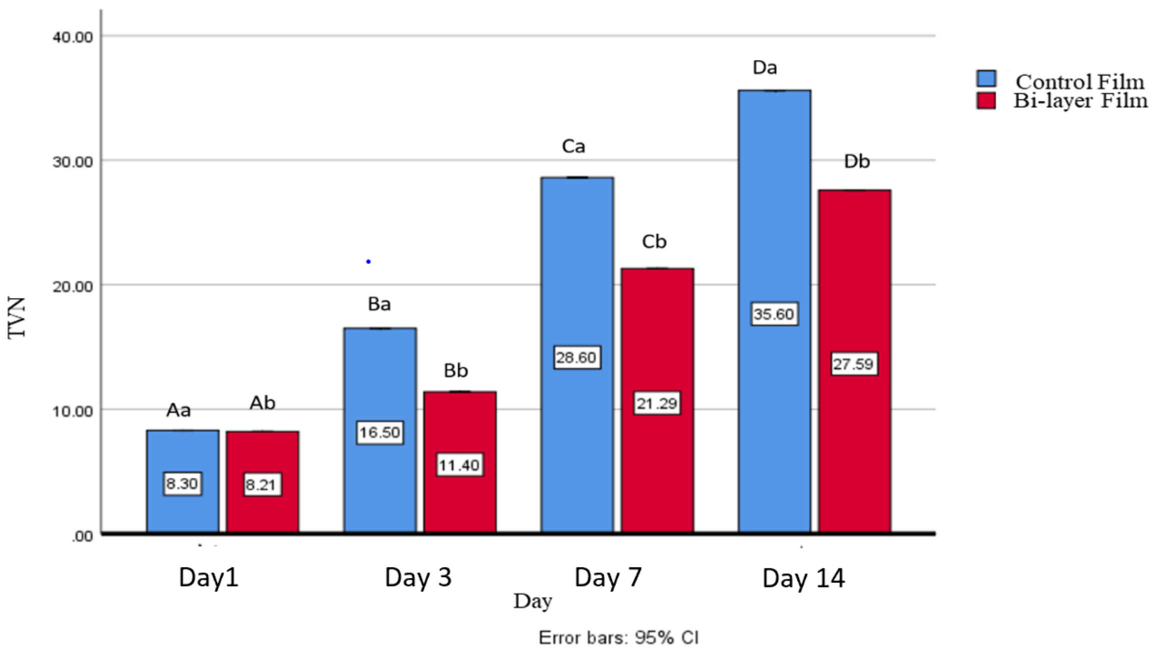

Fig. 6. Trend of TVN Changes During Storage of Fish Fillet Samples.
* Lowercase letters indicate significant differences between treatments, whereas uppercase letters indicate significant differences between days.

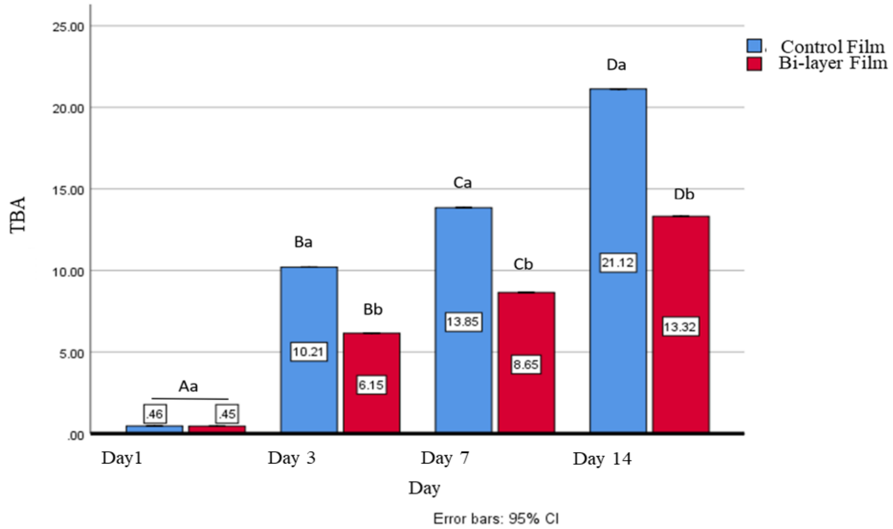

Fig. 7. Trend of Secondary Oxidation Product Levels Measured by TBA During Storage of Fish Fillet Samples.

* Lowercase letters indicate significant differences between treatments, whereas uppercase letters indicate significant differences between days.

the control samples. Rosemary extract reduced the fishy odor and improved the appearance of fish, thereby increasing their shelf life. The high capacity of chitosan to retain water delays moisture loss, which helps maintain the relative textural qualities of the fish samples. The results of the sensory evaluation were related to microbial and chemical assessments. Owing to high lipid oxidation and microbial growth, early spoilage in the control samples was observed, manifesting as off-odors, sliminess, and color changes. The antioxidant and antimicrobial effects of the bilayer film minimized oxidative effects while prolonging product quality, aligning with findings by Ozogul *et al*. (2017), who assessed the effects of plant-based nanoemulsions (rosemary, laurel, thyme, and sage) on the sensory, chemical, and microbiological qualities of rainbow trout (Yıldız, 2017; Ozogul *et al*., 2017). Additionally, Gedif *et al*. (2022) indicated that innovative coatings with antioxidant properties containing rosemary extract slowed the lipid oxidation processes in carp fillets during storage and protected the fish from oxidative rancidity during refrigeration. Thus, the presence of plant extracts, including rosemary, can delay chemical degradation and sensory quality reduction caused by oxidative breakdown (Derbew Gedif *et al*., 2022).

Conclusion

Maintaining the quality and freshness of food, especially for sensitive products such as fish, is of great importance. Active packaging is an innovative solution that plays a key role in preventing oxidation and microbial spoilage. Oxidation can lead to changes in the taste, odor, and color of fish, whereas microbial spoilage poses health hazards and reduces product quality. The use of two-layer packaging films containing rosemary extract, as an advanced technique, can extend the refrigerated shelf life of fish. Rosemary extract, owing to its antimicrobial and antioxidant properties, can reduce the growth of microorganisms and prevent the oxidation of unsaturated fats in fish. Enhancing the physical properties and barrier capabilities of packaging or integrating it with controlled atmospheres can provide better conditions for maintaining food quality and improving the current design. Ultimately, active packaging using chitosan nanoparticles and rosemary extract is an effective strategy for preserving the quality and freshness of fish and other food products. This method not only helps in reduction of food waste but also contributes to public health and increases consumer satisfaction.

Table 3. Average Sensory Evaluation Results of Fish Fillet Samples Packaged in Bilayer Film and Control Film, Evaluated by Four Assessors Based on: A. Color Evaluation B. Odor Evaluation C. Texture Evaluation D. Overall Acceptance

A	Sample	Average color evaluation by Twenty assessors
Day1	Control	5.0[a]
	Bilayer film	5.0[a]
Day3	Control	5.0[a]
	Bilayer film	5.0[a]
Day7	Control	3.0[b]
	Bilayer film	4.0[b]
Day14	Control	2.25[d]
	Bilayer film	3.0[c]

B	Sample	Average odor evaluation by Twenty assessors
Day1	Control	5.0[a]
	Bilayer film	5.0[a]
Day3	Control	4.0[b]
	Bilayer film	5.0[a]
Day7	Control	3.25[c]
	Bilayer film	4.0[b]
Day14	Control	1.25[e]
	Bilayer film	2.0[d]

C	Sample	Average texture evaluation by Twenty assessors
Day1	Control	5.0[a]
	Bilayer film	5.0[a]
Day3	Control	4.0[b]
	Bilayer film	4.75[a]
Day7	Control	3.0[c]
	Bilayer film	4.0[b]
Day14	Control	1.0[e]
	Bilayer film	2.5[d]

D	Sample	Overall acceptance rating by Twenty assessors
Day1	Control	5.0[a]
	Bilayer film	5.0[a]
Day3	Control	4.3[b]
	Bilayer film	4.9[a]
Day7	Control	3.1[d]
	Bilayer film	4.0[c]
Day14	Control	1.5[f]
	Bilayer film	2.5[e]

* Lowercase letters indicate a significant difference between treatments.

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