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Active Gelatin Films Containing Pumpkin Seed Oil and Rosemary Essential Oil for Extending the Shelf Life of Refrigerated Sardine Fillets

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Abstract

This study evaluated the effectiveness of gelatin-based edible films enriched with pumpkin seed oil and rosemary essential oil in preserving the quality and extending the refrigerated shelf life of sardine (*Sardina pilchardus*) fillets. Biodegradable films were prepared using gelatin plasticized with polyvinyl alcohol and glycerol, while bile acids extracted from chicken gallbladders were employed as a natural emulsifying agent to improve the incorporation and stability of the oils within the film matrix. Sardine fillets were assigned to uncoated (control) and coated treatments and stored at 4 ± 1°C for 15 days. The preservative efficacy of the developed films was assessed by monitoring physicochemical parameters, including pH, free fatty acids (FFA), peroxide value (PV), and thiobarbituric acid (TBA), together with microbiological quality indicators during refrigerated storage. All samples exhibited progressive increases in pH, lipid oxidation indices, and microbial counts throughout storage. However, fillets coated with the developed edible films showed significantly lower ($p < 0.05$) levels of chemical deterioration and microbial growth compared with the uncoated control. The films effectively limited oxygen and moisture transfer, thereby retarding oxidative reactions and inhibiting microbial proliferation. Among the tested formulations, rosemary essential oil-enriched films demonstrated the highest preservation efficacy, which can be attributed to the potent antioxidant and antimicrobial activities of their phenolic constituents. Moreover, coliform bacteria were not detected in any treatment during storage, indicating satisfactory hygienic handling and storage conditions. The findings suggest that gelatin-based edible films incorporated with rosemary essential oil or pumpkin seed oil constitute sustainable and eco-friendly active packaging systems with considerable potential to enhance the quality, safety, and refrigerated shelf life of fish products.

Keywords: Pumpkin seed oil, rosemary essential oil, edible films, microbiological quality, refrigerated storage.

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Introduction

Fish and fishery products are among the most perishable foods because of their high moisture content, abundance of unsaturated lipids, neutral pH, and the presence of autolytic enzymes and spoilage microorganisms. These characteristics accelerate lipid oxidation, microbial proliferation, and quality deterioration during refrigerated storage, resulting in shortened shelf life and substantial economic losses. Small pelagic fish such as sardine (*Sardina pilchardus*) are particularly susceptible to spoilage owing to their high content of polyunsaturated fatty acids, which readily undergo oxidative degradation and produce undesirable sensory changes. Therefore, developing effective preservation strategies for refrigerated sardine products remains a major challenge for the seafood industry (Nie *et al.*, 2025).

Conventional plastic packaging can delay quality deterioration to some extent; however, growing environmental concerns associated with the extensive use of petroleum-based plastics have stimulated interest in sustainable packaging technologies. In this context, edible films have emerged as promising alternatives because they are biodegradable, environmentally friendly, and can serve as carriers of functional compounds that actively enhance food preservation. Unlike edible coatings, which are directly applied onto food surfaces, edible films are prepared as self-supporting thin layers and subsequently wrapped around food products, providing an additional physical and functional barrier against quality deterioration (Athanasopoulou *et al.*, 2024).

Gelatin is one of the most extensively investigated biopolymers for edible packaging applications due to its excellent film-forming ability, transparency, biocompatibility, and biodegradability. Nevertheless, gelatin films exhibit relatively poor water-vapor barrier properties and limited resistance to moisture, restricting their practical application in high-moisture foods such as fish. To address these limitations, gelatin matrices have been modified with various bioactive compounds and hydrophobic substances to improve their mechanical performance and provide antioxidant and antimicrobial functions capable of retarding fish spoilage (Luo *et al.*, 2022).

Plant-derived oils and essential oils have attracted increasing attention as natural additives in active packaging systems because of their ability to inhibit lipid oxidation and microbial growth. Pumpkin seed oil is rich in unsaturated fatty acids and natural antioxidants, which contribute to its antioxidant and antimicrobial activities and suggest its potential as a natural preservative for lipid-rich foods (Hu *et al.*, 2024; Gedi, 2022; Rohman and Irnawati, 2020). Similarly, rosemary essential oil is recognized as an effective natural antioxidant and antimicrobial agent and has been widely reported to retard oxidative rancidity and suppress spoilage microorganisms in food systems (Rašković *et al.*, 2014).

Although gelatin films incorporated with plant extracts or essential oils have been investigated previously, studies examining the combined use of pumpkin seed oil and rosemary essential oil in gelatin-based edible films for preserving refrigerated sardine fillets remain scarce. Moreover, limited information is available regarding the synergistic effects of these two natural oils on the physicochemical, microbiological, and sensory stability of sardine during cold storage. The incorporation of pumpkin seed oil may improve the hydrophobicity and antioxidant potential of gelatin films, whereas rosemary essential oil may provide additional antimicrobial and antioxidative protection, potentially resulting in enhanced preservation efficacy (Nie *et al*., 2025).

Therefore, the present study aimed to develop a gelatin-based edible film fortified with pumpkin seed oil and rosemary essential oil and to evaluate its effectiveness in preserving the quality and extending the refrigerated shelf life of sardine (*Sardina pilchardus*) fillets. The novelty of this work lies in the development of a multifunctional biodegradable packaging system combining these two natural bioactive oils and in assessing its application specifically for the preservation of highly perishable sardine fillets during refrigerated storage.

Materials and Methods

Materials

Food-grade gelatin (Type B, Bloom strength 220 g), polyvinyl alcohol (PVA; molecular weight 85,000–124,000, 99% hydrolyzed), glycerol (≥99.5% purity), chloroform (HPLC grade), methanol (HPLC grade), diethyl ether, ethanol, potassium iodide, sodium thiosulfate, and all microbiological media were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Fresh rosemary (Rosmarinus officinalis L.) leaves and pumpkin (*Cucurbita maxima*) seeds were obtained from a local agricultural market in Basrah, Iraq.

Preparation of Pumpkin Seed Oil

Pumpkin seeds were cleaned, air-dried at room temperature (25 ± 2°C), ground into fine powder, and stored at 4°C until extraction. Crude pumpkin seed oil was extracted according to the method of Bligh and Dyer (1959) with slight modifications. Briefly, 100 g of pumpkin seed powder was homogenized with distilled water, chloroform, and methanol. The organic phase was separated, filtered, and concentrated under reduced pressure using a rotary evaporator at 40°C. The extracted oil was stored in amber glass bottles at 4°C until use.

Extraction of Rosemary Essential Oil

Rosemary essential oil was extracted by hydrodistillation using a Clevenger-type apparatus according to the method of the European Pharmacopoeia. Briefly, 100 g of dried rosemary powder was mixed with distilled water and subjected to hydrodistillation for 3 h. The obtained essential oil was dried over anhydrous sodium sulfate, collected in amber glass vials, and stored at 4°C until use.

Preparation and Characterization of Chicken Bile Emulsifier

Fresh chicken gallbladders were obtained from a local poultry slaughterhouse. The bile was aseptically collected, filtered through sterile gauze, and stored at 4°C. The pH and total bile acid content were determined before use. Preliminary experiments demonstrated that 1 mL of bile extract provided stable emulsification of the oils without adversely affecting film appearance.

Preparation of Gelatin-Based Films

Film-forming solutions were prepared according to El-Nemr et al. (2020) with modifications. Gelatin (1 g) and PVA (1 g) were dissolved separately in distilled water at 60°C and 90°C, respectively, and mixed thoroughly. Glycerol was added at 30% (w/w of total polymer content) as a plasticizer because preliminary experiments indicated that higher concentrations resulted in sticky films with poor handling properties.

For active films, pumpkin seed oil and rosemary essential oil were separately emulsified with chicken bile extract and incorporated into the film-forming solution under continuous stirring until homogeneous emulsions were obtained. The solutions were cast into 9-cm Petri dishes and dried at room temperature for 48 h.

Characterization of Film Properties

Film Thickness

Film thickness was measured at ten random locations using a digital micrometer (Mitutoyo, Japan) with a precision of 0.001 mm.

Water Vapor Permeability (WVP)

WVP was determined according to ASTM E96-95 using silica gel desiccators and expressed as g·mm/m²·day·kPa.

Mechanical Properties

Tensile strength (TS) and elongation at break (EAB) were measured according to ASTM D882 using a texture analyzer (Stable Micro Systems, UK).

Preparation of Sardine Fillets

Fresh sardine (*Sardina pilchardus*) were purchased from local fish markets in Basrah, Iraq, within 12 h after capture and transported to the laboratory under refrigerated conditions. A total of 60 fish specimens were used in this study. The fish had an average weight of 150–200 g and a total length of 20–25 cm. The fish were washed, headed, eviscerated, skinned, and filleted. The flesh was cut into pieces measuring approximately 4 × 3 × 2 cm.

Experimental Treatments

Four treatments were prepared:

1. Uncoated sardine fillets (control)
2. Sardine fillets coated with gelatin film
3. Sardine fillets coated with gelatin film containing pumpkin seed oil
4. Sardine fillets coated with gelatin film containing pumpkin seed oil and rosemary essential oil

Each treatment consisted of three independent replicates, and each replicate contained five fillets.

Storage Conditions

All samples were stored at 4 ± 1°C for 15 days. Analyses were performed on days 1, 3, 5, 10, and 15.

Physicochemical Analyses

The determination of pH, acid value (AV), free fatty acids (FFA), peroxide value (PV), and thiobarbituric acid value (TBA) was carried out according to the methods previously described by Vital *et al.* (2016), Pearson (1976), and Soltanizadeh and Ghiasi-Esfahani .(2015).

Microbiological Analyses

Total plate count, proteolytic bacteria, lipolytic bacteria, and total coliforms were determined according to APHA (2012). Results were expressed as log10 CFU/g.

Experimental Design and Statistical Analysis

The experiment was conducted using a completely randomized design (CRD). Data were analyzed using a factorial arrangement considering treatment and storage period as fixed factors. All measurements were performed in triplicate and expressed as mean ± standard deviation. Analysis of variance (ANOVA) was performed using GenStat software (Release 10.3DE), and significant differences among means were determined using Fisher's Least Significant Difference (LSD) test at $P \leq 0.05$.

Results and Discussion

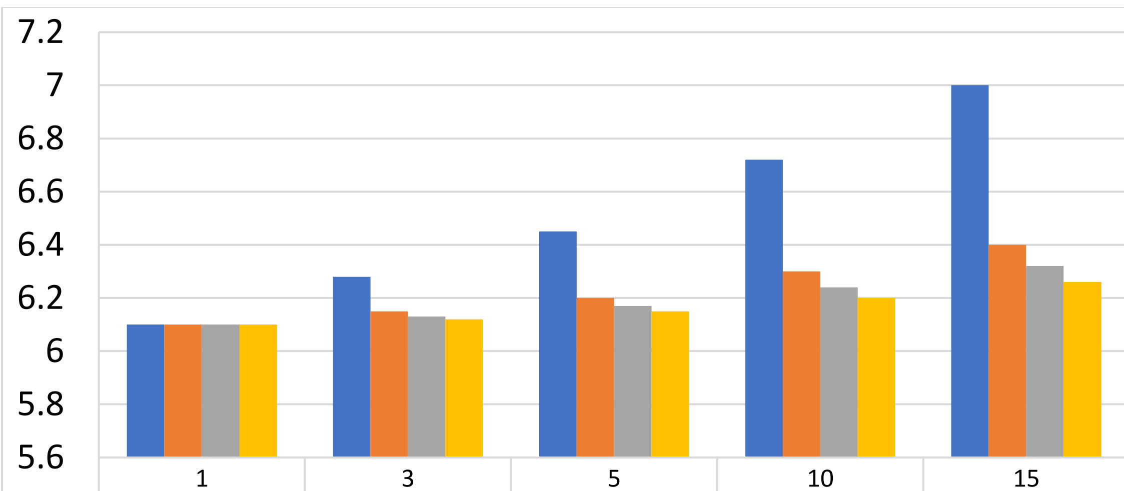
Changes in pH Values of Refrigerated Fish Fillets

The results presented in Figure (1) revealed significant differences ($P \leq 0.05$) among treatments as affected by film type, storage period, and their interactions. A gradual increase in pH values was observed throughout refrigerated storage. This increase was more pronounced in the control samples than in the coated treatments. The progressive increase in pH can be attributed to microbial activity and protein degradation, which lead to the formation of alkaline compounds such as ammonia and biogenic amines. The highest pH values were recorded in the uncoated control samples at the end of storage, indicating accelerated quality deterioration. These findings are consistent with those reported by Figueroa-Enríquez *et al.* (2024), who identified pH increase as a direct indicator of microbial spoilage in meat products.

Film type exerted a significant effect on pH stability. The active edible films effectively reduced the rate of pH increase during storage. Among all treatments, rosemary oil-fortified films maintained the lowest pH values, likely due to the antioxidant and antimicrobial activities of their phenolic constituents. Similar observations were reported by Akther *et al*. (2024), Antonino *et al*. (2023), and Ma *et al*. (2024), who demonstrated that essential oil-incorporated edible films improve barrier properties, inhibit spoilage microorganisms, and preserve food quality during refrigerated storage.

Storage duration also significantly affected pH values, which increased progressively over time, particularly in the control samples. This trend reflects the continued action of endogenous enzymes and spoilage microorganisms during refrigerated storage. The significant interaction between film type and storage period suggests that the protective effect of edible films depends on both film composition and storage duration. Rosemary oil-fortified films exhibited the greatest ability to maintain chemical stability and delay quality deterioration.

Overall, the results indicate that increasing pH values are closely associated with quality deterioration during refrigerated storage. The application of active gelatin-based edible films effectively reduced undesirable chemical changes, while rosemary essential oil provided the strongest protective effect among the tested treatments.



	1	3	5	10	15
T1	6.1	6.28	6.45	6.72	7
T2	6.1	6.15	6.2	6.3	6.4
T3	6.1	6.13	6.17	6.24	6.32
T4	6.1	6.12	6.15	6.2	6.26

Figure (1). Changes in pH values of uncoated fish fillets and fish fillets coated with gelatin-based films fortified and non-fortified with vegetable oils during refrigerated storage.

Each value represents the mean of two replicates.
LSD ($P \leq 0.05$) for the effect of film type on pH values: 0.003367
LSD ($P \leq 0.05$) for the effect of storage period on pH values: 0.003764
LSD ($P \leq 0.05$) for the interaction between film type and storage period on pH values: 0.007529

Free Fatty Acids (FFA%)

The results presented in Figure (2) demonstrated a significant ($P \leq 0.05$) gradual increase in free fatty acid (FFA) values throughout the refrigerated storage period of up to 15 days for all treatments. However, the rate of increase varied according to the type of film applied. The percentage of FFA was markedly influenced by the presence or absence of edible film coatings. Uncoated samples exhibited the highest FFA levels at the end of storage, whereas edible films fortified with vegetable oils effectively reduced lipid hydrolysis, particularly those containing rosemary oil, which showed the greatest ability to preserve lipid stability.

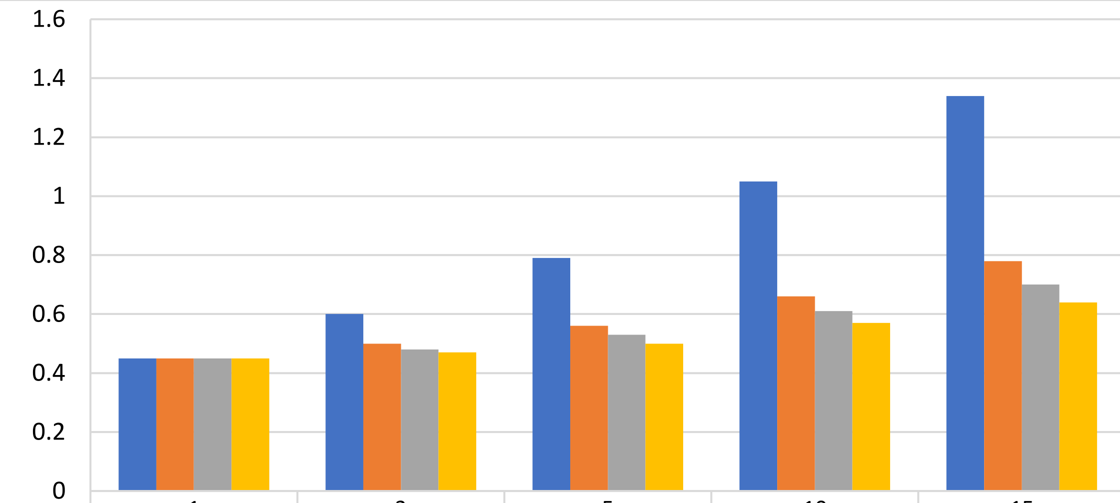
The increase in free fatty acid content is considered an important indicator of lipid deterioration, resulting primarily from lipase activity and microbial hydrolysis of triglycerides. The observed increase in FFA values may be attributed to the action of lipase enzymes, which hydrolyze the ester bonds linking fatty acids to glycerol molecules, thereby releasing free fatty acids as the final products of lipid hydrolysis. This process is mediated by lipolytic enzymes, including lipases and phospholipases, produced by lipolytic microorganisms (Yousif, 2014).

Statistical analysis revealed a significant effect of film type on FFA values, indicating genuine differences between coated and uncoated treatments. Films fortified with rosemary oil consistently exhibited the lowest free fatty acid levels throughout storage. This protective effect may be attributed to the presence of bioactive compounds with strong antioxidant properties capable of suppressing oxidative reactions and reducing the activity of lipid-degrading enzymes. These findings are in agreement with Kumar *et al.* (2021), who reported that the incorporation of essential oils into biodegradable packaging materials effectively reduces lipid degradation and limits the accumulation of free fatty acids in refrigerated meat products.

According to Domínguez *et al.* (2019), the susceptibility of lipids to deterioration during storage is strongly influenced by the degree of unsaturation of fatty acids, with highly unsaturated lipids being more vulnerable to oxidative degradation. Storage duration also exerted a significant effect on FFA values. A progressive increase was observed as storage time increased, particularly in control samples. This trend can be attributed to the continuous enzymatic and microbial activities occurring during refrigerated storage, which accelerate lipid hydrolysis over time. Similarly, Pagey *et al.* (2025) reported that prolonged refrigerated storage enhances lipolytic enzyme activity and promotes the accumulation of secondary oxidation products, especially in products lacking antioxidant-active packaging systems.

The interaction between film type and storage period was also significant. Vegetable oil-fortified films maintained greater lipid stability during extended storage periods compared with the control treatment. Mostafa *et al.* (2025) likewise demonstrated that bioactive edible films enriched with natural antioxidants effectively inhibit the formation of lipid oxidation products and retard lipid deterioration during refrigerated storage.

Overall, the findings indicate that gelatin-based edible films fortified with vegetable oils, particularly rosemary oil, effectively reduced lipid hydrolysis and preserved lipid stability in refrigerated fish fillets. Consequently, these films contributed to improved chemical quality and extended shelf life of the refrigerated products.



	1	3	5	10	15
T1	0.45	0.6	0.79	1.05	1.34
T2	0.45	0.5	0.56	0.66	0.78
T3	0.45	0.48	0.53	0.61	0.7
T4	0.45	0.47	0.5	0.57	0.64

Figure (2). Changes in free fatty acid (FFA%) values of uncoated fish fillets and fish fillets coated with gelatin-based films fortified and non-fortified with vegetable oils during refrigerated storage.

Each value represents the mean of two replicates
LSD ($P \leq 0.05$) for the Effect of Film Type on Free Fatty Acid Percentage= 0.003367
LSD ($P \leq 0.05$) for the Effect of Storage Period on Free Fatty Acid Percentage= 0.003764
LSD ($P \leq 0.05$) for the Interaction Effect between Film Type and Storage Period on Free Fatty Acid Percentage = 0.007529

Peroxide Value (PV) (meq O_2/1000 g Fish)

The results presented in Figure (3) demonstrated a gradual increase and significant differences ($P \leq 0.05$) among the experimental treatments in peroxide value (PV) throughout the refrigerated storage period. These findings indicate a genuine effect of the studied factors rather than random variation. The control samples (without coating) showed significantly higher PV values than all other treatments and consistently recorded the highest levels throughout storage, reflecting the continuous development of primary lipid oxidation products. This behavior is expected in refrigerated animal products due to the susceptibility of unsaturated fatty acids to oxidation when exposed to oxygen, light, and prolonged low-temperature storage conditions. The higher oxidation rate in the control samples can be attributed to direct exposure to oxygen and the absence of a protective barrier or antioxidant compounds, which accelerates the formation of primary hydroperoxides compared with samples coated with gelatin-based films and those fortified with antioxidant-rich vegetable oils (Domínguez *et al*., 2019).

In contrast, samples coated with gelatin films fortified with vegetable oils exhibited significantly lower PV values, particularly those enriched with rosemary oil, which consistently recorded the lowest oxidation levels during storage. This effect may be attributed to the antioxidant activity of the phenolic compounds present in rosemary oil, which effectively scavenge free radicals and interrupt lipid oxidation chain reactions. Recent studies have confirmed that essential oils, especially rosemary oil, possess a remarkable ability to enhance oxidative stability in refrigerated meat products and reduce the formation of oxidation products compared with untreated controls (Liu *et al*., 2012; Alansari, 2020).

Furthermore, active films composed of gelatin, polyvinyl alcohol, and glycerol have been reported to play an important role in reducing oxidation rates compared with uncoated samples by decreasing oxygen permeability and slowing oxidative reactions. In the present study, fish fillets coated with gelatin films fortified with rosemary oil showed significantly lower ($P \leq 0.05$) PV values than the other treatments. Although no significant differences were observed between rosemary oil treatments and some pumpkin seed oil treatments during the early storage periods, the statistical superiority of rosemary oil became more evident on days 10 and 15 of storage. This finding suggests a cumulative antioxidant effect over time, attributable to the ability of phenolic compounds to inhibit radical chain reactions and suppress the propagation phase of lipid oxidation. Similar observations have been reported in recent studies demonstrating that bioactive films enriched with plant-derived compounds effectively extend shelf life and reduce oxidative spoilage indicators in refrigerated meat products (Liu *et al*., 2020).

Storage period also exerted a highly significant effect ($P \leq 0.05$) on PV values. All treatments exhibited a progressive increase in peroxide values with storage time, and significant differences were observed among storage days (1, 3, 5, 10, and 15), indicating the continuous accumulation of primary oxidation products. Moreover, the interaction between film type and storage period had a significant effect, suggesting that the effectiveness of active films and incorporated vegetable oils in controlling lipid oxidation is strongly influenced by storage duration.

Overall, the results indicate that film type, incorporation of antioxidant vegetable oils, and refrigerated storage duration were all critical factors significantly affecting peroxide values. These findings highlight the importance of active packaging technologies in enhancing the oxidative stability of fish products and improving their quality and shelf life during refrigerated storage.

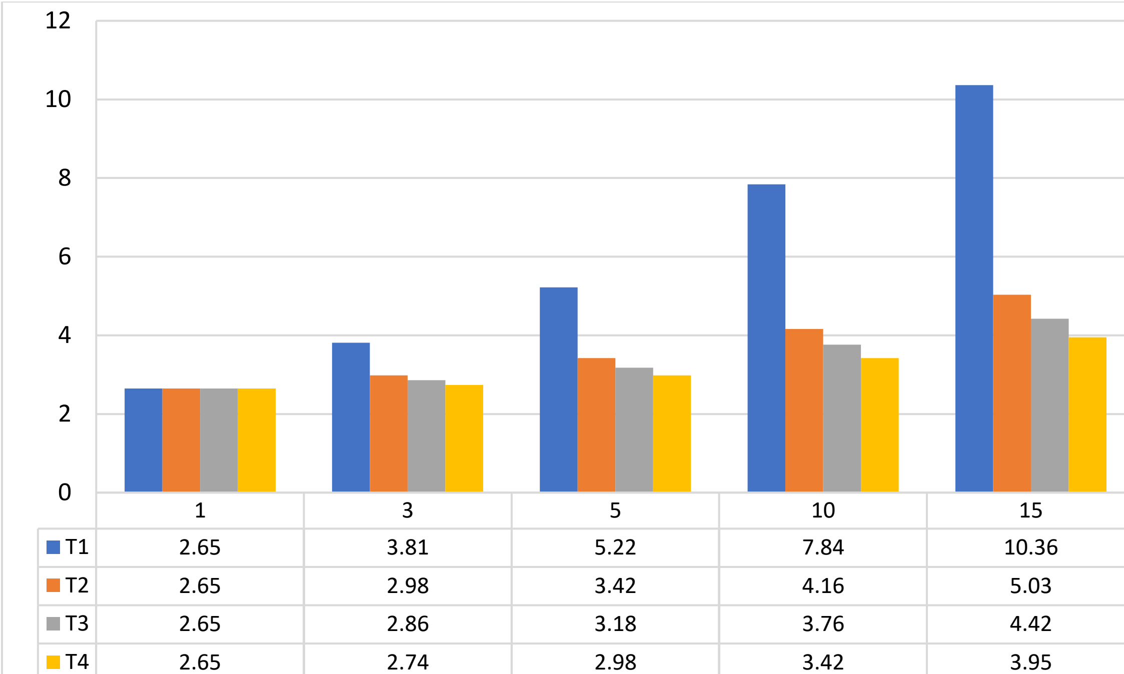


Figure (3): Changes in peroxide value (PV) of uncoated fish fillets and fish fillets coated with gelatin-based films fortified and non-fortified with vegetable oils during refrigerated storage.

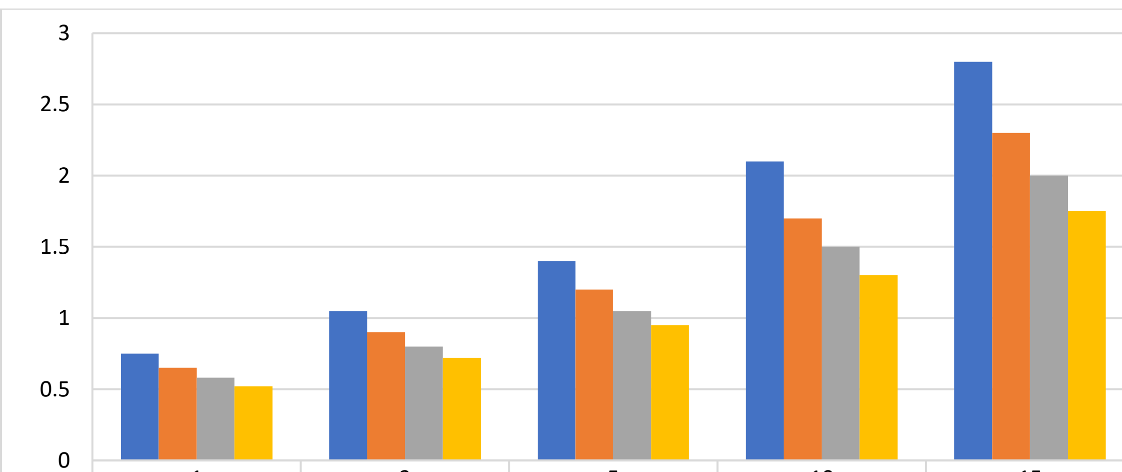
Each value represents the mean of two replicates.
LSD ($P \leq 0.05$) for the effect of film type on peroxide value (PV) = 0.003367
LSD ($P \leq 0.05$) for the effect of storage period on peroxide value (PV) = 0.003764
LSD ($P \leq 0.05$) for the interaction effect between film type and storage period on peroxide value (PV) = 0.007529

Thiobarbituric Acid Value (TBA) (mg Malondialdehyde/kg Fish Meat)

The results presented in Figure (4) revealed a gradual and significant increase (*$P \leq 0.05$*) in thiobarbituric acid reactive substances (TBARS) and TBA values in all refrigerated fish fillet samples throughout the storage period (1–15 days), indicating progressive lipid oxidation during refrigerated storage. Although refrigeration slows chemical reactions, it does not completely prevent the formation of secondary oxidation products, particularly malondialdehyde (MDA), which is the principal compound measured in the TBARS assay. These findings are consistent with those reported by Domínguez *et al*. (2019), who stated that refrigerated storage delays but does not entirely inhibit lipid oxidation processes.

In general, the observed increase in TBA values during refrigerated storage can be attributed to the accumulation of malondialdehyde, one of the major secondary products of lipid oxidation formed through the decomposition of lipid hydroperoxides in meat and meat products (O'Sullivan *et al*., 2004). Significant differences (*$P \leq 0.05$*) were observed among the coating treatments. The control samples (without coating) recorded the highest TBARS values compared with all coated treatments, whereas significantly lower values were observed in samples coated with gelatin-based films, particularly those fortified with rosemary oil. These results demonstrate the ability of gelatin films to reduce oxygen and moisture transfer, thereby inhibiting free radical formation and slowing oxidative reactions. This effect may be explained by the proteinaceous nature of gelatin films, which act as physical barriers that limit oxygen permeability. Similar findings were reported by Said and Sarbon (2022), who demonstrated that edible films significantly reduce lipid oxidation in refrigerated meat products.

From a practical statistical perspective, the incorporation of vegetable oils significantly affected TBA values. Samples coated with films fortified with rosemary oil exhibited lower TBARS values than those fortified with pumpkin seed oil or non-fortified films, confirming the existence of a genuine and statistically significant antioxidant effect rather than random variation. The superior performance of rosemary oil can be attributed to its high content of bioactive phenolic compounds, which function as potent antioxidants by scavenging free radicals and interrupting oxidative chain reactions. Nieto *et al.* (2018) reported that rosemary extracts are among the most effective natural antioxidants for suppressing lipid oxidation in food systems. Furthermore, the two-way analysis of variance revealed a significant interaction ($P \leq 0.05$) between film type and storage period. The rate of increase in TBARS values was markedly slower in coated samples than in the control treatment, indicating the effectiveness of edible films in reducing oxidative deterioration during refrigerated storage. This finding highlights the important role of active gelatin-based films, particularly those fortified with rosemary oil, in enhancing oxidative stability and extending the shelf life of refrigerated fish fillets.



	1	3	5	10	15
T1	0.75	1.05	1.4	2.1	2.8
T2	0.65	0.9	1.2	1.7	2.3
T3	0.58	0.8	1.05	1.5	2
T4	0.52	0.72	0.95	1.3	1.75

Figure (4): Changes in thiobarbituric acid TBA values of uncoated fish fillets and fish fillets coated with gelatin-based films fortified and non-fortified with vegetable oils during refrigerated storage.

Each value represents the mean of two replicates.
LSD ($P \leq 0.05$) for the effect of film type on thiobarbituric acid (TBA) values = 0.00948
LSD ($P \leq 0.05$) for the effect of storage period on thiobarbituric acid (TBA) values = 0.01060
LSD ($P \leq 0.05$) for the interaction effect between film type and storage period on thiobarbituric acid (TBA) values = 0.02119

Study of the Microbial Content of Uncoated Fish Fillets and Fish Fillets Coated with Gelatin-Based Films Fortified and Non-Fortified with Vegetable Oils During Refrigerated Storage

During refrigerated storage, certain psychrotrophic Gram-negative bacterial genera, particularly Pseudomonas and Acinetobacter, become active and contribute significantly to the spoilage of food systems. These microorganisms possess strong proteolytic and lipolytic activities, enabling them to degrade proteins and lipids and thereby accelerate quality deterioration in refrigerated products (Rajkumar and Dwivedi, 2011).

Total Plate Count (TPC)

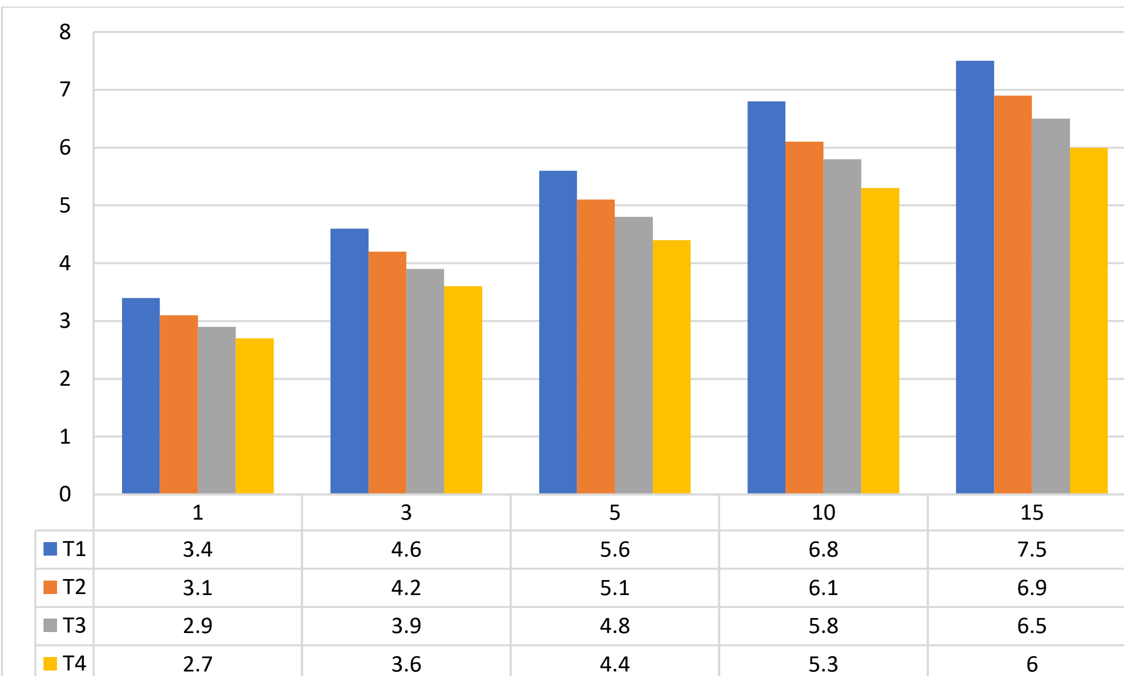
The results presented in Figure (5) showed a significant and gradual increase ($P \leq 0.05$) in total aerobic bacterial counts expressed as log10 CFU/g in both uncoated fish fillets (control samples) and fish fillets coated with gelatin/polyvinyl alcohol/glycerol films, whether fortified or non-fortified with vegetable oils, throughout the refrigerated storage period (1–15 days). This increase can be attributed to the continued microbial activity under refrigeration conditions. Although low temperatures reduce the rate of microbial growth, they do not completely inhibit bacterial proliferation. These findings are consistent with those reported by Orkusz *et al.* (2024), who indicated that refrigeration slows microbial growth but does not prevent microorganisms from eventually reaching the logarithmic growth phase during extended storage periods.

Statistical analysis revealed highly significant differences ($P \leq 0.05$) among treatments as affected by both film type and storage duration, in addition to significant interaction effects between these factors. Storage period exerted the greatest influence on bacterial counts, indicating that time is the most critical factor governing microbial proliferation and the subsequent deterioration of microbiological quality during refrigerated storage.

The results further demonstrated significant differences among treatment means. The control samples (without coating) recorded significantly higher bacterial counts ($P \leq 0.05$) than all coated treatments, highlighting the effectiveness of gelatin-based films in reducing microbial growth. This protective effect can be attributed to the ability of edible films to act as physical barriers that limit oxygen and moisture transfer, reduce external contamination, and restrict the environmental conditions favorable for bacterial proliferation. Similar findings were reported by Mariño-Cortegoso *et al.* (2025), who demonstrated that edible coatings significantly improve the microbiological stability of refrigerated food products.

Statistical comparisons also showed that treatments fortified with rosemary oil exhibited the lowest bacterial counts compared with pumpkin seed oil treatments and non-fortified films. This significant antimicrobial effect is primarily attributed to the high concentration of phenolic compounds present in rosemary oil, which possess strong antibacterial activity and can disrupt bacterial cell membranes and inhibit cellular metabolism. Similar observations have been reported by Kloy *et al.* (2020), who confirmed the antimicrobial efficacy of rosemary-derived bioactive compounds against spoilage microorganisms.

Moreover, the interaction between film type and storage period was statistically significant ($P \leq 0.05$), as bacterial growth proceeded at a considerably slower rate in coated samples than in the control treatment. All differences between rosemary oil-fortified treatments and the remaining treatments were significant, confirming the effectiveness of these antimicrobial coatings in enhancing the microbiological stability of fish products and extending their shelf life under refrigerated storage conditions.



	1	3	5	10	15
T1	3.4	4.6	5.6	6.8	7.5
T2	3.1	4.2	5.1	6.1	6.9
T3	2.9	3.9	4.8	5.8	6.5
T4	2.7	3.6	4.4	5.3	6

Figure (5): Changes in total aerobic bacterial count (TPC, log10 CFU/g) of uncoated fish fillets and fish fillets coated with gelatin-based films fortified and non-fortified with vegetable oils during refrigerated storage.

Each value represents the mean of two replicates.
LSD (P ≤ 0.05) for the effect of film type on total aerobic bacterial count (log10 CFU/g) = 0.01265
LSD (P ≤ 0.05) for the effect of storage period on total aerobic bacterial count (log10 CFU/g) = 0.01633
LSD (P ≤ 0.05) for the interaction effect between film type and storage period on total aerobic bacterial count (log10 CFU/g) = 0.03266

Proteolytic Bacteria Count

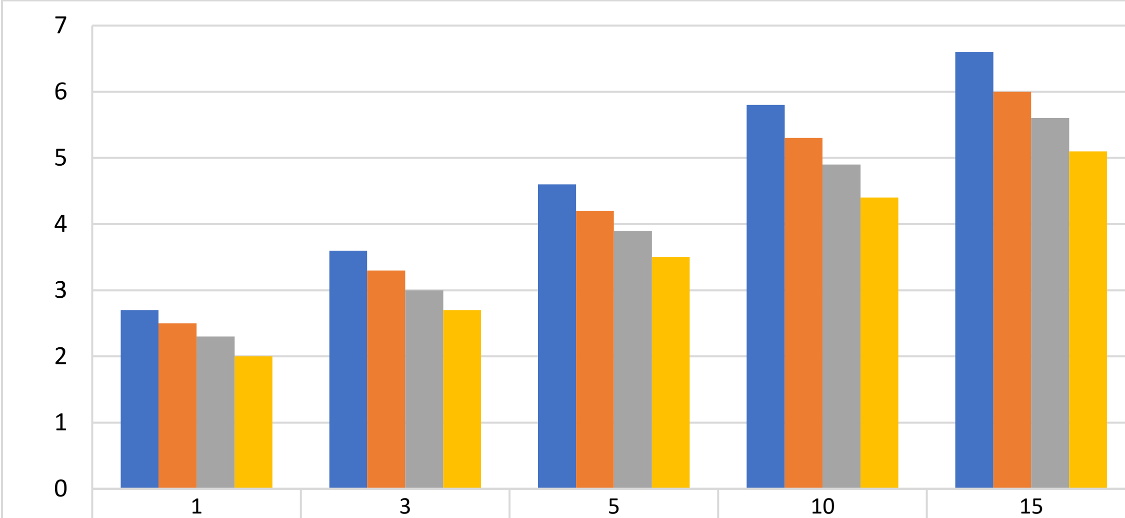
The results presented in Figure (6) showed a gradual and significant increase ($P \leq 0.05$) in the logarithmic counts of proteolytic bacteria in both uncoated fish fillets and fish fillets coated with gelatin/polyvinyl alcohol/glycerol films, whether fortified or non-fortified with vegetable oils, throughout the refrigerated storage period (1, 3, 5, 10, and 15 days). Bacterial counts increased continuously from the first day until day 15 in all treatments. This increase can be attributed to the ongoing metabolic activity of proteolytic bacteria, which secrete proteolytic enzymes capable of hydrolyzing proteins into peptides, amino acids, and simpler nitrogenous compounds. Such degradation processes contribute significantly to the deterioration of the quality attributes of meat and fish products. These findings are in agreement with those reported by Kang *et al.* (2026), who identified proteolytic bacteria as major microbial agents responsible for spoilage in refrigerated meat products due to their ability to utilize nitrogenous compounds as energy sources.

Statistical analysis revealed highly significant differences ($P \leq 0.05$) among treatments as affected by film type and storage duration, in addition to significant interaction effects between the studied factors. Significant differences were also observed among treatment means, indicating that the recorded variations were real and not attributable to random experimental error. The control samples (uncoated fillets) exhibited the highest proteolytic bacterial counts compared with coated treatments. This result may be attributed to the direct exposure of uncoated samples to oxygen, moisture, and environmental contamination, which create favorable conditions for the growth of proteolytic microorganisms.

In contrast, gelatin-based films played an important role in reducing bacterial counts by forming a protective barrier that limits oxygen and moisture transfer and minimizes surface contamination. These findings are consistent with those reported by Ahmed *et al.* (2012), who demonstrated the effectiveness of edible films in preserving refrigerated animal products by suppressing microbial growth.

The results further showed that treatments fortified with rosemary oil recorded the lowest proteolytic bacterial counts compared with pumpkin seed oil-fortified treatments and non-fortified films. These differences were statistically significant and can be attributed to the presence of bioactive phenolic compounds in rosemary oil, which exhibit strong antimicrobial activity through disruption of bacterial cell membrane permeability and inhibition of essential enzymatic systems. Similar findings were reported by Kloy *et al.* (2020), who observed comparable antimicrobial effects when rosemary extracts were incorporated into preservation systems for animal-derived foods.

Moreover, the interaction between film type and storage period was statistically significant ($P \leq 0.05$). Bacterial growth proceeded at a slower rate in coated samples than in the control treatment throughout the storage period, indicating the effectiveness of edible films in reducing microbial spoilage over time. Overall, the findings suggest that gelatin-based films fortified with natural antimicrobial agents, particularly rosemary oil, represent an effective strategy for improving microbiological stability and extending the shelf life of refrigerated fish and meat products by reducing proteolytic bacterial populations and minimizing protein degradation during storage.



	1	3	5	10	15
T1	2.7	3.6	4.6	5.8	6.6
T2	2.5	3.3	4.2	5.3	6
T3	2.3	3	3.9	4.9	5.6
T4	2	2.7	3.5	4.4	5.1

Figure (6): Changes in proteolytic bacterial counts (log10 CFU/g) of uncoated fish fillets and fish fillets coated with gelatin-based films fortified and non-fortified with vegetable oils during refrigerated storage.

Each value represents the mean of two replicates.
LSD ($P \leq 0.05$) for the effect of film type on proteolytic bacterial counts (log10 CFU/g) = 0.01461
LSD ($P \leq 0.05$) for the effect of storage period on proteolytic bacterial counts (log10 CFU/g) = 0.01633
LSD ($P \leq 0.05$) for the interaction effect between film type and storage period on proteolytic bacterial counts (log10 CFU/g) = 0.03266

Lipolytic Bacteria Count

The results presented in Figure (7) demonstrated a gradual and significant increase (*$P \leq 0.05$*) in the logarithmic counts of lipolytic bacteria (log10 CFU/g) in all treatments throughout the refrigerated storage period (1–15 days). However, uncoated fish fillets (control samples) exhibited significantly higher bacterial counts than fish fillets coated with gelatin/polyvinyl alcohol/glycerol films, whether fortified or non-fortified with vegetable oils. This increase can be attributed to the activity of lipolytic microorganisms capable of producing lipase enzymes, which catalyze the hydrolysis of triglycerides into free fatty acids and glycerol. Consequently, these reactions accelerate rancidity development and contribute to the deterioration of sensory quality. Lipolytic bacteria are considered among the principal spoilage microorganisms associated with refrigerated fish and meat products, particularly during extended storage periods. Similar findings were reported by Kang *et al.* (2026), who demonstrated a direct relationship between lipolytic bacterial growth and the increase in rancidity indicators in animal-derived foods.

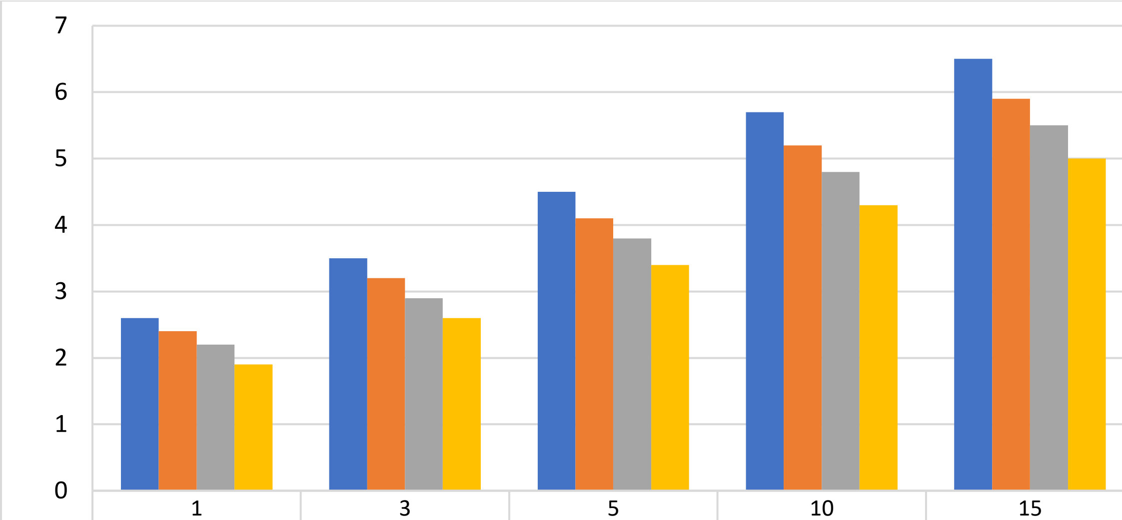
Statistical analysis revealed highly significant differences ($P \leq 0.05$) among the studied factors, including film type and storage period, as well as significant interaction effects between these variables. Storage duration exerted the greatest statistical influence, indicating that time was the most critical factor affecting bacterial proliferation. This observation reflects the cumulative effect of prolonged storage on microbial growth under refrigerated conditions.

The statistical comparisons further showed significant differences between the control treatment and coated samples. The control fillets recorded the highest lipolytic bacterial counts, whereas substantially lower values were observed in coated treatments, particularly those fortified with rosemary oil. This effect may be attributed to the ability of gelatin-based films to function as physical barriers that reduce oxygen and moisture transfer, thereby limiting the growth and metabolic activity of lipolytic bacteria. These findings are consistent with those reported by Mariño-Cortegoso *et al.* (2025), who demonstrated that edible coatings effectively reduce microbial spoilage and rancidity development in refrigerated food products.

Furthermore, treatments fortified with rosemary oil exhibited significantly lower lipolytic bacterial counts than pumpkin seed oil-fortified treatments and non-fortified films. The superior antimicrobial activity of rosemary oil can be attributed to its rich content of bioactive phenolic compounds, which possess both antioxidant and antibacterial properties. These compounds may inhibit lipase activity and disrupt bacterial cell membrane integrity, thereby reducing microbial proliferation. Similar observations were reported by Kloy *et al.* (2020), who confirmed the effectiveness of rosemary extracts in extending the shelf life of food products through their antimicrobial and antioxidant activities.

The interaction between film type and storage period was also significant ($P \leq 0.05$), with bacterial growth progressing at a slower rate in coated treatments than in the control samples throughout storage. This finding highlights the sustained protective effect of edible films against microbial spoilage during refrigerated storage.

Overall, the results indicate that the application of gelatin-based films fortified with natural antioxidants, particularly rosemary oil, represents an effective preservation strategy for controlling lipolytic bacterial growth, reducing rancidity development, and enhancing both the microbiological and sensory stability of refrigerated fish and meat products.



	1	3	5	10	15
T1	2.6	3.5	4.5	5.7	6.5
T2	2.4	3.2	4.1	5.2	5.9
T3	2.2	2.9	3.8	4.8	5.5
T4	1.9	2.6	3.4	4.3	5

Figure (7): Changes in lipolytic bacterial counts (log10 CFU/g) of uncoated fish fillets and fish fillets coated with gelatin-based films fortified and non-fortified with vegetable oils during refrigerated storage.

Each value represents the mean of two replicates.
LSD ($P \leq 0.05$) for the effect of film type on lipolytic bacterial counts (log10 CFU/g) = 0.01461
LSD ($P \leq 0.05$) for the effect of storage period on lipolytic bacterial counts (log10 CFU/g) = 0.01633
LSD ($P \leq 0.05$) for the interaction effect between film type and storage period on lipolytic bacterial counts (log10 CFU/g) = 0.03266

Total Coliform Bacteria Count

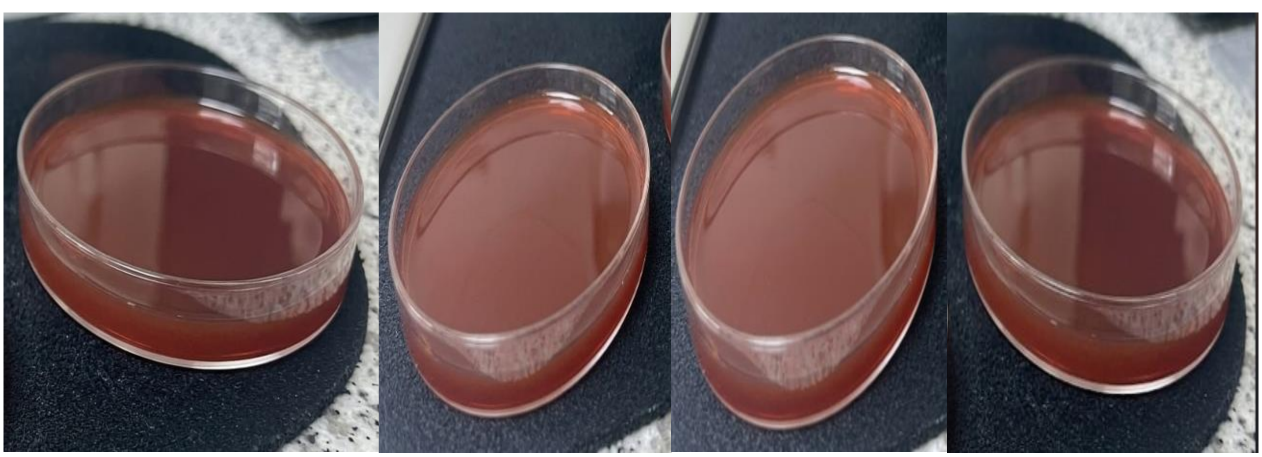
The microbiological examination results presented in Figure (8) revealed the absence of coliform bacteria (ND, Not Detected) in all fish fillet samples, including both the uncoated control treatment and the samples coated with gelatin/polyvinyl alcohol/glycerol films, whether fortified or non-fortified with vegetable oils. This finding represents an important indicator of product quality, hygienic processing efficiency, and the integrity of the refrigerated storage chain, all of which positively influence shelf life and the overall quality of refrigerated products (Hamid, 2020). Coliform bacteria are traditionally used as indicator microorganisms for environmental and fecal contamination in foods of animal origin (Mladenović *et al.*, 2021).

The complete absence of coliform bacteria in all treatments suggests that processing and handling operations were conducted under appropriate sanitary conditions, including slaughtering and processing according to Good Hygienic Practices (GHP), minimization of cross-contamination during filleting and packaging, and efficient refrigeration capable of suppressing microbial growth. Coliform bacteria are relatively sensitive to low temperatures; their growth is considerably slowed at refrigeration temperatures (0–4°C) and may cease under proper storage conditions. However, they can remain viable if initial contamination is present (Jay *et al.*, 2005). Therefore, their absence in the present study most likely reflects a low initial contamination load rather than merely the inhibitory effect of refrigeration.

These findings are consistent with numerous recent studies reporting that properly handled fresh refrigerated meats and fish products are frequently free of detectable coliform bacteria at the beginning of storage, whereas their presence is commonly associated with inadequate hygiene, temperature abuse, or contamination during handling and distribution. Coliforms are generally absent in freshly refrigerated meat products when sanitary slaughtering and processing standards are maintained but may appear later under improper storage conditions or elevated temperatures.

Furthermore, Sokra *et al.* (2026) emphasized that coliform bacteria serve as indirect indicators of fecal contamination and that their absence in refrigerated food products reflects effective implementation of Hazard Analysis and Critical Control Point (HACCP) systems. Consequently, the absence of coliform bacteria observed in this study constitutes a highly positive microbiological indicator, reflecting a low risk of contamination by enteric pathogens, effective sanitation and cleaning procedures, satisfactory hygienic quality, and compliance with food safety standards.

Moreover, the absence of coliform bacteria supports the findings obtained for the other microbiological indicators evaluated in this study, including total aerobic bacterial count, proteolytic bacteria, and lipolytic bacteria. These results confirm that the observed quality changes during refrigerated storage were primarily associated with the natural growth of environmental spoilage microorganisms rather than contamination of fecal origin.



T1 **T2** **T3** **T4**

Figure (8): Total coliform bacteria counts of uncoated fish fillets and fish fillets coated with gelatin-based films fortified and non-fortified with vegetable oils during refrigerated storage.

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الأغشية الجيلاتينية النشطة المحتوية على زيت بذور اليقطين وزيت إكليل الجبل العطري لإطالة العمر الخزني لشرائح سمك الحمام المبردة

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المستخلص

قيّمت هذه الدراسة فعالية الأغشية الغذائية المصنوعة من الجيلاتين والمُدعّمة بزيت بذور اليقطين وزيت إكليل الجبل العطري في الحفاظ على جودة شرائح الحمام (*Sardina pilchardus*) المبردة وإطالة مدة صلاحيتها. وتم تحضير أغشية قابلة للتحلل الحيوي باستخدام جيلاتين مُلدّن بكحول البولي فينيل والجلسرين، بينما استُخدمت الأحماض الصفراوية المُستخلصة من مرارة الدجاج كعامل استحلاب طبيعي لتحسين دمج الزيوت واستقرارها داخل بنية الغشاء. قُسّمت شرائح السردين إلى مجموعتين: مجموعة غير مُغلّفة (مجموعة ضابطة) ومجموعة مُغلّفة، وخُزّنت عند درجة حرارة 4 ± 1 درجة مئوية لمدة 15 يومًا. تم تقييم فعالية الأغشية المطورة كمادة حافظة من خلال مراقبة المعايير الفيزيائية والكيميائية، بما في ذلك الرقم الهيدروجيني، والأحماض الدهنية الحرة، وقيمة البيروكسيد، وحامض الثايوبربيتوريك، بالإضافة إلى مؤشرات الجودة الميكروبيولوجية أثناء التخزين المبرد. أظهرت جميع العينات ارتفاعًا تدريجيًا في الرقم الهيدروجيني، ومؤشرات أكسدة الدهون، وعدد الميكروبات طوال فترة التخزين. ومع ذلك، أظهرت شرائح السمك المغلفة بالأغشية الصالحة للأكل المطورة مستويات أقل بكثير ($p < 0.05$) من التلف الكيميائي ونمو الاحياء المجهرية مقارنةً بالعينات غير المغلفة. وقد حدّت الأغشية بشكل فعال من انتقال الأوكسجين والرطوبة، مما أدى إلى إبطاء التفاعلات التأكسدية وكبح تكاثر الاحياء المجهرية، ومن بين الاغشية المختبرة، أظهرت الأغشية الغنية بزيت إكليل الجبل العطري أعلى فعالية في الحفظ، وهو ما يُعزى إلى النشاط القوي لمضادات الأكسدة والاحياء المجهرية لمكوناتها الفينولية. علاوة على ذلك، لم يتم الكشف عن بكتيريا القولون في أي من المعاملات أثناء التخزين، مما يشير إلى ظروف صحية مرضية في التعامل والتخزين. تشير النتائج إلى أن الأغشية الغذائية المصنوعة من الجيلاتين والمُدعمة بزيت إكليل الجبل العطري أو زيت بذور اليقطين تُشكّل أنظمة تغليف فعّالة ومستدامة وصديقة للبيئة، ذات إمكانات كبيرة لتحسين جودة وسلامة وفترة صلاحية منتجات الأسماك في الثلاجة.

الكلمات المفتاحية: زيت بذور اليقطين، زيت إكليل الجبل العطري، الأغشية الغذائية، الجودة الميكروبيولوجية، التخزين المبرد.