Aquatic Food Studies 2(1), AFS92
https://doi.org/10.4194/AFS92

RESEARCH PAPER

Antimicrobial Activity of Ovotransferrin Loaded Fish Gelatin Electrospun Nanofibers Against Some Pathogens Originated from Fish Products

Esen Alp-Erbay[1,*], Ahmet Faruk Yeşilsu[1], Mustafa Türe[2]

[1]Central Fisheries Research Institute, Department of Food Technology, Trabzon/ Türkiye 61250
[2]Central Fisheries Research Institute, Department of Fisheries Health, Trabzon/ Türkiye 61250

How to cite

Alp-Erbay, E., Yeşilsu, A.F., Türe, M. (2022). Antimicrobial Activity of Ovotransferrin Loaded Fish Gelatin Electrospun Nanofibers Against Some Pathogens Originated from Fish Products. *Aquatic Food Studies*, 2*(1), AFS92*. https://doi.org/10.4194/AFS92

Article History
Received 17 June 2022
Accepted 01 July 2022
First Online 07 July 2022

Corresponding Author
Tel.: +904623411053
E-mail: esenalp2002@gmail.com

Keywords
Fish gelatin
Ovotransferrin
Electrospinning
Antimicrobial activity
Tensile strength
Contact angle

Abstract

Fish and fishery products rich in nutrients are sensitive to microbial spoilage even at cold temperatures. Cold water fish gelatin including various amounts of ovotransferrin was electrospun in order to obtain antimicrobial nanofibers. The obtained electrospun nanofibers were subjected to disc diffusion method to determine antimicrobial activity against *Shewanella putrefaciens, Pseudomonas* spp*., Flavobacterium psychrophilum* and *E. coli* originated from fish. Morphological characterization as well as mechanical strength of electrospun nanofibers were determined. High amounts of ovotransferrin exhibited elevated antimicrobial activity. Mostly inhibited bacteria by 15% ovotransferrin was *E. coli* with an inhibition zone diameter of 19±0.2 mm. Mats containing 15% ovotransferrin showed an inhibition zone diameter of 18±0.5 mm, 19±0.5 mm and 18±1.0 mm against *Pseudomonas florescens, Flavobacterium psychrophilum* and *Shewanella putrefaciens* respectively. Ovotransferrin content of 5% showed an inhibition zone diameter of 14±0.3 mm, 12±1.0 mm and 11±0.5 mm against *Pseudomonas florescens*, *Flavobacterium psychrophilum* and *Shewanella putrefaciens* respectively. Fish gelatin had a hydrophilic character according to the contact angle measurements which got worse by incorporating ovotransferrin. Ovotransferrin including nanofibers exhibited poor mechanical characterization which is attributed to weakened fish gelatin molecular bonding in presence of higher amounts of ovotransferrin. Neat gelatin had reasonable tensile strength comparing to other specimens. As a result, ovotransferrin incorporated fish gelatin electrospun nanofibers are promising in inhibiting fish originated microorganisms.

Introduction

Fish and fishery products are rich in valuable nutrients *i.e.,* long-chain omega–3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), essential amino acids, vitamins such as A, D and B2 (riboflavin), minerals including calcium, magnesium, phosphorus, iron, selenium, zinc, fluorine, and iodine (in marine fishes) (Ariño et al., 2012). Fish is a very perishable food product as a result of microbial activity and chemical oxidation or autolysis of lipids (Gram & Huss, 1996; Parlapani et al., 2014). However, the most important factor affecting the quality of fresh fish is the microbial spoilage mechanism (Parlapani et al., 2014). Many microorganism species and strains produce metabolites that cause fish spoilage under certain conditions. First mechanism of deteriorating fish is the microbial growth leading spoilage (Boziaris, 2020). Fish muscle are sterile at the beginning however, after caught the muscle

becomes flesh and flesh is contaminated with microorganisms on the fish skin (Comi, 2017). High amount of nonprotein nitrogenous compounds in fish flesh with high water activity and low acidity (pH > 6) induce microorganisms to grow rapidly (Sperber & Doyle, 2009). For instance, *Shewanella putrefaciens* reproduce in aerobic conditions subsequently fish caught from the northern seas even at cold temperatures, reducing trimethylaminoxide (TMAO) to TMA and causing the fish to deteriorate. *Shewanella putrefaciens* generates TMA, H_2S, CH_3SH, $(CH_3)2S$, Hx, and acids as spoilage compounds (Tavares et al., 2021). *F. psychrophilum* is associated with rainbrow trout fry syndrome and bacterial cold water disease which causes important mortalities in the hatcheries (Saticioğlu et al., 2019). The absence of an effective vaccination against *F. psychrophilum* leads antimicrobial treatments (Saticioğlu et al., 2019). *Pseudomonas* spp. also reproduce at low temperatures leading deterioration of Mediterranean fish species (Parlapani et al., 2013) generating CH_3SH, $(CH_3)2S$, ketones, esters, aldehydes, NH_3, and Hx as spoilage compounds (Tavares et al., 2021). Dalgaard (2003) and Gram & Huss (1996) reported that the metabolic activities of degrading microorganisms are mostly affected by temperature and the packaging type.

Packaging plays an important role in the preservation of food through production, processing, distribution and storage and in reaching the final consumer. When food packaging performs a function other than providing an inert barrier to external circumstances, it is referred to as active packaging. An oxygen scavenger or an antibacterial agent may be included in active packaging systems (Rooney, 1995). Electrospinning is a promising approach for creating active packaging materials and designing nanostructured food packaging layers. The submicron diameter, huge area/volume ratio, great sensitivity to changes in the surrounding atmosphere, and aptitude for encapsulating heat-sensitive active chemicals have all been cited as advantages of electrospun fibers as active packaging materials (Vega-Lugo & Lim, 2009). Antimicrobial chemicals that penetrate into the product and limit the growth of bacteria during storage are one sort of active packaging system. However, packaging materials that come into contact with food are acceptable to consumers when they are natural, efficient and non-toxic, but do not cause changes in the flavor of the food (Neo et al., 2013). Biopolymers obtained from food wastes are more preferred.

Gelatin which is generated from collagen after partial hydrolysis, obtained from bones, skin, hides, ligaments and cartilages (Mahmood Lubowa Muhammad et al., 2016) is one of the biopolymers substitutes petroleum based polymers. Gelatin derived from collagen extraction has been diversified to meet demands of ever-growing food and packaging industries (Gómez-Guillén et al., 2016). Gelatin is the mostly demanded biopolymer in food industry based on its physico-functional properties including rheological and thermal properties (Mahmood et al., 2016). Main gelatin source utilized in food industry is the mammalian gelatin however, fish gelatin has a potential to be an alternative. Fish gelatin has some disadvantages compared to mammalian gelatin in terms of less content of proline and hydroxyproline residues (Mahmood et al., 2016). Nevertheless, it has good film forming ability and can be improved for further process in food industry.

Recently, the use of bioactive plant extracts or natural compounds as antimicrobial agents in food packaging has been widely studied by researchers because natural antimicrobial substances have GRAS status and do not cause health and ecological concerns. Ovotransferrin, commonly known as conalbumin, is a monomeric transferrin glycoprotein that accounts for 13 % (170 µM in egg white) of total egg white protein (Legros et al., 2021). Transferrins are known to bind iron as an antimicrobial mechanism (Legros et al., 2021). Because iron is involved in many cellular activities such as respiration, DNA synthesis, redox-stress tolerance, and the tricarboxylic acid cycle (TCA), it is needed for all forms of life, including bacteria (Andrews et al., 2003). For optimal bacterial growth, concentrations ranging from 0.1 to 10 M are usually required (Andrews et al., 2003). Ovotransferrin has been linked to a number of antimicrobial mechanisms other than or in addition to iron restriction (Legros et al., 2021).

Hence, the objective of the present work was to develop fish gelatin electrospun nanofibers including ovotransferrin in subject to active food packaging materials. Various amounts of ovotransferrin incorporated electrospun nanofibers of cold water fish gelatin were produced and subsequently morphological characterization and mechanical strength were determined as well as antimicrobial efficiency.

Material and Methods

Material

Cold water fish gelatin, ovotransferrin, and food grade acetic acid were purchased from Sigma- Aldrich and Mueller Hinton Agar (MH, Himedia) was obtained from Oxoid. *Pseudomonas fluorescens* strains used in this study were previously isolated and identified from whiting (*Merlangius merlangus* euxinus) in the Eastern Black Sea coast of Turkey. *Flavobacterium psychrophilum*, *Shewanella putrefaciens* and *E. coli* strains were kindly provided from KTU, Faculty of Marine Science, Turkey. All strains were stored at -80°C in 15% to 20% glycerol containing tryptic soy broth (TSB, Merck) until the study.

Method

Solution preparation

Fish gelatin (2g) was dissolved 10 mL food grade acetic acid. The protein solution was stirred by a

magnetic stirrer at 50°C for 24 h. Then, ovotransferrin with various amounts regarding polymer content (0, 5, 10, 15% wt) was dispersed in the solution and kept stirring for 3 hours at room temperature (Alp-Erbay et al., 2019).

Electrospinning

The protein solutions either including ovotransferrin or not were transmitted to a plastic syringe connected through a polytetrafluoroethylene (PTFE) tube to a stainless steel needle and then processed by electrospinning using NE100 Single Nozzle Electrospinning device (İstanbul, Turkey). The solutions were electrospun for 3 h, under a constant flow-rate of 2,5 mL/h, setting a voltage of 18 kV and a tip-to-collector distance of 13 cm. All experiments were performed at room conditions, *i.e.,* 23 °C and 40% relative humidity (RH), in a controlled environmental chamber (Alp-Erbay et al., 2019).

Characterization

Nanofiber thickness

Nanofiber thickness was measured with a digital micrometer series S00014, having ±0.001 mm accuracy, from Mitutoyo Corporation (Kawasaki, Japan) at three random positions (Alp-Erbay et al., 2019a).

Morphological Characterization

A S-4800 microscope from Hitachi (Tokyo, Japan) was used to observe the morphology of the electrospun mats surfaces by scanning electron microscopy (SEM). All specimens were fixed to beveled holders using conductive double-sided adhesive tape, sputtered with a mixture of goldpalladium under vacuum, and observed using an accelerating voltage of 20 kV (Alp-Erbay et al., 2019a).

Mechanical Properties

The mechanical properties of the electrospun mats were determined by using a texture analyser (TA.XT plus, Stable Micro Systems, UK) with a 5 kg load cell equipped with tensile grips holder. The specimens were cut into a rectangular shape (0.5 – 2.5 cm). The thickness of the samples was ranged from 25-30 µm (Alp-Erbay et al., 2019a).

Contact angle

The contact angle was measured using a sessile drop method (5µL.s-1) by Dataphysics, OCA – Optical contact angle measurement and contour analysis systems, OCA-15 EC Filderstadt, Germany. A syringe was used to drop a 5 µL water drop onto the membrane surface. At ambient temperature, contact angles at five distinct places on each sample were measured and analysed using SCA 20 and SCA 22 software. The average of the five points was determined (Alp-Erbay, 2021).

Antimicrobial activity of ovotransferrin loaded fish gelatin electrospun nanofibers

Antimicrobial activity of electrospun mats were determined by using an optimized method of Kirby-Bauer Disc diffusion method by (Hudzicki, 2012). Each bacterial strain was incubated in 5mL nutrient broth (NB) at 100 rpm, 22-24°C during a night. Nanofiber mats were Sterilized fifilms, under ultraviolet light for 10 min, were cut into 1 × 1 cm^2 discs and placed onto Muller Hinton Agar plates, previously seeded with 0.1 mL of inoculum by swabbing with approximately 10^5 CFU/mL of the tested bacteria. Inhibitory concentrations for each strain generated a clear zone around disc specimens. All experiments were conducted 2 times for each bacterial strain (Alp-Erbay et al., 2019).

Statistical Analysis

The one-way ANOVA, post-hoc Tuckey´s multiple comparisons as post-test ($p < 0.05$), and the mean SD were conducted. Data generated were analysed using SPSS software, version 22 for Windows.

Results and Discussion

Morphology of Electrospun Nanofibers

Scanning electron microscope images of fish gelatin electrospun nanofibers with and without ovotransferrin at various contents were shown in Figure 1a. Neat gelatin electrospun nanofibers exhibited rough morphology with mean fiber diameter of 756±111 nm. Fiber diameter is affected by concentration, viscosity, surface tension and conductivity (Alp-Erbay et al., 2019). In the case of increasing viscosity, fiber diameter tends to increase however, decrease in concentration and viscosity generally leads reduced fiber diameter with beads (Khan et al., 2015). Surface tension, one of the solution parameters affecting fiber morphology and diameter changes by both concentration and applying voltage and the distance between nozzle and collector (Fatimah et al., 2020). In Figure 1b, images of fish gelatin/Ov. 5% showed closer morphology to neat fish gelatin electrospun nanofibers with mean fiber diameter of 711±132 nm. Ovotransferrin content affected fiber morphology substantially. In Figure 1c, images of fish gelatin/Ov. 10% were displayed and bead formation can be seen obviously. The fiber morphology was also as rough as neat gelatine electrospun nanofibers. Mean fiber diameter of fish gelatin/Ov. 10% was 695±122 nm. Fish gelatin/Ov. 10% SEM image was shown in Figure 1d. Mean fiber diameter of fish gelatin/Ov. 15% was 632±158 nm. Fiber morphology was rough and beady.

Thickness and Mechanical Properties

Mechanical strength of electrospun nanofiber mats mostly depends on nanofiber assembly and density of fiber contact points (Tarus et al., 2020). According to the results, mean thickness of fish gelatin nanofibers was 131±10 µm, 129±13 µm, 127±11 µm, and 126±10 µm for fish gelatin/Ov. 5%, fish gelatin/Ov. 10% and fish gelatin/Ov. 15% respectively (Table 1). Tensile strength was measured as 0.841±0.26 Mpa for neat fish gelatin fibers. Ovotransferrin content altered the tensile strength markedly. Increase in the ovotransferrin content lead a decrease in tensile strength, *i.e.,* fish gelatin/Ov. 5% was 0.744±0.33 Mpa and fish gelatin/Ov. 10% was 0.691±0.27 Mpa. Fish gelatin/Ov. 15% had 0.658±0.12 Mpa tensile strength displaying insignificant difference with fish gelatin/Ov. 10%. Breaking strain of the specimens were determined as 44.12±1.33 % for neat fish gelatin, 52.02±2.78 % for fish gelatin/Ov. 5%, 51.03±1.36 % for fish gelatin/Ov. 10% and 55.54±2.0 % for fish gelatin/Ov. 15%. Toughness of the electrospun mats were measured as 0.088±0.1 MJ m^{-3} for neat fish gelatin, 0.256±0.01 MJ m^{-3} for fish gelatin/Ov. 5%, 0.211±0.1 MJ m^{-3} for fish

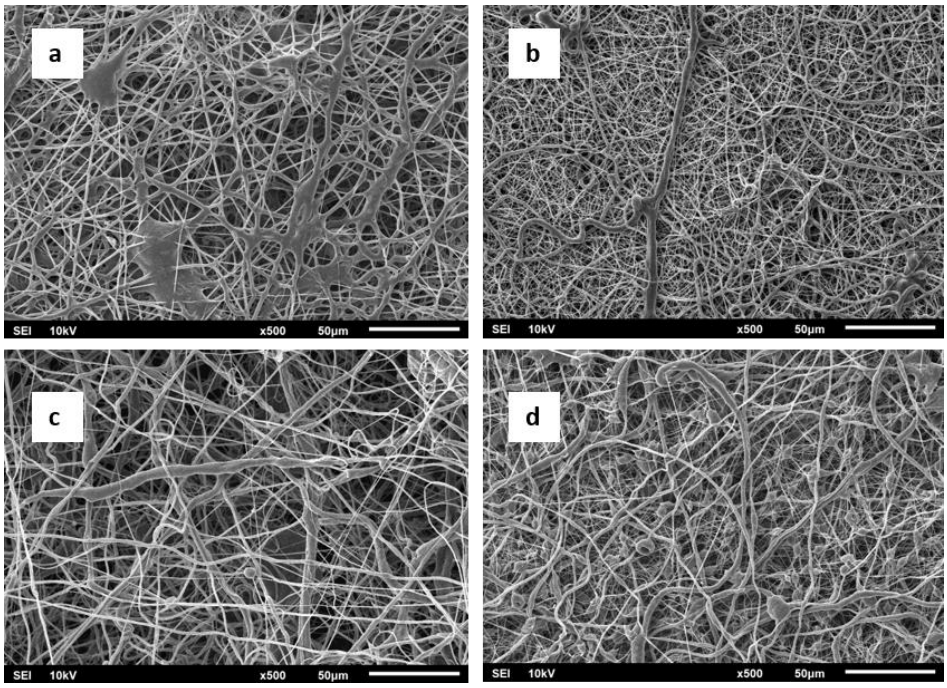

Figure 1. Scanning electron microscope images of **a.** Fish gelatin electrospun nanofibers, **b.** Fish gelatin/Ov. 5% electrospun nanofibers, **c.** Fish gelatin/Ov. 10% electrospun nanofibers **d.** Fish gelatin/Ov. 15% electrospun nanofibers.

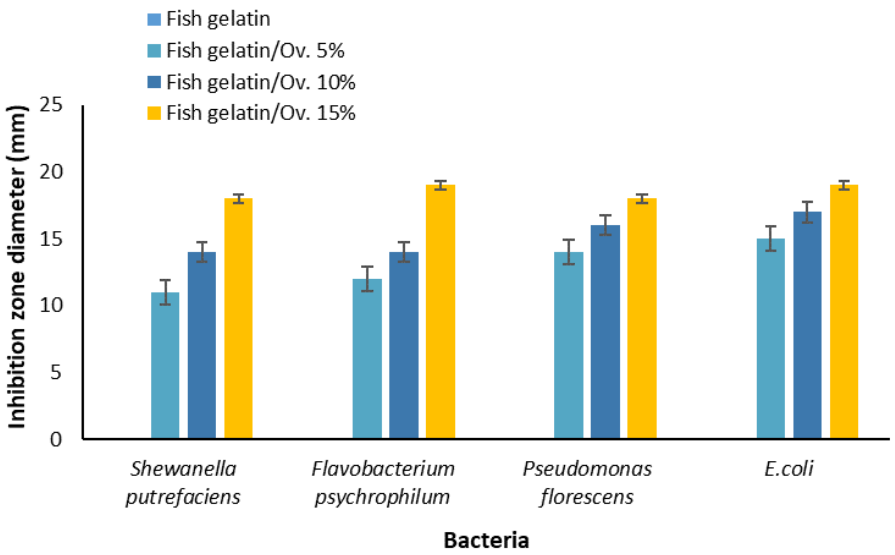

Figure 2. Inhibition zone diameter (mm) of fish gelatin electrospun nanofibers, fish gelatin/Ov. 5% electrospun nanofibers, fish gelatin/Ov. 10% electrospun nanofibers and fish gelatin/Ov. 15% electrospun nanofibers against *Pseudomonas fluorescens*, *Flavobacterium psychrophilum*, *Shewanella putrefaciens* and *E. coli*.

gelatin/Ov. 10% and 0.201±0.1 MJ m^{-3} for fish gelatin/Ov. 15%. Mechanical strength of neat gelatin and ovotransferrin incorporated gelatin electrospun nanofiber mats exhibited poor characterization similar with An et al., 2010. Poor tensile strength was related to weakened fish gelatin molecular bonding in presence of higher amounts of ovotransferrin. Increase in breaking strength in higher contents of ovotransferrin could be attributed to the reduced fiber diameter (Wendorff et al., 2010).

Antimicrobial activity of ovotransferrin loaded fish gelatin electrospun nanofibers

Ovotransferrin incorporated fish gelatin electrospun nanofiber mats exhibited elevated antimicrobial activity in parallel to the high content (Figure 3). As expected neat fish gelatin nanofiber mats did not show antimicrobial activity. Fish gelatin/Ov. 5% nanofiber mats exhibited antimicrobial activity mostly against *E. coli* with an inhibition zone diameter of 15±1.0 mm. Ovotransferrin content of 5% showed an inhibition zone diameter of 14±0.3 mm, 12±1.0 mm and 11±0.5 mm against *Pseudomonas florescens, Flavobacterium psychrophilum* and *Shewanella putrefaciens* respectively. Higher amounts of ovotransferrin provided elevated antimicrobial activity against the selected pathogens originated from fish and fishery products. Ovotransferrin content of 10% exhibited inhibition zone diameter of 16±0.3 mm, 14±1.0 mm and 14±0.5 mm against *Pseudomonas florescens, Flavobacterium psychrophilum* and *Shewanella putrefaciens* respectively. Mostly inhibited pathogen was *E. coli* with an inhibition zone diameter of 17±0.3 mm. Fish gelatin/Ov. 15% nanofiber mats showed the highest antimicrobial activity among the experimental groups. Mats containing 15% ovotransferrin showed an inhibition zone diameter of 18±0.5 mm, 19±0.5 mm and 18±1.0 mm against *Pseudomonas florescens, Flavobacterium psychrophilum* and *Shewanella putrefaciens* respectively. As expected mostly inhibited pathogen by 15% ovotransferrin was *E. coli* with an inhibition zone diameter of 19±0.2 mm. It is reported that, antimicrobial mechanism of ovotransferrin is attributed to its iron-restriction/ binding activity (Legros et al., 2021). However, authors declared that antimicrobial mechanism of ovotransferrin is not limited with iron-binding which provides a large spectrum of

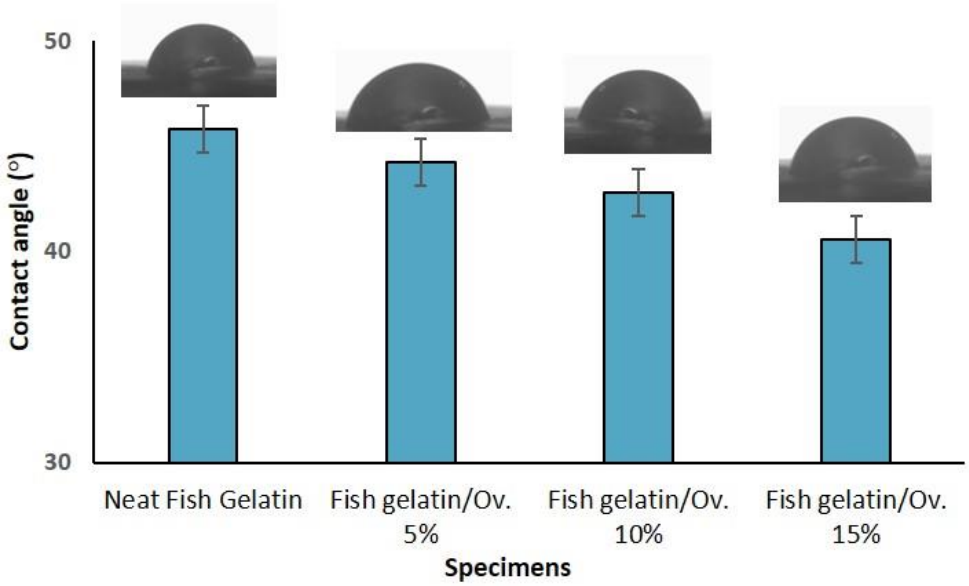

Figure 3. Contact angle measurements of fish gelatin electrospun nanofibers, fish gelatin/Ov. 5% electrospun nanofibers, fish gelatin/Ov. 10% electrospun nanofibers and fish gelatin/Ov. 15% electrospun nanofibers.

Table 1. Mechanical characterization of electrospun nanofibers.

	Thickness (µm)	Tensile Strength (Mpa)	Breaking Strain (%)	Toughness (MJ m^{-3})
Fish gelatin	131±10[a]	0.841±0.26[a]	44.12±1.33[a]	0.088±0.1[a]
Fish gelatin/Ov. 5%	129±13[a]	0.744±0.33[b]	52.02±2.78[b]	0.256±0.01[b]
Fish gelatin/Ov. 10%	129±11[a]	0.691±0.27[c]	51.03±1.36[b]	0.211±0.1[b]
Fish gelatin/Ov. 15%	128±10[a]	0.658±0.12[c]	55.54±2.0[b]	0.201±0.1[b]

Different letters (a-c) in the same column indicate the difference between the groups (P<0.05).

inhibition (Legros et al., 2021). Ovotransferrin can also form thermally stable complexes with Fe^{3+}, Al^{3+}, Cu^{2+}, or Zn^{2+} (Jalili-Firoozinezhad et al., 2020).

Contact Angle

Fish gelatin exhibited contact angle of 45.8° similar with (Tang et al., 2019). Gelatin is known to be a hydrophilic material which has some limitations in food packaging applications. Generally, hydrophobic materials are blended with gelatin to improve hydrophilic properties but in this case, ovotransferrin incorporation induced a worse hydrophilic characteristic. (Renkler et al., 2021) incorporated egg white protein to polycaprolactone electrsopun nanofibers and reported that egg white protein converted hydrophobic character of polycaprolactone nanofibers (90°) to hydrophilic (70°). Ovotransferrin lowered the neat gelatin's contact angle from 45.8° to 44.26°, 42.8° and 40.56° for ovotransferrin content of 5%, 10% and 15% respectively (Figure 2).

Conclusion

Gelatin is a biopolymer derived from collagen of various sources. Fish gelatine is an alternative to mammalian gelatin for some reasons; *i.e.,* higher viscosity and lower immunogenicity. However, some limitations are still challenging in industrial applications which are required to be improved by further studies. The water solubility and poor mechanical characteristics have restricted gelatin's implementations especially in food packaging. Antimicrobial food packaging is one of the most demanded type of packaging generally in fish and fishery products which are perishable due to the rapid bacterial growth. Natural compounds as antimicrobials such as plant extracts and/or bioactive compounds are more preferred by consumers due to the health issues. For this reason, ovotransferrin known as conalbumin was loaded in to fish gelatin electrospun nanofibers in order to obtain antimicrobial nanofibers. Ovotransferrin, one of the transferrin family member is a natural antimicrobial compound found in egg white protein at a content level of approximately 13%. The antimicrobial mechanism of ovotransferrin is attributed to either iron binding capability or other sort of mechanisms alters due to the bacterial strain. Those obtained nanofibers were subjected to the antimicrobial tests against *Shewanella putrefaciens, Pseudomonas* spp., *Flavobacterium psychrophilum* and *E. coli* originated from fish and fishery products. As a result, ovotransferrin loaded nanofibers at 15% level exhibited strong antimicrobial activity against *E. coli, Flavobacterium psychrophilum, Shewanella putrefacien s*, and *Pseudomonas florescens* respectively. In all levels of ovotransferrin, mostly inhibited microorganism was *E. coli*. Contact angle measurements showed that gelatin has a highly hydrophilic character which ought to be improved by blending hydrophobic materials such as biopolymers at various levels. Ovotransferrin at whole levels also lowered the contact angle. Tensile strength of the nanofibrous membranes were lower in ovotransferrin loaded speciemens comparing to neat gelatine nanofibers. However, gelatin nanofibers with ovotransferrin at 5% level had reasonable tensile strength and antimicrobial activity against *Pseudomonas florescens* and *E. coli*. Hence, ovotransferrin encapsulated cold water fish gelatin electrospun nanofibers are promising in inhibition of fisheries originated microorganisms in subject to develop active food packaging materials.

Ethical Statement

There is no need for ethical declaration in this study.

Funding Information

This study was funded by Republic of Türkiye Ministry of Agriculture and Forestry General Directorate of Agricultural Research and Policies (Grand number TAGEM/HSGYAD/17/A03/P05/133).

Author Contribution

EAE: Formal Analysis, Data Curation, Investigation, Methodology, Visualization and Writing -original draft;

AFY: Formal Analysis, Conceptualization, Writing - review and editing;

MT: Formal Analysis, Conceptualization, Writing - review and editing;

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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