Received: 22 January 2021 | Revised: 20 February 2021 | Accepted: 12 March 2021

DOI: 10.1111/are.15239

Aquaculture Research WILEY

ORIGINAL ARTICLE

Impact of gelatin nanogel coating containing thymol and nisin on the microbial quality of rainbow trout fillets and the inoculated *Listeria monocytogenes*

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Abstract

This study aimed to assess the effects of edible gelatin nanogel coating containing thymol and nisin on the inhibition of microbial flora and inoculated *Listeria monocytogenes* in fish fillets during a 12-day storage period at the temperature of 4°C. Data were collected and analysed on several factors of microbiological change, including total viable count, total psychrophilic count, lactic acid bacteria and hydrogen sulphide-producing bacteria count, and the inhibitory effects of the coating on inoculated *Listeria monocytogenes* were determined during 3-day intervals. The highest reduction rate of total viable bacteria (0.95 log CFU/g), total psychrophilic (0.92 log CFU/g), Hydrogen sulfide producing bacteria (0.66 log CFU/g), lactic acid bacteria (0.95 log CFU/g) and *L. monocytogenes* count (0.65 log CFU/g) were observed in gelatin nanogel containing thymol and nisin in comparison with control samples. Furthermore, the separate use of these compounds led to the significant growth inhibition of the bacteria compared with the control. Therefore, it was concluded that the gelatin nanogel coating without an antimicrobial agent could not be effective in bacterial growth control, and the added thymol and nisin would be beneficial in the preservation of foods such as fish and its products.

KEYWORDS
gelatin, *Listeria monocytogenes*, nanogel, nisin, thymol

1 | INTRODUCTION

Rainbow trout is a beneficial food product on the global market considering the fast growth and production rate, favourable quality, remarkable nutritional value and significant feed efficiency ratio (Feng et al., 2017). However, this natural seafood must be preserved with care in the storage period as it could perish rapidly, thereby leading to chemical, enzymatic and microbiological changes induced by the high content of polyunsaturated fatty acids (Jasour et al., 2015). When fish and fish products become infected, foodborne pathogens promptly increase in the nutrient contents. *Listeria monocytogenes* frequently causes bacterial infection in fish and plays a key role in the sporadic listeriosis of seafood (Zhaveh et al., 2015). *L. monocytogenes* is a Gram-positive and psychrotrophic pathogen, accounting for 30% mortality in patient populations (Rajaei et al., 2017). Recent findings have indicated that perishable foods could be effectively preserved by edible coatings at varied concentrations (Hosseini et al., 2016). Some of the important advantages of edible coatings are protection against oxygen and moisture; mechanical modification; and prevention of chemical, biological and physical decay (Nowzari et al., 2013). Nanogels are nano-scale delivery systems with remarkable release properties, stability, biocompatibility, loading capacity and water dispersibility. Nanogels are produced by several compounds, including

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lipids, proteins and polysaccharides (Beyki et al., 2014). Collagen hydrolysis yields numerous beneficial products, one of the most important of which is gelatin (Sarika et al., 2015). Some of the key advantages of gelatin are non-toxicity, biocompatibility, affluence, biodegradability and low production costs, which have encouraged its application in food processing (Sarika et al., 2016). According to the literature, the quality changes in edible coatings, which mainly occur during storage, could be minimized by the addition of phenolic compounds and antimicrobial agents (e.g. bacteriocins) (Rohani et al., 2011). One of these effective phenolic compounds is thymol (2-Isopropyl-5-methylphenol), which is extracted from thyme and the essential oil of oregano as a key constituent of the herbal essential oils of the *Lamiaceae* family. Thymol has notable antimicrobial effects on multiple foodborne microorganisms, including fungal and bacterial food spoilage agents and pathogens (Ramos et al., 2014). Nisin is a natural bacteriocin and a polypeptide with cationic and hydrophobic properties, which is extracted from *Lactococcus lactis* strains. This compound has been reported to disrupt the membrane of sensitive cells through pore formation, thereby causing the efflux of cellular components (Govaris et al., 2010). Various studies have investigated the use of gelatin coating in meat products (Sun et al., 2019)(Jridi et al., 2020) (Cardoso et al., 2019) (Andevari & Rezaei, 2011) (Antoniewski et al., 2007), while limited research has been focused on the applications of nanogel for increasing the shelf life of these products (Rajaei et al., 2017) (Hadian et al., 2017), and no studies are available in the current literature to clarify the protective effects of natural antimicrobial-based nanogel coatings against foodborne pathogens in seafoods. The present study aimed to produce an edible nanogel coating with gelatin, nisin and thymol via ultrasound, assess its antibacterial effects on inoculated *L. monocytogenes*, maintain the microbial quality in fish fillets and evaluate the function of the active coating solution of the prepared gelatin nanogel.

2 | MATERIALS AND METHODS

2.1 | Experimental materials

In this study, the used gelatin had been obtained from cold-water fish skin, which was purchased by the researchers from Sigma-Aldrich Chemical. In addition, analytical-grade thymol and nisin were provided by Sigma-Aldrich Chemical. The current experiment required four lyophilized *L. monocytogenes* strains, including ATCC: 7644, 82119, CIP: 7834 and NCTC: 10671, which were provided by the Iranian Biological Resource Center (Tehran, Iran). Notably, the culture media were obtained from Merck (Darmstadt, Germany).

2.2 | Preparation of coating solutions

At this stage, nanogel preparation was initiated by using nisin and thymol as the main antibacterial components, while the formula of the nanogel also contained glycerol (0.75 ml/g) and gelatin (5% w/v), which were immersed in bi-distilled water. Afterwards, the gelatin solution was interspersed with nisin and thymol (10% in ethanol), so that the solution would reach 250 and 500 ppm. Following that, a laboratory T25 digital ULTRA-TURRAX mixer (IKA, Staufen, Germany) was employed for the blending of the mixture at 1120 *g* for two minutes. An ultrasound device (Sonopuls, Bandelin, Berlin, Germany) was also used for the ultrasound exposure of the emulsion with the pulse duration of 45 seconds (on-time) and 15 seconds (off-time), continuing for six minutes at the amplitude of 50%, which finally resulted in the formation of the nanogel. Finally, a water bath containing ice was used to refrigerate the product at the interaction chamber outlet in order to maintain the product's temperature at <20°C (Salvia-Trujillo et al., 2013).

2.3 | Particle size measurement

To determine the droplet size distribution, the polydispersity index (PDI) and mean droplet size (Z diameter) were initially measured. To do so, the backscattered light was measured via dynamic light scattering using the Zetasizer Nano-ZS laser diffractometer at 633 nanometres and the temperature of 25°C; the device also consisted of a backscatter detector (Nano S, Marven, England). At this stage, the sample ratio of 1:100 was considered to determine and resolve the multiple scattering effects on the polystyrene cuvettes by double-distilled water.

2.4 | Fish sample preparation

In the present study, we purchased fresh rainbow trout fillets (*Oncorhynchus mykiss*) from a market in Mashhad city (Iran). The samples weighed 550–600 grams and were instantaneously sent to the food hygiene and aquaculture laboratory of Ferdowsi University of Mashhad in insulated polystyrene ice flasks. After washing off the slime and blood of the fish at the laboratory, the fillets were dried and sectioned into samples with the diameters of 2 × 4 × 1 centimetres and weighed to be 10 grams.

2.5 | Treatment of the fillets samples

The fillet samples were randomly divided into six groups, which are shown in Table 1. The control samples were preserved in polyethylene bags, and the treatment samples were placed in various coating solutions for two minutes. For draining, the samples were maintained within an airflow at room temperature on a biological containment hood for 30 minutes. After the samples were placed inside zipped polyethylene bags, they were preserved at the temperature of 4°C for the further quality assessment on days zero, three, six, nine and 12.

TABLE 1 List of treatments in the present study

	Treatments	Description
1	CON	Samples without any coating
2	GEL	Samples coated with gelatin 5% (w/v)
3	NG	Samples coated with nano gelatin 5% (w/v)
4	NG-T	Samples coated with gelatin nanogel containing thymol (500 ppm) (w/v)
5	NG-N	Samples coated with gelatin nanogel containing nisin (250 ppm) (w/v)
6	NG-T-N	Samples coated with gelatin nanogel containing thymol (500 ppm) and nisin (250 ppm) (w/v)

2.6 | Microbiological analysis

To determine the microbial count, 10 grams of the fillet samples were prepared at the volume of 90 millilitres using 0.1% sterile peptone water, followed by homogenization by a stomacher (Seward Medical, London, UK) for one minute. The drop plate method was applied for the bacterial counting (Sharifi et al., 2017), and the total viable counting was also carried out after the incubation of the fillet samples at the temperature of 37°C for 48 hours on a nutrient agar (AOAC, 2002). Furthermore, the plate count agar was used to determine the psychrotrophic bacterial count, with the incubation of the plates performed at the temperature of 7°C for 10 days (Raeisi et al., 2016). On the same note, *Shewanella putrefaciens* and *Pseudomonas fluorescens* were considered as the hydrogen sulphide-producing bacteria, and their count was determined at the temperature of 30°C for 72 hours on iron agar (Raeisi et al., 2016). The enumeration of lactic acid bacteria was achieved on the De Man, Rogosa and Sharpe agar plates via incubation in anaerobic conditions at the temperature of 25°C (Sani et al., 2017). Notably, the counting of the microorganisms was obtained in terms of log 10 CFU/g.

2.7 | Bacterial inoculation of the samples

At this stage, the samples were sectioned into 10-gram parts, followed by ethanol spraying (70% v/v) and the removal of the surface micro-organisms by burning (Sani et al., 2017). Afterwards, four *L. monocytogenes* strains were combined and cultured on nine millilitres of the Brain Heart Infusion Broth, followed by incubation at the temperature of 37°C for 24 hours. The 24-hour culture was used to obtain a bacterial suspension and prepare the turbidity standard (0.5 McFarland) via spectrophotometry, which contained approximately 1.5 × 108 CFU/ml, and its dilution (1:100) was performed to the density of 1.5 × 107 CFU/ml. At the next stage, 100 microliters of the bacterial suspension was inoculated into all the samples using micropipettes (1.5 × 107 CFU/ml), and the final concentration was estimated to be 106 CFU/g. To determine the count of the inoculated bacteria, 10 grams of the samples was prepared at the final volume of 90 millilitres using 0.1% sterile peptone water, followed by homogenization for two minutes using a stomacher (Seward Medical, London, UK). Finally, decimal dilutions were prepared, and the drop method was deployed to culture 10 microliters of the homogenate serial dilutions on the palcam agar, as well as incubation for 48 hours at the temperature of at 37°C (Abdollahzadeh et al., 2014).

2.8 | Statistical analysis

Data analysis was performed in SPSS version 21 using repeated-measures analysis of variance (ANOVA), Dunnett T3 test and Bonferroni post hoc test, and the p-value of less than 0.05 was considered significant in all the statistical analyses. Notably, the experiments in the present study were performed in triplicate.

3 | RESULTS AND DISCUSSION

3.1 | Gelatin nanogel coating characterization

Ultrasonic emulsification was performed for six minutes, and the measured mean diameter of the nanogelatin coating droplet was 140 nanometres. This is consistent with the study by Sarika et al., (2015), which indicated the size of 120–147 nanometres for alginic aldehyde and gelatin nanogel (Sarika et al., 2015). In another research, Koul et al., (2011) calculated the mean size of nanogel to be 249 nanometres (Koul et al., 2011). Nanogels are made of different gelatin types with smaller sizes than 200 nanometres. In the present study, the nanogelatin coating had PDI of 0.36, which is in line with the results obtained by Abreu et al., (2012) (Abreu, Oliveira, Paula, and de Paula, 2012). Previous studies have denoted that if PDI tends to zero, size distributions are homogeneous, while the PDI values tending to one are indicative of heterogeneous size distributions (Acevedo-Fani et al., 2015).

3.2 | Total viable count

Figure 1a depicts the growth of the total viable count (TVC) in the treatment groups after the 12-day storage period. According to the

findings, the mean TVC was estimated at 4.36 ± 0.09 log CFU/g, which was within the range of the TVC values denoted by Hosseini et al., (2016; 3.2 log CFU/g) (Hosseini et al., 2016). In the current research, the bacterial count increased during storage in all the groups, while the CON and GEL samples had significantly higher rates of respective growth compared with the other study groups ($p < 0.05$); these values were observed to increase to 8.36 and 8.23 log CFU/g after storage respectively. Our findings in this regard are consistent with the studies conducted by Andevari and Rezaei (2011) and Moreno et al. (2018), which demonstrated the significant inhibition of TVC by gelatin coating in chicken meat and rainbow trout fillets respectively (Andevari & Rezaei, 2011). As is depicted in Figure 1a, inhibitory effects were exerted by the NG-T, NG-N and NG-T-N treatments within the first six days of the storage period, which could be attributed to the antibacterial properties of nisin and thymol. After the storage period, the TVC value was observed to increase to more than 7 log CFU/g, which is the maximum permissible limit for raw fish. This is in line with the study by Mastromatteo et al. (2010), which investigated the impact of thymol and modified atmosphere packaging on the control of mesophilic bacterial count in ready-to-use peeled shrimps (Mastromatteo et al., 2010). In another investigation, Gao et al. (2014) applied combined rosemary extract with nisin, observing the reduced TVC of stored fish fillet ($p < 0.05$) (Gao et al., 2014). According to the information in Table 2, TVC decreased most significantly (~ 1 log CFU/g) in NG-T-N treatment samples compared with the CON samples; this finding is in congruence with the study conducted by Ettayebi et al., (2000) regarding the synergy of nisin and thymol and their role in the antibacterial effects against *Bacillus subtilis* and *L. monocytogenes* (Ettayebi et al., 2000). Furthermore, Rajaei et al., (2017) experimented chitosan–myristic acid nanogel encapsulated with clove essential oil, which was observed to significantly increase the shelf life of beef cutlet (Rajaei et al., 2017).

3.3 | Total psychrophilic count

Aerobically stored fish products could be rapidly spoiled by psychrotrophic bacteria during cold storage. Figure 1b illustrates the total psychrophilic count (TPC) growth in the fish fillets during the 12-day storage period. TPC was initially estimated at ca. 4.11 log CFU/g and increased significantly throughout the storage period in all the fish fillets; this is in line with the research by Khanzadi et al. (Khanzadi et al., 2020). In the present study, the highest TPC values belonged to the CON samples (8.52 CFU/g) after the storage period. According to the information in Table 2, the GEL and CON samples had no significant difference in terms of the gelatin coating as it exerted no antibacterial effects independently. This is consistent with the findings of Andevari and Rezaei (2011), which demonstrated that the quality of fresh rainbow trout was not affected by gelatin coating alone during refrigerated storage, and the coating exerted no significant antibacterial effects as well (Andevari & Rezaei, 2011). According to the findings of the current research, the impact of the NG-N samples on the growth of the psychrotrophic bacteria was more significant than the CON samples. Pore formation plays a pivotal role in the antibacterial properties of nisin, which occurs in the targeted cytoplasmic bacterial membrane (especially Gram-positive psychrotrophic bacteria) when the proton motive force is disseminated, causing the efflux of solutes with a low molecular weight, such as potassium ions and amino acids (Behnam et al., 2015). In the present study, NG-T-N was considered to be the most effective treatment as it results in the most significant TPC reduction rate (~1 log CFU/g); this is in line with

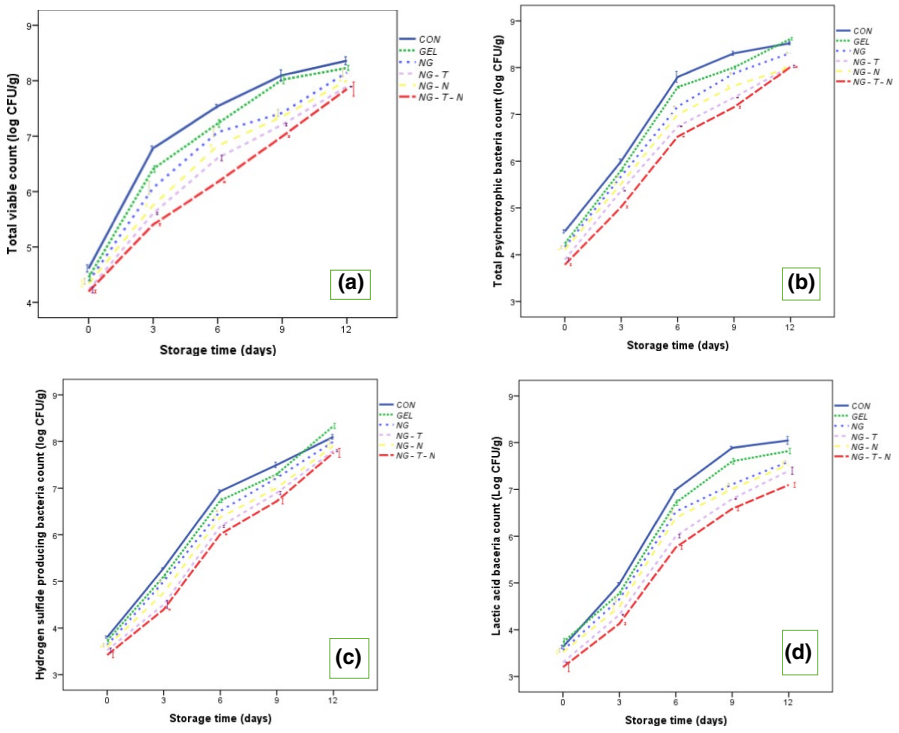

FIGURE 1 Changes in the total viable (a), total psychrotrophic (b), Hydrogen sulfide producing bacteria (c) and lactic acid bacteria (d) (log CFU/g) of rainbow trout samples in different treatments during 12 days of storage at 4°C. Data are expressed as mean ± SD (n=3)

TABLE 2 Average reduction rate of total viable bacteria, total psychrotrophic bacteria, hydrogen sulphide-producing bacteria, lactic acid bacteria and *Listeria monocytogenes* count (log CFU/g) among treatments when compared together during 12 days of storage[a]

		Mean Difference				
Attributes		**GEL**	**NG**	**NG-T**	**NG-N**	**NG-T-N**
Total viable bacteria	CON	0.21*	0.46*	0.77*	0.62*	0.95*
	GEL		0.25	0.56*	0.41*	0.74*
	NG			0.31*	0.16	0.49*
	NG-T				0.14	0.18*
	NG-N					0.33*
Total psychrotrophic bacteria	CON	0.17	0.38*	0.74*	0.57*	0.92*
	GEL		0.21*	0.57*	0.40*	0.75*
	NG			0.36*	0.19*	0.54*
	NG-T				0.16*	0.17*
	NG-N					0.34*
Hydrogen sulphide-producing bacteria	CON	0.08	0.25*	0.54*	0.39*	0.66*
	GEL		0.16*	0.45*	0.31*	0.57*
	NG			0.27*	0.14	0.41*
	NG-T				0.14	0.12
	NG-N					0.26*
Lactic acid bacteria	CON	0.17	0.41*	0.74*	0.51*	0.95*
	GEL		0.24*	0.56*	0.34*	0.77*
	NG			0.32*	0.10	0.53*
	NG-T				0.22*	0.20
	NG-N					0.43*
Listeria Monocytogenes	CON	0.11	0.26	0.52*	0.39*	0.65*
	GEL		0.15	0.40*	0.28*	0.54*
	NG			0.25	0.12	0.38*
	NG-T				0.12	0.13
	NG-N					0.25

[a]Data are expressed as mean ±SD (n = 3).; *Indicate a statistically significant difference ($p < 0.05$).

a previous study performed on shrimp in cold storage (Shirazinejad et al., 2010).

3.4 | Hydrogen sulphide-producing bacterial count

Previous studies have investigated and enumerated H_2S-producing bacteria, such as *Pseudomonas fluorescens* and *Shewanella putrefaciens*, with the latter causing an off-odour in food and reducing trimethylamine oxide to trimethylamine, which in turn leads to H_2S production. Figure 1c shows the increment in the enumerated hydrogen sulphide-producing (HSC) bacteria in the samples in the storage period. Initially, the HSC value was estimated to be ca. 3.61 log CFU/g, which increased significantly in samples throughout storage. This is in congruence with the study by Raeisi et al. (Raeisi et al., 2015). In the current research and after the storage, the highest HSC values were observed in the CON and GEL groups (8.10 and 8.33 log CFU/g respectively), which is consistent with an investigation conducted by Sun et al., (2019) regarding the impact of fish gelatin coating on grass carp fillets (*Ctenopharyngodon idellus*) (Sun et al., 2019). According to the information in Table 2, the HSC values did not differ significantly between the NG and NG-N treatment groups, which could be due to the bactericidal properties of nisin Gram-positive bacteria. On the same note, the findings of Gogus et al., (2006) demonstrated that using only nisin had no significant effect on the growth inhibition of Gram-negative bacteria. In the current research, the NG-T-N samples had the highest HSC values (~0.5 log CFU/g) compared with the controls, and the higher growth inhibition rate could be attributed to the role on thymol in enhancing the penetrative power of nisin into Gram-negative bacteria through the decomposition of the cell wall faster than nisin. This is in line with the results obtained by Gogus et al. (Gogus et al., 2006).

3.5 | Lactic acid bacterial count

Lactic acid bacteria (LAB) are Gram-positive, anaerobic, facultative bacterial strains with the unique growth ability in both aerobic and anaerobic conditions. Figure 1d shows the LAB growth rate and enumeration in the studied samples during the 12-day

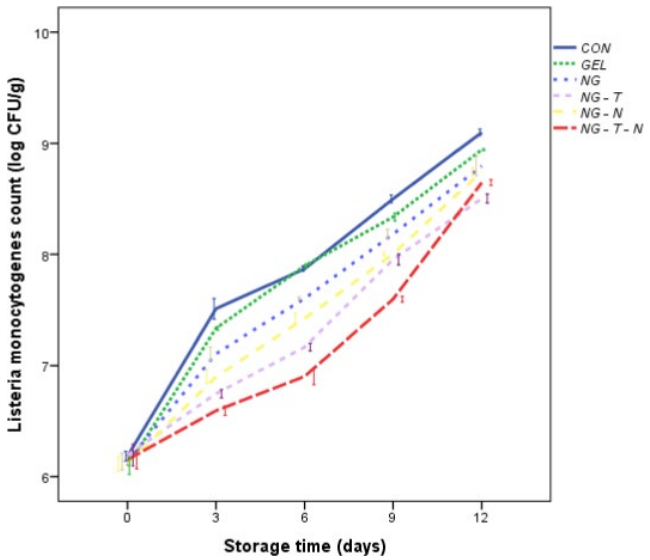

FIGURE 2 Changes in *Listeria monocytogenes* count (log CFU/g) of rainbow trout samples in different treatments during 12 days of storage at 4°C. Data are expressed as mean ± SD (n=3). Data are expressed as mean ± SD (n=3)

storage period. According to our findings, the LAB value was initially calculated to be ca. 3.46 log CFU/g, steadily incrementing in the study groups throughout the storage period. This is consistent with the study conducted by Alparslan et al., (2014) (Alparslan et al., 2014). According to the results of the present study, the lowest LAB count belonged to the NG-T-N (7.09 log CFU/g), NG-T (7.4 log CFU/g) and NG-N treatment samples (7.53 log CFU/g) after the storage (Figure 1d), showing the reduction in the bacterial count by 0.5–1 log CFU/g in the treatment groups compared with the controls ($p < 0.05$; Table 2). Furthermore, our findings demonstrated that the combination of nisin and thymol could effectively delay LAB growth throughout the storage period, maintaining the bacterial population at 7 log CFU/g after this period. According to the study by Arfat et al., (2014), LAB growth inhibition occurred with the use of fish skin gelatin films inoculated with 3% zinc oxide nanoparticles (Arfat et al., 2014). Moreover, the authors claimed that the addition of 1% laurel essential oil to the gelatin film could decrease the enumerated LAB by 2.9 log CFU/g compared with the controls ($p < 0.05$) (Alparslan et al., 2014). In another research, Kallinteri et al., (2013) confirmed the reductive effects of nisin and/or natamycin on the lactobacilli and lactococci in *Galotyri* cheese (Kallinteri et al., 2013).

3.6 | *L.monocytogenes*Count

Figure 2 depicts the *L. monocytogenes* growth affected by the treatments during the 12-day storage. The initial count of *L. monocytogenes* was estimated at ca. 6.23 ± 0.17 log CFU/g, significantly increasing in the study groups throughout the storage period. This finding is in congruence with the previous studies in this regard (Sharifi et al., 2017) (Ryser & Marth, 2007). In our research, the CON and GEL samples had a significantly higher growth rate compared with the other groups ($p < 0.05$), with the rate surging to 9.1 and 8.94 log CFU/g after the storage period respectively. On the other hand, the NG-T-N treatment samples were observed to have the lowest *L. monocytogenes* bacterial count (8.63 log CFU/g), which demonstrating the antibacterial effects of the nisin–thymol combination. In a similar research, Govaris et al., (2010) confirmed the synergy between nisin and oregano essential oil in the protection of cells against *Salmonella enteritidis* in minced mutton in cold storage (Govaris et al., 2010). Furthermore, Hadian et al., (2017) encapsulated *Rosmarinus officinalis* essential oil in chitosan–benzoic acid nanogel, effectively decreasing the inoculated *Salmonella typhimurium* in beef cutlet (Hadian et al., 2017). Meanwhile, Azizian et al., (2019) inoculated the nanogel/emulsion of chitosan coating with *Ziziphora clinopodioides* essential oil and nisin, and the combination proved to be an effective coating in the removal of *E. coli* O_{157}:H_7 from beef samples at the temperature of 4°C (Azizian et al., 2019).

4 | CONCLUSION

According to the results, the quality of the rainbow trout fillet samples could be preserved by using the prepared gelatin nanogel coating, and the effects were more sustainable compared with the controls in the storage conditions with the temperature of 4°C. Furthermore, the growth of the inoculated microbial flora and *L. monocytogenes* into the fillet samples was effectively inhibited by the gelatin nanogel coating incorporated with the nisin–thymol combination at the same temperature, while no impact was observed on the microbial growth compared with the controls. Therefore, the gelatin nanogel coating inoculated with the nisin–thymol combination is recommended as a viable option for the quality and safety improvement of various food products, as well as the elimination of foodborne pathogens, in order to optimize food safety.

ACKNOWLEDGEMENTS

This study was supported by the department of nutrition, faculty of medicine, Mashhad university of medical sciences, Mashhad, Iran. The Authors would like to thank Mrs. S. Khajenasiri for its kind cooperation.

AUTHOR CONTRIBUTION

Fatemeh Mohajer: Writing-original draft, Methodology. Saeid Khanzadi: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. Kobra Keykhosravy: Methodology, Writing-original draft. Seyyed Mohammad Ali Noori: Methodology, Conceptualization. Mohammad Hashemi: Conceptualization, Methodology, Project administration, Resources, Visualization, Roles, Writing – review & editing. Mohammad Azizzadeh: Data curation, Formal analysis, Software, Validation.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

Data is available upon request

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How to cite this article: Mohajer F, Khanzadi S, Keykhosravy K, Noori SM, Azizzadeh M, Hashemi M. Impact of gelatin nanogel coating containing thymol and nisin on the microbial quality of rainbow trout fillets and the inoculated *Listeria monocytogenes*. *Aquaculture Research*. 2021;00:1–8. https://doi.org/10.1111/are.15239