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Influence of Nisin and Lysozyme on the Shelf Life of Hot-smoked Rainbow Trout Fillets (*Oncorhynchus mykiss*) during Storage at 4 °C

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Hot-smoked rainbow trout (*Oncorhynchus mykiss*) fillets with vacuum packaging are an important export source and the manufacturers recommend a shelf life of 21 d at 4 °C. This time is very short in terms of the shelf life of packaged foods. In this study, nisin and lysozyme (0.5 and 1% w/v, respectively) were used in order to prolong the shelf life of hot-smoked rainbow trout by adding them to the brine (8–10 ˚C, 10 h). Five treatment groups were formed, including the control group. Samples (105) were hot-smoked at 72 ˚C and vacuum-packaged. The effect of nisin and lysozyme (0.5 and 1% w/v) on the chemical [(pH, total volatile basic nitrogen (TVB-N), peroxide, thiobarbituric acid reactive substances (TBARS), free fatty acids (FFAs)], microbiological [total number of aerobic mesophilic bacteria (TMAB), number of lactic acid bacteria (LAB), total number of coliform bacteria (TCB), total number of psychrophilic bacteria (TPB)], and sensorial properties were investigated during storage at 4 °C for 42 d. TVB-N, TBARS, and FFA values were evaluated. The most favorable results among the treatment groups for the chemical analysis were observed in groups treated with nisin (1 and 0.5%, respectively) followed by lysozyme-treated groups (1 and 0.5%, respectively), and the control group. Nisin and lysozyme showed the most important effect microbiologically and extended the shelf life of the samples from 21 d to 42 d at 4-˚C storage. The sensory analyses suggested that nisin and lysozyme treatments did not have a negative impact on the organoleptic properties of samples. Natural antimicrobials (nisin and lysozyme) reduced the TMAB, TCB, and TPB. As a result, it was determined that nisin and lysozyme can be used to increase the quality characteristics and shelf life of hot-smoked rainbow trout fillets instead of synthetic antioxidants and additives.

Keywords: lysozyme, nisin, rainbow trout, shelf life, smoking

INTRODUCTION

Fish and fish products have an important place in today's world as a healthier and easily perishable alternative to animal origin foods with their high nutritional value. The increasing world population, limited resources, and decreasing agricultural areas increase the preference for seafood as a protein source. Sustainable harvest from fishing cannot be increased from the current level, but the demand for fish of the human population is higher and increasing. Aquaculture has been the most rapidly growing agriculture industry for the past forty years and now accounts for more than half of the fish consumed worldwide (Stankovic *et*

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al. 2015). The Food and Agriculture Organization of the United Nations estimates that the main increase in fish production will come from aquaculture and reach 109 million in 2030, with an average annual growth rate of 4% to date (Kobayashi *et al.* 2015).

Rainbow trout (*Oncorhynchus mykiss*) is the most cultivated fish species in the world with good meat quality, short incubation period, easy adaptation to environmental conditions, high ability to benefit from artificial feeds, and resistance to diseases (EUMOFA 2019).

Rainbow trout consumption has increased in recent years as it ranks first among the fish farmed in Turkey and Europe (TÜIK 2020; EC). In parallel with the rapid increase in trout farming, the storage period of the fish is gaining importance. In this context, methods such as salting, drying, canning, marinating, and smoking are used to keep the fish for a longer time period (Yildiz 2003). Rainbow trout is available worldwide as white, pink, or orange; whole or filleted; and fresh or smoked.

Smoking is a process that gives the fish a different aroma by drying the fish and releasing the chemicals in the fume as it leads to preserving the fish for a longer time. The smoking process can be applied as "hot," "warm," and "cold" smoking (Hassan 1988). In order to prevent denaturation of proteins, cold smoking is performed below 25 °C and the temperature used in the cold smoking process varies depending on the species of fish but is usually between 15–23 °C. Warm smoking is carried out by applying a temperature between 25–50 °C while hot smoking is performed at 50–80 °C for 20–60 min. With this technique, the fish becomes ready to consume and gains desired aroma (Arvanitoyannis and Kotsanopoulos 2012).

Lysozyme, a small protein, has been of great interest because of its unique properties against a wide range of Gram-positive bacteria by hydrolyzing the glycosidic bonds of peptidoglycans of bacteria (Chung and Hancock 2000). Lysozyme is the only antimicrobial enzyme that has gained commercial application as a food preservative. Hen egg lysozyme is classified as GRAS (generally recognized as safe) in the United States and is approved in Europe where it has the food additive number E1105 (Baines and Seal 2012).

Nisin, which is a biopreservative bacteriocin released by *Lactococcus lactis* subsp. *lac* is commercially used in food and beverage products. Nisin has been verified as a GRAS substance by the World Health Organization and the United States Food and Drug Administration as a natural preservative additive; it is likewise allowed in 50 countries worldwide. Nisin is a polypeptide that is effective against Gram-positive bacteria, especially those that produce heat-resistant spores (Baines and Seal 2012; Gharsallaoui *et al.* 2016).

Smoked fish products – which are rich in essential amino acids, minerals, fat-soluble vitamins, and unsaturated fatty acids – are considered healthy ready-to-eat products. Turkey provides 99.3% of non-EU (European Union) imports (3.477 tons) for smoked rainbow trout in the EU market (EUMOFA 2019). Hot-smoked fish in the vacuum-packaged form at refrigerated temperature is very susceptible to deterioration as its shelf life varies from 3–4 wk (Rehbronn and Rutkowski 1982). Therefore, the shelf life of hot-smoked rainbow trout is reported to be 21 d at 4 °C by manufacturers and this is applied in several retail markets in EU countries (İnanli *et al.* 2018). With the improvements made on the shelf life of hot-smoked rainbow trout, it will be possible to ensure that this economically valuable fish will reach new markets with minimum losses.

In this study, lysozyme and nisin (0.5 and 1% w/v, respectively) were added to the brine of hot-smoked rainbow trout and the effect on the shelf life of the vacuum-packaged product was investigated during the storage at 4 °C. Nisin and lysozyme were used in order to extend the shelf life of hot-smoked rainbow trout while improving its chemical, microbiological, and sensorial properties.

MATERIALS

Raw Material and Sample Preparation

Rainbow trout, weighing an average of 200 ± 20 g, obtained from an aquaculture farm in Samsun (Bafra), Turkey was brought to the fish processing facilities along with the cold chain using a refrigerated truck. Nisin was obtained from Danisco (Nisaplin) and lysozyme from Sigma Aldrich (lyophilized powder, protein ≥ 90%, ≥ 40,000 units/ mg protein. Nisin and lysozyme ratios were derived from previous studies (Nattress and Baker 2003; Conte *et al.* 2011; Gao *et al.* 2014; Behnam *et al.* 2015; Uçar 2018). On the same day, the samples were kept in brines containing 0.5 and 1% nisin, 0.5 and 1% lysozyme, and 8% salt; prepared according to the production procedure; and left in brine for a night at 8–10 ºC. Following the draining, smoking was performed with oak smoke at 72 ºC using Kerres 2850 (Backnang, Germany) smoking house. After the smoking step, rainbow trout fillets were vacuum-packaged and subjected to blast freezing treatment. Fillets were filled into the styrofoam boxes with ice covered and were brought to the Ondokuz Mayis University's Food Engineering research laboratory. Samples were labeled N1 (0.5% nisin-treated samples), N2 (1% nisin-treated samples), L1 (0.5% lysozyme-treated samples), L2 (1% lysozyme-treated samples), and C (control samples). Samples were analyzed at 7, 14, 21, 28, 35, and 42 d of storage at 4 °C.

METHODS

Physicochemical Composition of Hot-smoked Rainbow Trouts

Moisture content. Moisture content analysis was measured according to AOAC procedures (2000). Approximately 2 g of sample was weighed into aluminum containers and dried at 105 °C in the oven until a constant weight was reached. Moisture content was determined by calculating the weight difference resulting from the evaporation of water.

Ash content. Homogenized samples between 3–5 g were weighted into crucibles and burned at 550 °C for about 4 h until a light gray color was obtained. Crucibles were cooled to room temperature in a desiccator and weighed on a sensitive balance. The ash content was calculated as followed:

$$Ash\ content\ \% = \frac{Wt\ after\ ashing - Tare\ wt\ of\ crucible}{Original\ sample\ wt} \times 100 \quad (1)$$

Crude fat content. Crude fat analysis was performed according to the method of Undeland *et al.* (1998). Following homogenization, 10 g of sample was weighed, after which 120 mL of 1:2 methanol chloroform mixture was added to samples and homogenized by ULTRA-TURRAX® (IKA T25). 20 mL of a 0.4% $CaCl_2$ solution was added to these homogenized samples and filtered through a filter paper. Filtered solutions were transferred into flasks, which were then sealed with parafilm and kept in a dark environment for one night; on the next day, the methanol + water layer was removed by using a separating funnel. From the chloroform + lipid portion of the remaining solution in the flask, chloroform was evaporated at 60 °C using a rotary evaporator. The flasks were left in the oven for 1 h at 90 °C to allow the chloroform to evaporate completely and finally cooled to room temperature in a desiccator and weighed on a scale. Crude fat content was determined by calculating the weight difference.

Crude protein content. Crude protein analysis was performed according to the Kjeldahl method (AOAC 2000). Onto 1-g homogenized sample in Kjeldahl tubes, two catalyst tablets and 20-mL H_2SO_4 were added and the samples were burned for about 3 h in the incineration unit until a green-yellow transparent color was obtained. Kjeldahl tubes were cooled to room temperature and 75 mL of distilled water was added. 25 mL of 4% boric acid (H_3BO_3) solution was added to the flask and Kjeldahl tubes were placed in the Kjeldahl distillation apparatus. Distillation was performed with 40% (w/v) NaOH for 6 min. The solution in the flasks from the distillation apparatus was titrated with 0.1M HCl until the color was clear. The amount of HCl used was recorded and protein amounts were calculated with the following formula:

$$\%\ nitrogen = \frac{14.01 \times N\ H_2SO_4 \times Vol\ H_2SO_4}{Sample\ wt \times 10} \quad (2)$$

$$\%\ crude\ protein = \%\ nitrogen \times 6.25 \quad (3)$$

Chemical analysis. Samples were diluted 1:10 (v/v) with distilled water and homogenized with ULTRA-TURRAX® (IKA T25). pH values were measured with a pH meter (Starter 2100, OHAUS), which was calibrated with buffer solutions of 4.00 and 7.00 before measurement.

TBARS analysis was performed according to the procedures of Lemon (1975). 1 g of fish sample was mixed into 6 mL of extraction solution containing EDTA (ethylenediaminetetraacetic acid) and propyl gallate. Samples were homogenized for 15 s and filtered through Whatman® no. 1 filter paper. The filtrate (1 mL) was mixed with 1 mL of thiobarbituric acid and then vortexed. This mixture was then heated at 100 °C for 40 min and after the cooling step, the sample was centrifuged at 2000 rpm for 5 min. Absorbance was measured at 532 nm. TBARS concentration was calculated from the regression equation obtained from standard solutions.

Peroxide analysis was performed according to the method modified according to the International Dairy Federation standards (Undeland *et al.* 1998). For the blank experiment, 5 mL of high-purity ethanol, 100 µL of isohexane, 100 µL of Fe^{2+}, and 100 µL of 30% (w/v) ammonium thiocyanate was used. Readings were performed at 500 nm using the Agilent G6860A Cary 60 UV-Vis instrument.

FFA analysis was performed according to the method of the American Oil Chemists' Society (1994). The percentage of free acid in oleic acid was calculated by the following formula:

$$\%\ free\ fatty\ acid = \frac{(C - B) \times 2{,}805}{W} \quad (4)$$

where C is the amount of 0.1M NaOH spent for sample, B is the amount of 0.1M NaOH spent for the blind, W is the sample weight, and 2.805 is the conversion factor.

TVB-N analysis was performed by distillation based method applied by Antonacopoulos and Vyncke (1989). Calculation of the amount of TVB-N was performed according to the following formula:

$$mg\ TVB\text{-}N/\ 100\ g = A \times 1.4 \times 100/B \quad (5)$$

where A is the amount of acid consumed in mL and B is the weight of the sample.

Microbiological Analysis

LAB. The appropriate dilutions were transferred to the Petri dishes containing MRS (de Man, Rogosa, and Sharpe) agar by transferring 1 mL of sample to each dish. Petri dishes were incubated in a CO_2 anaerobic incubator at 30 ºC for 72 h. At the end of the incubation, the number of colonies formed was taken into consideration and the result was calculated in numbers.

TCB. The appropriate dilutions were transferred to one Petri dish containing VRBD (violet red bile dextrose) agar by transferring 1 mL of sample, and the Petri dishes were incubated in an anaerobic incubator with CO_2 at 37 ºC for 48 h. At the end of the incubation, colony counts were made and the result was calculated as log cfu (colony-forming unit) per g sample.

TMAB. Cultivation was carried out by transferring 1 mL of sample into Petri dishes containing protocatechuic acid (PCA) from appropriate dilutions, and the Petri dishes were incubated at 30 ºC for 48 h. At the end of incubation, colony counts were made and the result was given as log cfu/g.

TPB. The method used for the determination of TMAB was applied. For this purpose, bacteria grown in PCA medium after 10 d of incubation at 5 ˚C were evaluated as psychrophiles.

Sensory Analysis

Sensory analyses of hot-smoked rainbow trout were made according to the modified quality index method (Bonilla *et al.* 2007). Sensory analyses were performed by ten experienced panelists in the Ondokuz Mayis University's Food Engineering Department. Sensory analyzes were carried out on the last day of the 21-d shelf life recommended by the company.

Statistical Analysis

Analyses were calculated with mean and standard deviation. A two-way analysis of variance was applied using SPSS (MAC OS X SPSS 22). The significance level was taken as $p < 0.05$.

RESULTS AND DISCUSSION

The composition analysis of the rainbow trout used in the study, chemical degradation analysis, and microbiological quality analysis results are given in Table 1. Furthermore, the nutritional value results of hot-smoked trout samples are given in Table 2.

The highest moisture value was found in the control group of hot-smoked rainbow trout. Significant differences ($p < 0.05$) were found between the treated groups. The amount of moisture decreased in all groups after applications of nisin and lysozyme to the brine. In smoked fish, water loss due to salting, smoking, and drying is an expected

Table 1. Characteristics of fresh rainbow trout fillets.

Moisture (%)	Protein (%)	Fat (%)	Ash (%)
73.42 ± 0.54	18.86 ± 0.49	5.78 ± 0.12	1.43 ± 0.27

pH	TBARS (mg MDA/ kg)	Peroxide value (meq O_2/ kg oil)	FFAs (%)	TVB-N (mg/ 100 g)
6.85 ± 0.03	0.45 ± 0.05	5.45 ± 0.15	4.90 ± 0.10	18.45 ± 0.15

TMAB (log cfu/ g)	LAB (log cfu/ g)	TCB (log cfu/ g)	TPB (log cfu/ g)
4.40 ± 0.05	1.14 ± 0.07	2.43 ± 0.04	2.85 ± 0.06)

Results are mean ± standard deviation and three replicates were performed.

Table 2. Compositional profile of hot-smoked rainbow trout fillets treated with nisin (0.5 and 1% w/v), lysozyme (0.5 and 1% w/v), and control samples.

Sample	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)
L1	66.63 ± 0.87^{bc}	23.35 ± 0.82^{a}	7.01 ± 0.07^{c}	1.70 ± 0.25^{a}
L2	64.03 ± 0.72^{a}	23.82 ± 0.95^{a}	6.86 ± 0.06^{bc}	1.94 ± 0.07^{ab}
N1	67.15 ± 1.59^{bc}	24.14 ± 1.31^{a}	6.81 ± 0.05^{b}	1.78 ± 0.15^{a}
N2	66.02 ± 1.22^{abc}	24.52 ± 1.09^{a}	6.76 ± 0.12^{ab}	1.82 ± 0.15^{ab}
Control	67.67 ± 1.12^{c}	23.42 ± 0.87^{a}	6.77 ± 0.15^{ab}	1.87 ± 0.24^{ab}

Means marked with different letters (a, b, c) are statistically different from each other ($p < 0.05$); values represent mean ± SD of three replicates in duplicate.

result. The amount of protein, fat, and ash in rainbow trout increased and the moisture content decreased after the smoking process.

It was reported that the product may lose moisture during the salting process applied before or after the smoking process (Bhuiyan *et al.* 1986; Adepoju *et al.* 2018). No significant differences ($p > 0.05$) were observed between the treatments in terms of protein content. Significant ($p < 0.05$) differences were found in moisture, ash, and fat content of treatment groups. Brining and smoking process applied to raw rainbow trout also increased the amount of protein determined in the samples from 18.86% to 23.42–24.52%. This situation is thought to be caused by the transfer of salt to fish meat during the brining step and the removal of water during the smoking process. In a study done by Oğuzhan *et al.* (2006), proximate composition of rainbow trout was reported as 19.23% of crude protein, 7.02% of crude lipid, 1.54% of crude ash, and 72.06% moisture. Tokur (2007) reported that the moisture content of smoked and raw trout was 70.67 and 61.14%, the protein content was 26.53 and 21.23%, the lipid content was 6.04 and 3.88%, and the crude ash content was 1.71 and 1.48%, respectively. Smoked rainbow trout had 63.93% moisture, 24.45% protein, 3.95% fat, and 2.98% ash in a study done by Mutlu and Bilgin (2016).

Chemical Analysis

Post-mortem changes in fish muscle vary depending on the fish species, size, and ambient temperature, and lipid oxidation continues as amino acids breakdown.

The pH value in fish meat usually varies between 5.7–6.6. The pH of fresh fish is close to neutral. With the effect of lactic acid with glycolysis, some decrease in pH is observed and once the fish starts to deteriorate, an increase in pH occurs again (Ruiz-Capillas and Moral 2001). The pH value in fresh rainbow trout was determined as 6.85. It was observed that the pH value of the smoked rainbow trout samples in this study decreased with the effect of the salting process during the smoking process. Salt has a significant linear decreasing effect at pH values explained by the increase of ionic strength of the solution (Leroi and Joffraud 2000). CO_2 and CO released during the smoking process react with the water present in fish meat and forms carbonic acid. This results in the pH value determined in fresh trout (6.85) to fall between 6.36–6.57 values after smoking. As shown in Table 3, the lowest pH value at the beginning of storage (Day 0) was 6.34 and the highest was 6.60; on Day 42, the lowest value was 6.46 and the highest was 6.54. Immersion in antimicrobial solutions caused significant ($p < 0.05$) changes in the pH values of the fish. At the end of storage, pH value was found to be lower in N2 and L2 groups compared to control group. It is thought to be caused by the inhibitory effect of nisin and lysozyme on the growth of microorganisms at 1% solution concentration and, thus, delaying the formation of volatile compounds.

TVB-N is produced by the destruction of proteins and non-protein nitrogen compounds as a result of microbial activity. TVB-N is an analysis showing the total amount of compounds such as trimethylamine, dimethylamine, monoethylamine, indole, scatol, and cadaverine – which shows the conversion of amino acids to carbonyl components, especially ammonia, by bacterial degradation (Sallam *et al.* 2007). TVB-N value in raw rainbow trout was determined as 18.45 ± 0.15 mg N/ 100 g. TVB-N values increased in all groups following the smoking process. This increase was significantly influenced by weight loss after smoking. At the beginning of the storage period, TVB-N values varied from 19.08 mg N/ 100 g (N1) to 22.41 mg N/ 100 g (N2). The average values of the TVB-N amount for 42 d of storage in our study increased, as shown in Table 3, excluding 1% nisin treatment. Fluctuations were observed during the storage period. The TVB-N value in nisin-treated groups did not create a statistically significant difference during 42 d of cold storage. The lowest mean TVB-N values at the end of storage were found to be 20.27 and 21.20 mg/ 100 g in samples treated with 0.5 and 1% nisin, respectively. Uçar (2018) reported that the application of nisin provided statistically ($p < 0.05$) less TVB-N value in his study where vacuum-packed sea bass fillets were stored at 4 ˚C for 12 d by immersing them in 0.2, 0.4, and 0.8% nisin solutions. The TVB-N value ranged from 30–35 mg/ 100 g for fish is generally regarded as the limit of acceptability (Varlık *et al.* 1993). In the control group, nisin, and lysozyme groups, the limit value of 35 mg/ 100 g was not reached. Microbial activity during storage is considered as one of the sources of nitrogen production. The increase of TVB-N amount in fish muscle during storage at 4 ± 2 °C is probably due to deamination of amino acids. It was determined that the amount of TVB-N in our samples was lower in nisin-treated groups and that the amount of TVB-N varied depending on the concentration of nisin used. Lysozyme was not effective in retarding the TVB-N value. Nisin treatments were found to be effective in extending the shelf life of hot-smoked rainbow trout samples.

The first step of oxidation forms peroxides by the addition of O_2 to the double bonds of unsaturated fatty acids. Peroxides are odorless and tasteless and may occur when there is no deterioration in the organoleptic appearance of fish. The initial peroxide value in raw trout was found to be 5.45 meq O_2/ kg. Significant differences ($p < 0.05$) were observed between the control group and nisin- and lysozyme-treated groups during the storage period ($p <$

Table 3. The changes in pH, TVB-N, PV, TBARS, and FFA content of hot-smoked rainbow trout fillets during 42 d of storage at 4 ˚C (n = 3).

Storage days	Treatments				
	Lysozyme 0.5% (L1)	Lysozyme 1% (L2)	Nisin 0.5% (N1)	Nisin 1% (N2)	Control (C)
pH values					
0	6.49^{a,A} (0.00)	6.36^{a,A} (0.03)	6.45^{a,BC} (0.00)	6.43^{a,B} (0.01)	6.54^{a,D} (0.06)
7	6.51^{ab,A} (0.04)	6.46^{b,A} (0.02)	6.46^{a,B} (0.01)	6.49^{b,A} (0.01)	6.66^{b,C} (0.00)
14	6.55^{bc,A} (0.01)	6.55^{c,A} (0.01)	6.63^{d,B} (0.01)	6.57^{c,A} (0.01)	6.61^{b,B} (0.02)
21	6.58^{c,A} (0.02)	6.60^{e,A} (0.00)	6.59^{c,A} (0.05)	6.58^{c,A} (0.02)	6.61^{b,A} (0.01)
28	6.55^{bc,A} (0.01)	6.56^{cd,A} (0.01)	6.58^{c,B} (0.01)	6.58^{c,B} (0.00)	6.61^{b,C} (0.01)
35	6.51^{ab,A} (0.04)	6.59^{de,BC} (0.01)	6.58^{c,BC} (0.01)	6.56^{c,B} (0.03)	6.62^{b,C} (0.02)
42	6.53^{abc,B} (0.01)	6.48^{b,A} (0.02)	6.53^{b,B} (0.01)	6.49^{b,A} (0.00)	6.54^{a,B} (0.00)
TVB-N content					
0	22.10^{ab,A} (1.50)	19.98^{ab,A} (2.87)	19.08^{a,A} (0.77)	22.41^{a,A} (2.41)	20.55^{ab,A} (1.48)
7	20.31^{a,A} (1.28)	20.65^{ab,A} (4.57)	18.78^{a,A} (2.52)	20.40^{a,A} (1.99)	22.91^{bc,A} (1.82)
14	20.93^{a,B} (0.16)	17.89^{a,A} (0.78)	21.17^{a,B} (2.28)	20.89^{a,B} (0.22)	20.87^{ab,B} (1.41)
21	23.10^{abc,C} (2.03)	18.57^{a,A} (0.75)	20.91^{a,ABC} (0.30)	22.68^{a,BC} (2.28)	19.95^{a,AB} (2.03)
28	24.21^{bc,B} (1.40)	19.83^{ab,A} (0.21)	20.60^{a,A} (0.33)	20.20^{a,A} (0.64)	21.11^{ab,A} (0.51)
35	24.96^{cd,C} (2.28)	19.59^{ab,A} (1.09)	20.19^{a,AB} (0.97)	20.29^{a,AB} (0.34)	22.06^{ab,B} (0.43)
42	22.10^{ab,A} (1.50)	23.39^{b,B} (0.47)	21.17^{a,A} (0.67)	21.51^{a,A} (1.04)	24.72^{c,B} (0.99)
PV values					
0	7.65^{a,B} (1.66)	11.42^{a,C} (1.78)	7.00^{a,B} (0.95)	4.55^{a,A} (0.93)	5.92^{a,AB} (0.32)
7	8.60^{a,AB} (3.47)	12.59^{ab,BC} (6.65)	7.97^{a,AB} (2.62)	10.54^{b,AB} (1.94)	5.23^{a,A} (1.99)
14	12.46^{b,AB} (2.62)	17.46^{bc,C} (2.70)	7.83^{a,A} (2.35)	16.21^{cde,BC} (1.26)	14.43^{b,BC} (3.56)
21	9.41^{ab,A} (0.99)	9.40^{a,A} (1.19)	8.57^{a,A} (0.78)	15.21^{cd,B} (2.63)	13.95^{b,B} (1.60)
28	16.72^{c,BC} (0.88)	17.28^{bc,C} (1.20)	14.22^{b,AB} (1.17)	13.21^{bc,A} (2.17)	15.99^{bc,BC} (1.38)
35	16.85^{c,AB} (2.00)	17.32^{bc,AB} (0.81)	18.04^{c,AB} (1.31)	16.52^{de,A} (0.97)	19.42^{c,B} (1.07)
42	20.46^{d,AB} (1.06)	20.58^{c,AB} (1.05)	18.58^{c,A} (0.75)	18.80^{e,A} (1.51)	22.13^{d,B} (2.41)
TBARS content					
0	1.37^{a,A} (0.38)	1.29^{a,A} (0.08)	1.40^{a,A} (0.13)	1.37^{a,A} (0.37)	1.65^{a,A} (0.11)

Storage days	Treatments				
	Lysozyme 0.5% (L1)	Lysozyme 1% (L2)	Nisin 0.5% (N1)	Nisin 1% (N2)	Control (C)
7	$3.92^{bc, C}$ (0.68)	$1.60^{a, A}$ (0.29)	$2.32^{b, AB}$ (0.28)	$2.44^{b, B}$ (0.18)	$2.68^{b, B}$ (0.30)
14	$3.30^{b, A}$ (0.72)	$3.11^{b, A}$ (0.30)	$4.25^{c, B}$ (0.44)	$3.66^{c, AB}$ (0.18)	$3.38^{c, A}$ (0.31)
21	$4.58^{cd, B}$ (0.32)	$3.93^{c, A}$ (0.14)	$4.82^{d, B}$ (0.16)	$3.90^{c, A}$ (0.05)	$3.65^{c, A}$ (0.24)
28	$4.84^{de, BC}$ (0.28)	$4.42^{d, A}$ (0.19)	$4.96^{d, C}$ (0.09)	$4.46^{d, A}$ (0.17)	$4.54^{d, AB}$ (0.25)
35	$5.35^{de, B}$ (0.31)	$4.74^{de, A}$ (0.12)	$5.45^{e, B}$ (0.19)	$5.29^{e, B}$ (0.16)	$5.84^{e, C}$ (0.15)
42	$5.45^{e, AB}$ (0.08)	$5.08^{e, A}$ (0.31)	$5.76^{e, BC}$ (0.06)	$5.29^{e, AB}$ (0.07)	$6.19^{e, C}$ (0.14)
FFA content					
0	$7.02^{a, C}$ (0.56)	$5.29^{a, AB}$ (0.54)	$4.22^{b, A}$ (0.74)	$4.42^{a, AB}$ (0.70)	$5.75^{a, BC}$ (1.26)
7	$7.28^{a, D}$ (0.60)	$5.57^{a, C}$ (0.45)	$3.30^{a, A}$ (1.01)	$4.24^{a, AB}$ (1.02)	$8.47^{b, D}$ (0.40)
14	$8.78^{b, C}$ (0.31)	$7.80^{ab, B}$ (0.64)	$7.64^{b, B}$ (0.39)	$6.02^{c, A}$ (0.28)	$5.62^{b, A}$ (0.27)
21	$8.52^{bc, CD}$ (0.47)	$8.07^{b, C}$ (0.44)	$7.29^{d, B}$ (0.25)	$6.50^{bc, A}$ (0.22)	$9.16^{b, DE}$ (0.14)
28	$9.29^{cd, C}$ (0.25)	$8.90^{c, C}$ (0.22)	$8.22^{e, B}$ (0.20)	$7.34^{cd, A}$ (0.25)	$10.17^{c, D}$ (0.18)
35	$9.47^{d, BC}$ (0.16)	$9.13^{c, B}$ (0.08)	$8.54^{e, B}$ (0.05)	$7.62^{d, A}$ (0.21)	$10.31^{c, CD}$ (1.06)
42	$9.68^{d, B}$ (0.30)	$5.29^{a, AB}$ (0.54)	$4.22^{b, A}$ (0.74)	$4.42^{a, AB}$ (0.70)	$5.75^{a, BC}$ (1.26)

Values represent mean ± SD of three replicates; the upper case indicates the statistical difference between groups, whereas the lower case indicates the statistical difference between storage days in same group ($p < 0.05$).

0.05). At the end of 4 °C storage for 42 d, the peroxide value was determined as 22.13 meq O_2 / kg in the control group. This value was found to be statistically different ($p < 0.05$) from lysozyme- and nisin-treated groups and it was the highest peroxide value determined at the end of storage. On the 42th day of storage, the lowest peroxide values were determined as 18.58 meq O_2/ kg in 0.5% nisin-treated group and 18.80 meq O_2/ kg in the 1% nisin-treated group. It is believed that this is due to the antioxidant properties of the compound, especially with the ability to chelate metal ions or remove reactive O_2 species (Behnam *et al.* 2015). In addition, Reale *et al.* (2008) reported that one of the factors that increase lipid oxidation is microbial enzymes. The higher peroxide content in the control group is associated with the conversion of lipid hydroperoxides to reactive alkoxy radicals with the presence of both iron or trace elements and greater microbial growth in the control group. It has been suggested that the deterioration in flavor will occur when the peroxide number reaches a value between 20–40 meq O_2/ kg. In our study, 20 meq O_2/ kg was not reached in 42 d of storage in nisin-treated groups. The group treated with the lysozyme and the control group exceeded the consumable limit on the 42nd day of storage (Table 3).

TBARS analysis is an important quality parameter in the determination of secondary lipid oxidation. As a result of the oxidation of fats, a bitter taste occurs and this change – which is known as oxidative rancidity – is mostly seen in fatty fish. Oxidation in fish meats varies depending on species, fat content, processing and storage conditions, and technology applied (Sallam *et al.* 2007). Malondialdehyde (MDA) is formed by the multiple separations of cyclic internal hydroperoxides from fatty acids with three or more double bonds during lipid oxidation (Careche and Colmenero 1988). At the beginning of the storage period, the lowest TBARS value was determined in the L2 group followed by L1 and N1 groups, and the values increased with the storage period. At the end of storage, TBARS values were significantly lower ($p < 0.05$) in nisin- and lysozyme-treated groups compared to the control group. This could be attributed to metal ion chelating or scavenge

of reactive oxygen species from the antioxidant activity of nisin and lysozyme (Lin and Yen 1999; Sarmadi and Ismail 2010; Behnam *et al.* 2015) TBARS value is reported as 3 mg MDA/kg in very good material and at most 5 mg MDA/kg in good material, whereas the limit value for acceptability is between 7–8 mg MDA/kg (Erdem *et al.* 2005; Sallam *et al.* 2007). According to this evaluation, this value was not reached in all of the sample groups (Table 3).

dFFAs occur as products of enzymatic hydrolysis of esterified lipids. Hydrolytic degradation is generated by lipase enzymes and triggers the formation of free FFAs from triglycerides (Pacheco-Aguilar *et al.* 2000). Significant differences ($p < 0.05$) were observed between groups treated with nisin and lysozyme during storage. At the beginning of storage, FFA values were found as 5.75, 7.02, 5.29, 4.22, and 4.42% in the control group; 0.5 and 1% in the lysozyme group; and 0.5 and 1% in the nisin group, respectively. In all of the treatment groups and the control group, the FFA value increased during storage. At the end of storage, FFA was determined at least in the samples where nisin (1%) was applied. Uçar (2018) reported FFA values for the control and nisin treatment groups (0.2, 0.4, and 0.8%) as 3.51, 2.62, 1.95, and 2.04%, respectively at the start of the storage at 4 ˚C for seabass. At the end of 12 d of storage, these values reached 8.19, 7.72, 7.02, and 6.22% respectively.

Microbiological Analyses

Figure 1 shows the microbiological changes of hot-smoked rainbow trout fillets treated with/without nisin and lysozyme during 42 d of storage at 4 °C.

The TMAB in raw rainbow trout was determined as 4.40 log cfu/g, indicating a good quality of fish. The smoking and brining process significantly reduced the microbial load of rainbow trout. Initial microbial load for rainbow trout was reported as between 3–4 log cfu/g in other studies, and this value increased with the advancement of the duration of cold storage (Rezaei and Hosseini 2008; Ozogul *et al.* 2013, 2017).

TMAB count was less than 1 log cfu/g on the first day of storage in all sample groups. Behnam *et al.* (2016) reported 3.15 and 3.10 log cfu/g TMAB in the filets treated with and without nisin, respectively.

TMAB value in our study was determined as 1.84 log cfu/g in the L2 group on the 7th day of storage. From the 14th day of storage, the number of TMAB in the control group reached 2.59 log cfu/g. Dondero *et al.* (2004) reported that increases in aerobic mesophyll numbers were associated with storage time and temperature. In groups treated with 1% lysozyme, 0.5% nisin, and 1% nisin, TMAB was

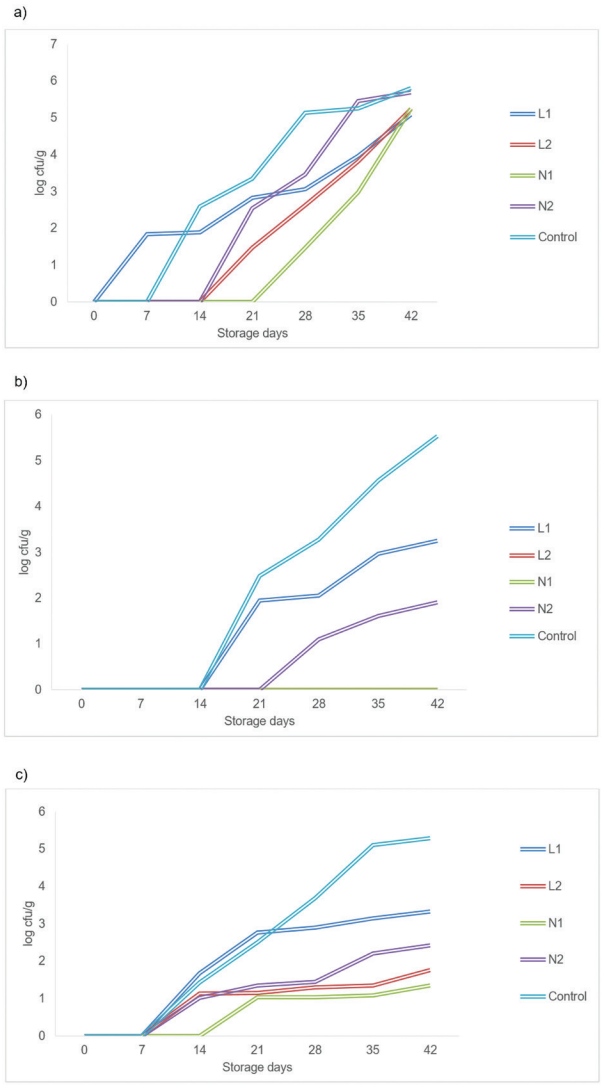

Figure 1. Microbiological changes of hot-smoked rainbow trout treated with lysozyme and nisin: a) TMAB; b) TCB; c) total psychrotrophic bacteria.

detected below 1 log cfu/g on the 14th day of storage. On the 21st day of storage, in all groups except for 0.5% nisin application, TMAB was determined above 1 log cfu/g and each sample group showed a statistically significant difference ($p < 0.05$). On the 28th day of storage, TMAB was determined above 1 log cfu/g in all sample groups and all groups were statistically different ($p < 0.05$). 7 log cfu/g is regarded as a consumable limit for seafood products (ICMSF 1986). The TMAB value of the samples did not exceed this limit value in any sample group. On the last day of storage, on the 42nd day, the lowest TAMB value was determined in the group treated with 0.5% lysozyme, and the highest TMAB value was determined in the control group.

Lactic acid bacteria are among the bacteria that spoil vacuum-packaged seafood. In our study, 1.14 log cfu/g LAB was found in raw rainbow trout. The LAB in hot-smoked rainbow trout fillets was determined as 1.69 log cfu/g in the control group and less than 1 log cfu/g in the nisin and lysozyme treatment groups at the beginning of storage. At the end of 42-d storage at 4 ºC, these values were determined below 1 log cfu/g for the control group and L1, L2, N1, and N2 treatment groups (LAB values are not given in figure). All application levels of nisin and lysozyme treatment showed an inhibitory effect on lactic acid bacteria in hot-smoked rainbow trout and the LAB was detected less than 1 log cfu/g at the end of storage. The bactericidal activity of nisin was mostly on Gram-positive bacteria, and the trend observed in the LAB count of the samples was an expected result. The effect of nisin against lactic acid bacteria has been reported to result from its interaction with the phospholipid membrane without a specific receptor (Nattress and Baker 2003). Gao *et al.* (2002) reported that lysozyme was effective in controlling the growth of the indigenous flora of LAB. Lysozyme has been proposed to act as muramidase, which is enzymatically breaking the sugar linkages of peptidoglycan, thus causing cells to burst due to the weakened peptidoglycan (Chung and Hancock 2000).

Coliform bacteria are Gram-negative, non-spore-producing, facultative anaerobes in the Enterobacteriaceae family that produce gas within 48 h at 35 ºC (Jay *et al.* 2005). In our study, the TCB in raw rainbow trout was determined as 2.43 log cfu/g. The TCB at the beginning of storage was detected below 1 log cfu /g in all sample groups. The TCB in the raw material decreased with the effect of salting, heating during smoking, drying, and antibacterial compounds in the smoke composition. The organic compounds in the smoke generally consist of phenol, aldehyde, ketone, organic acid, alcohol, ester, hydrocarbon, and various heterocyclic compounds and provide microbiological protective effect by formaldehyde and acids (Adeyeye 2019). As shown in Figure 1 after 21 d of storage, rapid bacterial growth was observed in the control group. In the nisin and lysozyme treatment groups, the limit value (2 log cfu/g) was not reached during the 42-d storage period except for the 0.5% lysozyme group (ICMSF 1986). Significant increases were observed for TCB throughout the storage. The highest increase was seen in the control group, whereas the N1 group generally had a lower TCB compared with other treatment groups during the storage at 4 ˚C. The main antimicrobial activity of nisin is to create pores in the cytoplasmic membrane, resulting in the loss of small intracellular molecules and ions and the collapse of the proton motive force (Gharsallaoui *et al.* 2016). Lysozyme is reported to have bactericidal activities against Gram-positive and -negative bacteria with muramidase activity and its cationic, hydrophobic properties (Pellegrini *et al.* 1992).

The additives used in the study were found to be effective in the development of TCB.

Psychrophilic bacteria are bacteria that can develop at refrigeration temperature and are effective in the deterioration of food stored in cold. The microflora of hot-smoked fish are psychotropic Gram-negative, rod-shaped bacteria of *Pseudomonas* and *Aeromonas* (Lund *et al.* 2000). In our study, TPB counts detected in our samples during storage are given in Figure 1. The TPB determined in the raw material was 2.85 log cfu/g. After brining and smoking process, the number of TPB decreased to less than 1 log cfu/g. The TPB increased in the control group and treatment groups over storage time. In the control sample, TPB was detected less than 1 log cfu /g at the beginning of storage. On Day 42 of storage, the number of TPB increased to 5.28 log cfu/g. While the number of TPB was determined to be less than 1 log cfu/g until the 7th day of storage in the samples treated with lysozyme, a statistically significant difference ($p < 0.05$) was observed between the two groups from the 14th day of storage to the 42nd day of storage. TPB increased in number to 3.33 log cfu/g in the 0.5% lysozyme group and 1.76 log cfu /g in the 1% lysozyme group. In the sample groups treated with 0.5 and 1% nisin, the number of TPBs was determined to be less than 1 log cfu/g until the 7th day of storage. On the 14th day of storage, 1.01 log cfu/g TPB was determined in the group treated with 1% nisin. The number of TPBs increased in both groups during storage. On the 42th day of storage, 1.35 log cfu/g TPB was determined in the group treated with 0.5% nisin and 1.35 log cfu /g TPB in the group treated with 1% nisin. To summarize, nisin and lysozyme applications slowed down the development of TPB.

Sensory Analysis

Sensory properties in fish are one of the important factors in consumer purchasing preference.

The sensory properties of smoked fish mainly depend on processing conditions (hot or cold smoking), initial microbial load, packaging type, storage temperature, and conditions during the transportation.

Results of sensory analysis of hot-smoked rainbow trout fillets treated with/without nisin and lysozyme are given in Table 4.

The most admired group was the L1 and the control group in terms of taste and texture according to sensory analysis results. The most preferred odor was that of L1, and significant differences were observed in groups. The highest mean score in color attribute was 8.55 for L2

Table 4. The sensory evaluation of hot-smoked rainbow fillets**.**

Characteristics	Treatments				
	L1	L2	N1	N2	Control
Color	7.66 ± 1.00^{ab}	8.55 ± 0.72^{b}	7.44 ± 1.50^{a}	7.44 ± 1.13^{a}	8.55 ± 0.53^{b}
Shape	8.33 ± 1.11^{a}	8.55 ± 0.52^{a}	8.33 ± 1.65^{a}	8.11 ± 1.16^{a}	8.77 ± 0.44^{a}
Odor	8.77 ± 0.44^{b}	8.11 ± 0.60^{ab}	7.55 ± 1.13^{a}	8.11 ± 0.78^{ab}	8.00 ± 0.86^{ab}
Texture	7.66 ± 0.70^{a}	7.44 ± 1.33^{a}	7.22 ± 1.39^{a}	7.44 ± 0.88^{a}	7.66 ± 0.70^{a}
Taste	8.77 ± 0.44^{b}	8.44 ± 0.88^{ab}	7.66 ± 1.22^{a}	8.22 ± 0.83^{ab}	8.11 ± 0.92^{ab}

Values represent mean ± SD of three replicates in duplicate.

and the control group. The control group had the highest score (8.77) and N2 had the lowest (8.11) for the shape properties of the samples. As a result, nisin and lysozyme treatment did not result in negative sensory properties and they can be preferred in the industry.

CONCLUSIONS

In this study, rainbow trout treated with nisin (0.5 and 1%) and lysozyme (0.5 and 1%) were subjected to hot smoking, vacuum-packed, and examined for 42 d at 4 °C for chemical and microbiological changes with the sensory properties. Nisin and lysozyme treatments were successful in delaying the microbial growth of TMAB, TCB, and TPB as the highest values were determined in the control group. The lowest microbial growth in TMAB, TCB, and TPB was seen in the N1 treatment group – followed by N2, L2, and L1 respectively. Nisin treatment led to lower TVB-N, PV, TBARS, and FFA values compared with lysozyme treatment and the control group. Both treatments did not have a negative impact on the sensory properties of samples. The shelf life of hot-smoked rainbow trout is given as 21 d at 4 ˚C storage by the manufacturers and markets. According to microbiological results, the shelf life of hot-smoked rainbow trout could be extended to 42 d at 4 ˚C with the use of nisin and lysozyme.

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Compliance with Ethical Standards
The authors declare that they have no conflict of interest.

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