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Prevalence of *Listeria* spp. in Seafood Samples and Control of *Listeria monocytogenes* with Using LISTEX™ P100 Bacteriophage Applications in Smoked Rainbow Trout

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ABSTRACT
This study was carried out to determine the presence of *Listeria* spp. in seafood and determine the effect of LISTEX™ P100 bacteriophage applications (incorporated into the sodium alginate based film and applied directly to the surface) on the smoked trout. In this study, *Listeria* spp. was isolated in 40 of the 100 products analyzed. Among the *Listeria* isolates, 8% correspond to *L. monocytogenes*, 15% to *L. innocua*, 6% to *L. seeligeri*, 10% to *L. welshimeri*, and 15 to *L. grayi*. *L. ivanovii* was not detected in any of the products analyzed. LISTEX™ P100 bacteriophage as antimicrobial compounds was incorporated into the sodium alginate based film for the first time. Bacteriophage in sodium alginate based film and direct bacteriophage applications in smoked trout were found to be effective in *L. monocytogenes* inactivation during storage. In addition, the preservation of phage stability of the two groups during storage indicates that in the smoked products can use in the control of *L. monocytogenes*.

Keywords: *Listeria monocytogenes*, Bacteriophage, LISTEX™ P100, Alginate film, Biopreservation, Seafood

1. Introduction

Listeria species are Gram-positive, non-spore-forming, facultative anaerobic bacteria widely distributed in the natural environment (Amajoud et al. 2018). Among *Listeria* genus, *Listeria monocytogenes* is most significant species because it causes listeriosis one of the most severe foodborne illness, whereas *Listeria innocua*, *Listeria welshimeri*, *Listeria ivanovii*, and *Listeria seeligeri* are associated with sporadic human infections (Amajoud et al. 2018).

In terms of food-borne illness, Ready-to-eat (RTE) foods are risky products for *L. monocytogenes* (Chibeu et al. 2013). Because, RTE are consumed directly, without a bacterial inactivation processing (Guenther et al. 2009). The salt content (2-8%), moisture contents (65-78%), pH (5.9-6.3) and water activity value (0.95-0.98) of the smoked seafood products, which are RTE products, facilitate the development of *L. monocytogenes* (Hwang et al. 2009). Therefore, smoked fish products are classified as risky products for listeriosis (Rørvik 2000).

The preservation methods and procedures applicable to minimally processed RTE foods are insufficient to ensure complete control of this microorganism (Guenther et al. 2009). The use of bacteriophage within these approaches is a good alternative (Oliveira et al. 2014), because the use of bacteriophage to prevent the development of *L. monocytogenes* in foods is very effective (Soni & Nannapaneni 2010; Rossi et al. 2011; Perera et al. 2015). Lytic bacteriophages are virus that infect bacterial cells and disrupt bacterial metabolism, causing bacterial lysis (Garcia et al. 2008). There are several commercially available phage products such as ListShield, EcoShield, Agriphage, SalmoFresh and LISTEX™ P100 (Hagens & Offerhaus 2008; Gálvez et al. 2014; Oliveira et al. 2014). The U.S. Food and Drug Administration (FDA) approved LISTEX™ P100 for use in foods to struggle with *L. monocytogenes* contamination (Soni and Nannapaneni 2010).

There are many studies in which bacteriophages are added directly to the surface of seafood products (Soni et al. 2009; Soni & Nannapaneni 2010; Galarce et al. 2014; Soni et al. 2014; Perera et al. 2015). As a result of these studies, it was reported that direct phage application to surface of products was effective in seafood. However, there are few studies in which a bacteriophage is incorporated into the film or within the absorbent pads (Gouvêa et al. 2015; Gouvêa et al. 2016). In these studies, the effects of phage containing films and pads on pathogen bacteria were investigated in vitro. There is only one study investigating the effect of bioactive packaging material containing bacteriophage (LISTEX™ P100) on *L. monocytogenes* in RTE turkey meat (Lone et al. 2016). However, in this study, we investigated the effect of sodium alginate-based film containing LISTEX™ P100 bacteriophage on *L. monocytogenes* in smoked trout fillets.

In this study was aimed to detect the presence of *Listeria* spp. in seafood sold in Turkey and to determine the effect of LISTEX™ P100 bacteriophage applications (incorporated to the film and applied directly to the surface) on *L. monocytogenes* in the smoked trout fillets.

2. Material and Methods

2.1. Investigation of Listeria spp. in seafood

In this study, a total of 100 raw and processed seafood products were purchased from supermarkets and restaurants in Turkey. These samples were investigated for the presence of *Listeria* spp. ISO (International Standards Organization 11290-1) method was used for the isolation of *Listeria* spp. (Anon 2000; ISO 2017). According to this method, the isolation process was carried out by pre-enrichment, selective enrichment, planting of solid medium and evaluation of colonies.

2.2. L. monocytogenes strains and *Bacteriophage source and plaque forming assay*

In this study, *Listeria monocytogenes* ATCC 7644 strain was used. The LISTEX™ P100 bacteriophage used in the study was obtained from Micreos (The Netherlands). Phage P100 stock solution in buffered saline had an approximate concentration of 10^{11} PFU (Plaque-forming units) mL^{-1}. To determine the phage titer of LISTEX^{TM} commercial suspension by double layer agar.

To determine the titre;

Phage titer (PFU mL^{-1}): N × 1/DF × 1/V

N: Number of plaques of lysis counted on a plate, DF: Dilution factor, V: Volume

2.3. Preparation of sodium alginate based film containing bacteriophage and evaluation of antimicrobial activity of film

Two types of film were prepared for evaluation of antimicrobial activity of sodium alginate based film containing bacteriophage. First one was sodium alginate film with phage P100 incorporated, other was control film not included phage P100.

1% sodium alginate based film solution was prepared; 1 g of sodium alginate is dissolved in 50 mL of sterile distilled water and mixed until dissolved in magnetic stirrer at 80 °C. The solution was cooled to 50 °C, it was added to 50 ml of 10^8 PFU mL^{-1} LISTEX™ P100 bacteriophage in film solution to achieve 1% concentration of solution. In this way, group of film containing bacteriophage was formed. For group of control film not containing bacteriophage; it was added to 50 mL of physiological salt water in film solution to achieve 1% concentration of solution. The films were poured into sterile petri dish to measure their antimicrobial activity. 3 mL of 0.05 M and 12.8 M $CaCl_2$ solutions used by Brachkova et al. (2012) were added to the films to strengthen the film (Han et al. 2018). To evaluate the antimicrobial activity of sodium alginate based films, a 1.75 cm diameter films were cut with a sterile lid.

The 24 hour cultures of *L. monocytogenes* (ATCC 7644) were diluted with PSW to standard concentration of 0.5 Mac Farland, and 100 µL of bacterial culture (10^8 CFU mL^{-1}) was spread to petri dishes containing Muller Hilton Agar (MHA, Merck). Films of 1.75 cm diameter have been placed on this plate. After plates' incubation, the diameter (mm) of the inhibition zones was recorded.

2.4. Effect of LISTEX™ P100 phage on L. monocytogenes (ATCC 7644) *in smoked trout*

2.4.1. Application of liquid smoked process to trout fillets

The fresh rainbow trout were obtained from a commercial company. The fish were filleted after harvest and immediately transported to the laboratory under a cold chain. Liquid smoke condensate (Red Arrow, SmokEz-C-10, mesguite water based) purchased from a food additive company (GMT Food Ingredients) was used to smoke the trout fillets. 100 mL liquid smoke condensate was mixed in 36% salt solution. Raw trout fillets were kept in this solution for 4 hours at 4 °C. At the end of this period, fillets were removed from this solution and dried for 25 min. Fillets were baked at 120-130 °C until their internal temperature reached 80 °C (Alçiçek 2010; Alcicek and Atar 2010). After cooking, the smoked trout were made into small fillets of 10 g.

2.4.2. Inoculation of smoked trout fillets with L. monocytogenes (ATCC 7644)

500 µL of *L. monocytogenes* culture (ATCC 7644) (6 log CFU g^{-1}) were poured on each side of raw trout sample (10 g). Total of 1000 µL of *L. monocytogenes* culture were used for contaminating both sides of fillets. After inoculation, the smoked fillets were allowed to air dry for 15 min in a Biosafety Level 2 laminar flow hood.

Experimental groups

Four different experimental groups were designed in this study.

Group 1: Group of smoked trout fillets inoculated with *L. monocytogenes* (ATCC 7644) coated with alginate film containing bacteriophage **(BF)**
Group 2: Group of smoked trout fillets inoculated with *L. monocytogenes* (ATCC 7644) coated with alginate film not containing bacteriophage **(CF)**
Group 3: Group of applied bacteriophage directly to the surface of smoked trout fillets inoculated with *L. monocytogenes* (ATCC 7644) **(B)**
Group 4: Group of smoked trout fillets inoculated with *L. monocytogenes* (ATCC 7644) **(C)**

2.4.3. Bacteriophage applications in smoked trout fillets inoculated with L. monocytogenes (ATCC 7644)

Smoked trout fillets inoculated with *L. monocytogenes* (ATCC 7644) were coated by dipping method with BF and CF film solutions that prepared as described in "Preparation of sodium alginate based film containing bacteriophage" section. And $CaCl_2$ solutions were added to the films to strengthen the film (Brachkova et al. 2012; Han et al. 2018).

For fillets applied bacteriophage directly to the surface (B), LISTEX™ P100 bacteriophage was inoculated 500 µL (8 log PFU g^{-1}) to two side of smoked trout fillets inoculated with *L. monocytogenes* (ATCC 7644).

In control group (C), smoked trout fillets were only inoculated with *L. monocytogenes* (ATCC 7644). Fillets (BF, CF, B and C) were stored in refrigerator at 10 °C for 7 days. The number of *L. monocytogenes* and bacteriophages were determined on the fillets at 0., 24., 48. and 96. hours and 7th day of storage.

2.4.4. Determination of L. monocytogenes and bacteriophage count in smoked trout fillets inoculated with L. monocytogenes during storage

For *L. monocytogenes* counting, each smoked trout fillet sample was aseptically homogenized and diluted. Serial dilutions of bacterial culture were spread on PALCAM Agar plates. They were incubated at 30 °C for 24-48 hours. At the end of the incubation, colonies were counted and the number of bacteria was determined as log CFU g^{-1}. For the determination of the number of bacteriophages, double layer agar method was used. They were incubated at 30 °C for 24 hours. At the end of the incubation, plaques of lysis were counted and the number of phages expressed as log PFU g^{-1}.

2.5. Statistical analysis

The obtained data were analyzed using Statistical Analyses Software Package (version 22, SPPS, Inc., Chicago, IL). The experimental data were subjected to T-test and One-way analysis of variance (ANOVA). The means comparison was performed by Duncan Multiple Comparison with the level of significant set at $P<0.05$.

3. Results and Discussion

3.1. The prevalences of Listeria spp. in seafood

Listeria spp. was isolated in 40 of the 100 products analyzed. Among the *Listeria* isolates, 8% correspond to *L. monocytogenes*, 15% to *L. innocua*, 6% to *L. seeligeri*, 10% to *L. welshimeri*, and 15 to *L. grayi*. *L. ivanovii* was not detected in any of the products analyzed (Table 1).

Table 1- Distribution of *Listeria* spp. in raw and processed seafood samples (%)

Listeria species	*Raw seafood*	*Processed seafood*	*Total*
L. monocytogenes	2	6	8
L. innocua	10	5	15
L. ivanovii	0	0	0
L. seeligeri	2	4	6
L. welshimeri	6	4	10
L. grayi	0	1	1

At the end of this study, *L. monocytogenes* was isolated in 8% of the seafood samples and this prevalence is 2% for raw seafood samples; 6% for processed seafood. Similarly, In the results of Fallah et al. (2013) and Yamazaki et al. (2000) it was found that *L. monocytogenes* number is higher in ready to eat and processed seafood products compared to raw seafood. The results show that preservation methods applied to processed seafood products and Ready-to-eat foods seem to be insufficient to prevent *Listeria monocytogenes* contamination and growth (Guenther et al. 2009).

Our results show that *L. monocytogenes*, *L. seeligeri* and *L. grayi* prevalence were higher in processed seafood products compared to raw seafood. The high prevalence detected in these products could be due to many factors such as cross contamination and insufficient cooking process (Jamali et al. 2013).

3.2. Antimicrobial activity of sodium alginate based film containing bacteriophage

Films incorporated with bacteriophage are promising for future application in food packaging (Gouvêa et al. 2015). It was been observed to not develop of bacterial cells around the sodium alginate based film containing LISTEX™ P100 bacteriophage. This condition was caused by the lysis caused by bacteriophage. No lysis was observed around the film in the control film group. The sodium alginate based film containing bacteriophage was determined to be 4.3±0.11 mm in inhibition zone against *L. monocytogenes* in MHA (Figure 1). Gouvêa et al. (2016) used absorbent food pads containing bacteriophage, similar to our results; the researchers reported that these pads were effective on the pathogen bacteria in the medium against *Salmonella Typhimurium* pathogen.

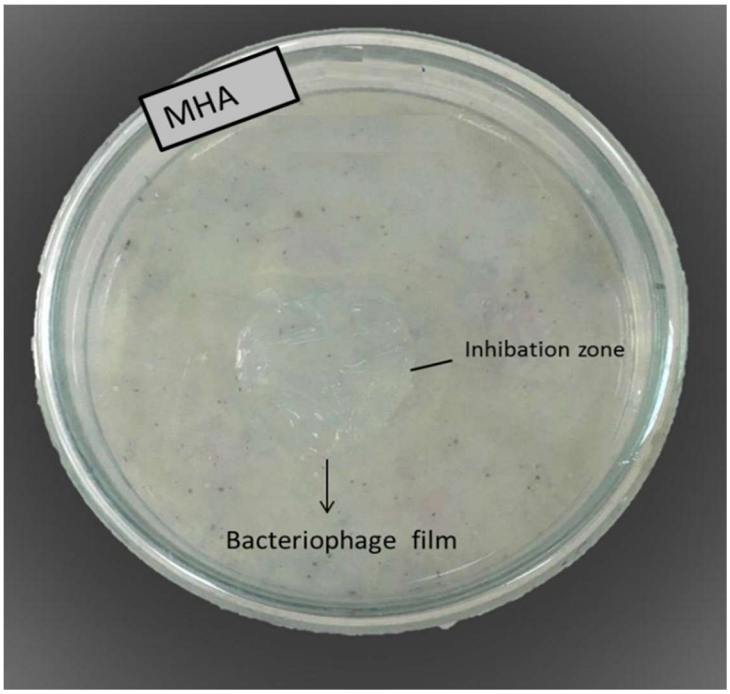

Figure 1- Antimicrobial activity of sodium alginate based film containing bacteriophage against *Listeria monocytogenes* on MHA plates

3.3. The effects of bacteriophage applications on L. monocytogenes in smoked trout fillets

The effect of LISTEX™ P100 on *L. monocytogenes* contaminating smoked trout fillets during storage at 10 °C was dependent on the way of application (Figure 2).

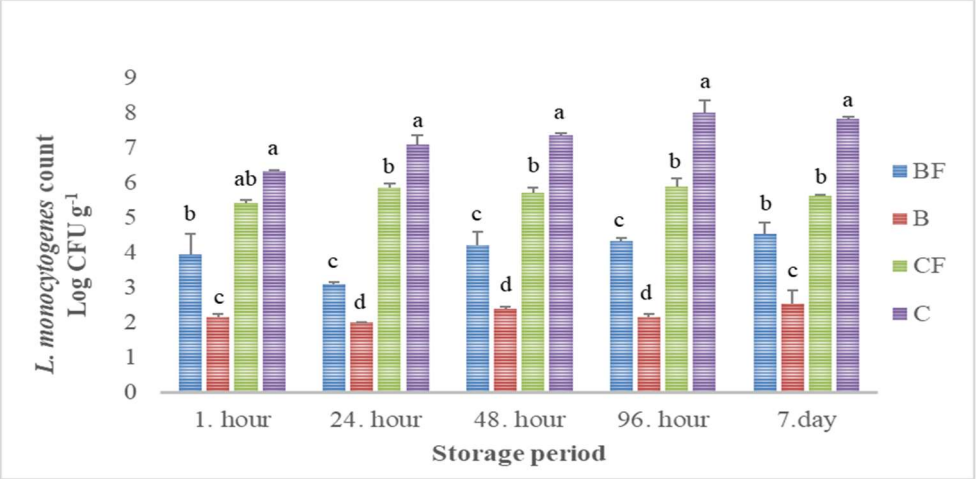

Figure 2- The effects of bacteriophage on *L. monocytogenes* in smoked trout fillets stored at 10 °C (log CFU g^{-1}) BF: Film containing bacteriophage, B: Applying of bacteriophage directly to the surface, CF: Film not containing bacteriophage, C: phage not added *Different letters (a, b) represent statistical differences among groups in same storage period (P<0.05)

The number of *L. monocytogenes* in BF Group provided 1.48 log CFU g^{-1} reduction compared to the CF Group in 1st hour of storage, however, this decrease was not found to be statistically significant (P>0.05). The number of *L. monocytogenes* in BF Group provided 2.37 log CFU g^{-1} reduction compared to C Group (P<0.05). During 7 days of storage, although the most effective bacteriophage application to inactivation of *L. monocytogenes* was the direct bacteriophage application (B) (P<0.05), application of film containing bacteriophage (BF) was found to be effective compare to the control group (C) (P<0.05).

Soni & Nannapaneni (2010) investigated the effect of 10^8 PFU g^{-1} bacteriophage on *L. monocytogenes* in raw salmon fillets during 10 days of storage. During storage, researchers determined similarly our results that the number of *L. monocytogenes* in fillet was lower in the group that were applied bacteriophage compared to the group that were not applied bacteriophage (P<0.05).

Gutiérrez et al. (2017) reported that the 10^9 PFU mL^{-1} LISTEX™ P100 bacteriophage application in dried hams was effective (below the detectable limit) in reducing the number of *L. monocytogenes* (10^3, 10^4, 10^5 CFU cm^{-2}) in storage at 4 ºC and 12 ºC at the end of 24 hours.

In this study performed by us, it determined that direct bacteriophage application at the end of the 24th hour resulted in a decrease of 5 log CFU g^{-1} in the number of *L. monocytogenes* in the fillets compared to the control group and it determined that the number of *L. monocytogenes* at 24 hours in the fillets was 2.15 log CFU g^{-1}. This difference between the two studies is thought to be caused by the difference between the numbers of *L. monocytogenes* and bacteriophages initially added. It was reported by Carlton et al. (2005) that the differences in the number of added phages were effective on the number of *L. monocytogenes.* The researchers stated that low dose phage (1.5 x10^8 PFU g^{-1}) application on the development of *L. monocytogenes* on cheese yielded 2-3 log reduction in bacterial count, whereas high dose phage application was effective in inactivating *L. monocytogenes* completely. Similarly, Perera et al. (2015) reported that cold smoked trout fillets that were inoculated with *L. monocytogenes* detected a 0.4 and 1.0 log reduction in the number of bacteria in the fillets at the 24th hour following low-dose (10^5 PFU g^{-1}) and high-dose (10^6 PFU g^{-1}) bacteriophage (ListShield) application.

Although the number of *L. monocytogenes* in bacteriophage application applied directly to the surface of smoked trout fillets resulted in an average reduction 1.78 log (P<0.05) compared to the number of *L. monocytogenes* in application bacteriophage in alginate films over 7 days of storage, application of bacteriophage in alginate films resulted in a 1.67 log reduction in storage compared to the control film without the addition of bacteriophage. Similarly, Lone et al. (2016) reported that the phage-based material they developed had a significant antimicrobial effect on contaminated foods.

In this study, *L. monocytogenes* could not be completely eliminated with bacteriophage applications. But the smoked trout fillets were contaminated with high levels of bacteria in this study, unlikely to be encountered in real life. However, LISTEX™ P100 applications (incorporated into the sodium alginate based film and applied directly to the surface) were generally found to be effective in inactivation of *L. monocytogenes* during 7 days of storage. For this reason, it is possible to say that it will be more effective to the phages both directly applied to the fillets and incorporated into the sodium alginate films in commercial food processing facilities.

3.4. Stability of phage LISTEX™ P100 in smoked trout fillets

The stabiliy of bacteriophages during storage at 10 °C for 7 days in smoked trout fillets contaminated with *L. monocytogenes* is shown in Figure 3.

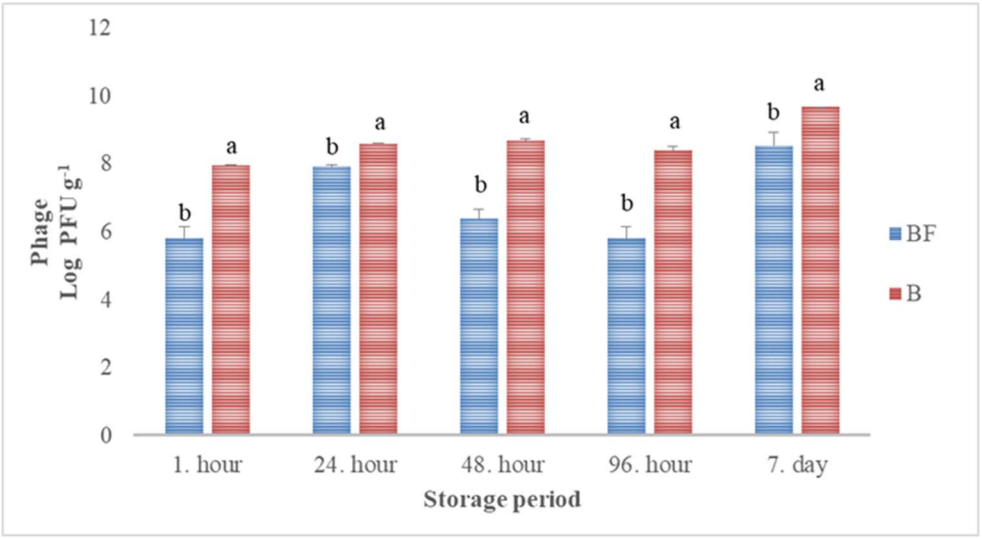

Figure 3- Stability of phage LISTEX™ P100 during the 7 days shelf life of smoked trout fillets stored at 10 °C (log PFU g^{-1})
BF: Film containing bacteriophage, B: Applying of bacteriophage directly to the surface *Different letters (a, b) represent statistical differences among groups in same storage period (P<0.05).

The incorporation of phages into the sodium alginate films resulted in a phage titer of 5.80 log PFU g^{-1} in trout fillets after 1 hour of storage at 10 °C, and the titer increased up to 8.54 log PFU g^{-1} at day 7 of storage (P<0.05). The phage titer was higher when the phage product was directed applied on the trout fillets. Indeed, 7.95 log PFU g^{-1} and 9.67 PFU g^{-1} were detected at the first hour and the day 7, respectively (P<0.05).

At the end of this study, the number of phages increased in the two groups in which bacteriophage was applied compared to the beginning of storage (P<0.05). This is the result of the natural phage cycle (which starts with one phage infecting one bacterial cell and resulting in 100–200 phages) (Moye et al. 2018). Unlike our findings, Soni & Nannapaneni (2010) reported that phage stability remained constant at 4 °C for 10 days and that the number of 10^8 log PFU g^{-1} bacteriophages initially added at the end of storage was about 8 log PFU g^{-1}. In this study, they have demonstrated that bacteriophage LISTEX™ P100 was able to reduce *L. monocytogenes* counts in a model broth system and on raw salmon fillet tissue.

4. Conclusions

Phage biocontrol is increasingly accepted as a natural and green technology, effective at specifically targeting bacterial pathogens in various foods, in order to safeguard the food chain. Because of the specificity of bacteriophages, phage biocontrol offers a unique opportunity to target pathogenic bacteria in foods without disturbing the normal microflora of foods (Moye et al. 2018). In this study, LISTEX™ P100 bacteriophage applications (incorporated into the sodium alginate based film and applied directly to the surface) were found to be effective in *L. monocytogenes* inactivation during 7 days storage. Also in this study, it was concluded that LISTEX™ P100 bacteriophage addition into sodium alginate film for the first time was be effective in control of *L. monocytogenes* in smoked trout fillets. Considering the results obtained, it may be concluded that the applications of LISTEX™ P100 bacteriophage could be very effective for the specific biocontrol of *L. monocytogenes* in smoked rainbow trout.

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