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INHIBITION OF LISTERIA *MONOCYTOGENES* ON RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) USING TROUT SKIN GELATIN EDIBLE FILMS CONTAINING NISIN

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A B S T R A C T

Contaminated food can pose high risk of listeriosis for susceptible populations. *Listeria monocytogenes* has been isolated from fresh, frozen and processed seafood products. Our goal in this study was to develop nisin-containing edible films using trout skin gelatin and test their effectiveness against *L. monocytogenes* on raw trout fillets (stored at 4 °C and 10 °C) as a strategy for reducing the risk of contamination. Films with 18 µg nisin/cm^2 showed consistent inhibition and were chosen for storage studies conducted at 4 and 10 °C for 30 days. Trout fillets were challenged with 2 log CFU *L. monocytogenes*/g before or after coating with nisin-containing films. Films with nisin reduced *L. monocytogenes* counts below the detection limit (0.3 log CFU/g) at 4 °C. At 10 °C, a 0.3 to 1.1 log reduction was observed compared to controls by the end of storage. The effectiveness of treatments depended upon the concentration of nisin and storage temperature. The developed edible films have the potential to reduce pathogens on seafood and can be incorporated with a variety of antimicrobials.

Keywords: *Listeria monocytogenes,* nisin, trout, antimicrobial packaging, gelatin.

INTRODUCTION

Listeriosis causes over 1600 cases annually in the USA with a projected average cost per case of $1 million for patient care and the associated costs of product recalls and market withdrawals (CDC 2011; Cagri *et al.,* 2002). An average of 132 cases of listeriosis is reported to occur annually in Canada (Public Health Agency of Canada, 2012). In 1999, 26 listeriosis cases with 7 deaths were reported in France due to contaminated pork tongue in jelly (WHO, 2013). Recent recalls have included seafood, salads, produce and dairy products. During a two month period between July and September 2012, *L. monocytogenes* was the cause of 30 food related recalls (FDA 2012). High risk population including pregnant women, infants, and people with compromised immune system are at greater risk of contracting listeriosis (Rocourt and Cossart, 1997). There is zero tolerance policy for this microorganism in ready-to-eat foods in the United States (FDA 2011). An FDA/USDA risk assessment included seafood in the list of foods at high risk of causing human listeriosis on a per annum basis (Datta *et al.,* 2008; Brett *et al.,* 1998; Ericsson *et al.,* 1997; Farber *et al.,* 2000). Up to 25% of frozen seafood, smoked salmon, and raw fish may be contaminated with *L. monocytogenes* (Norton *et al.,* 2000; Hoffman *et al.,* 2003) including rainbow trout (Rocourt and Cossart, 1997). In one study, 17% of cold-smoked and 50% of salt-cured rainbow trout tested were contaminated with *L. monocytogenes* (Johansson *et al.,* 1999). In a more recent study with fish roe products, 11.4% of cod roe (tarako) and 10% of salmon roe (ikura) were found to be contaminated with *L. monocytogenes* (Handa *et al.,* 2005). Presence of *L. monocytogenes* in seafood is often traced back to contamination in processing environments (Shin *et al.,* 2008; Vogel *et al.,* 2001; Rorvik *et al.,* 1995). Numerous factors contribute to the incidence of *L. monocytogenes* in seafood, including methods of processing, cleaning and sanitation in processing facilities and worker food handling practices. This indicates that improved sanitation controls resulting from the implementation of HACCP programs are critical and that greater efforts are needed to reduce risk. There has been a great deal of interest in methods

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To control foodborne pathogens using naturally occurring antimicrobial agents and minimizing the use of synthetic chemical preservatives. Approaches to control *L. monocytogenes* on seafood include the addition of lactic acid bacteria (LAB) cultures which are capable of producing bacteriocins with antilisterial activity (Nilsson *et al.,* 2004; Tome *et al.,* 2008), addition of organic acid salts (Neetoo *et al.,* 2008a) and bacteriocins (Al-Holy *et al.,* 2005; Szabo and Cahill 1999). Selected LAB produce several compounds including organic acids, hydrogen peroxide, diacetyl, bacteriocins, antibiotics, acetaldehydes, oxygen metabolites and carbon dioxide which result in the inhibition of *L. monocytogenes* (Montville and Winkowski, 1997). However, LAB can reduce the shelf life of products by resulting in higher volatile nitrogen concentrations (Tome *et al.,* 2006). Use of powdered bacteriocins is limited by their low solubility (Nilsson *et al.,* 2004). Organic acids impart undesirable sensory attributes to the product (Shin *et al.,* 2008). Since the approaches listed here have limitations, alternative control methods against *L. monocytogenes* are necessary. Control of several foodborne pathogens, including *L. monocytogenes,* with edible films containing antimicrobial compounds has been investigated. Different natural antimicrobials, either singly or in combination with others, can be incorporated into edible films to enhance their antimicrobial properties. Edible films can improve food safety by reducing the risk of microbiological contamination by prolonging antimicrobial activity on food surfaces. Edible films can modulate the diffusion of antimicrobial agents to the surface of food. Spraying of antimicrobial agents onto the surface of a food is usually less effective because of the rapid diffusion of the antimicrobial agent into the bulk of the food and the limited contact time with the food surface (Pranoto *et al.,* 2005). Growth of microorganisms can be prevented or minimized by antimicrobial edible films during storage of perishable seafood products. Min *et al.* (2005) showed that whey protein films incorporating a lactoperoxidase system could inhibit the growth of *L. monocytogenes* on the surface of smoked salmon and could preserve smoked salmon for 35 days at 4 °C and for 14 days at 10 °C. Similar antilisterial challenge studies were conducted on cold-smoked salmon by using alginate films and nisin-chitosan-coated plastic films (Neetoo *et al.,* 2008[a]; Neetoo *et al.,* 2008[b]; Ye *et al.,* 2008). Antilisterial activity of whey protein isolate films with nisin and glucose oxidase was demonstrated by Murillo-Martinez *et al.* (2013). Chitosan coatings with cinnamon oil were used to enhance the microbiological shelf life of rainbow trout with a one log reduction of psychrotrophs and a two log reduction in total viable counts (Ojagh *et al.,* 2010). Nisin and sodium lactate were shown to control *L. monocytogenes* on rainbow trout stored at 8 °C for 17 days achieving a 1.5 log reduction (Nykanen *et al.,* 2000). Growth of *L. monocytogenes* was reduced by 2 logs on cold-smoked salmon stored at 4 °C using potato peel waste-based films containing oregano oil (Tammineni *et al.,* 2013).

Nisin is the most popular bacteriocin and is produced by some strains of *Lactococcus lactis*. Since the 1950's, nisin has been used commercially in the UK and other countries, including the United States, where it was approved as a food additive in 1988. Nisin belongs to the Group I bacteriocins which are small peptides with sulfhydryl rings (Montville and Winowski, 1997). Nisin is used as a food preservative for processed cheeses, dairy desserts and canned foods. It has anti-bacterial activity against Gram-positive bacteria such as *Bacillus* spp., *Staphylococcus* spp. (Ray and Daeschel, 1994) and *Listeria* spp. (Brewer *et al.,* 2002, Schillinger *et al.,* 2001). Nisin can bind to negatively charged cell membranes creating pores and leakage of cell contents leading to cell death (Bruno *et al.,* 1992).

There are numerous studies reported on the use of edible films to increase shelf life of meat products. However, from the scope of literature review, such studies are limited with aquatic food products. The objective of this study was to investigate the effect of trout gelatin films containing natural antimicrobial nisin on the growth of *L. monocytogenes* inoculate onto the surface of trout fillets.

MATERIALS AND METHODS

Gelatin extraction: For extraction of gelatin from trout skins, a modified method of Chiou *et al.* (2006) was followed. Trout skins (Big Bend Trout Inc. Buhl, ID) were thawed and washed three times with ice-cold water (2 to 5 °C) and with ice-cold 0.8 N NaCl. The ratio of weight of skins to volume of wash solution in each step was 1:6. Skins were stirred for 40 min in ice-cold 0.2 N NaOH and washed with tap water. This step was repeated three times. After NaOH wash, skins were washed using ice-cold 0.2 N H H_2SO_4 and ice-cold 0.7% citric acid in a

similar manner as with the NaOH solution. After completion of all the washings, the skins were totally immersed in distilled water at 45 °C for overnight. The skins were removed from the solution and the solution was filtered using Whatman filter #4 and subsequently freeze-dried (Freeze Mobile 600L, Virtis Company, Gardiner, NY) to obtain gelatin.

Preparation of gelatin films containing nisin: Film formation protocol was adopted from Krishna *et al.* (2012). Gelatin solution (6.75% w/w) was stirred for 30 min at room temperature. The solution was denatured for 30 min in a 90 °C water bath and cooled to room temperature. Glycerol (Sigma, St. Louis, MO, USA) was added as a plasticizer (gelatin:glycerol ratio = 1:0.2). Nisin (MP Biomedicals, Solon, OH, USA), at different concentrations, was dissolved in sterile deionized water and added to the film-forming solution. The solution was degassed under vacuum until no visible air bubbles were present. The degassed solution was poured onto Teflon plates (16 cm internal diameter, 4 mm groove depth and 5 mm thickness) and left to dry for 24 h at 25 °C, 30% RH. To attain a uniform film thickness of 0.1 mm in all films, a constant dry solid content of 3 g was maintained. The area of each film was approximately 200 cm^2. The prepared films were stored in a 50% RH chamber at room temperature until further use.

Bacterial strains, growth conditions and enumeration: A cocktail of *L. monocytogenes* strains ATCC 19114, ATCC 7644 and ATCC 19113 was prepared. The strain 19114 was originally isolated from animal tissue and the strains 7644 and 19113 are human clinical isolates (ATCC, 2012) selected for their importance in foodborne outbreaks and adaptation to low temperature conditions. Each strain was grown to an A_{600} value of 0.8 to 1 at 37 °C in 10 mL Tryptic Soy Broth with 1% yeast extract (Becton Dickinson, Sparks, MD, USA). Equal volumes (10 mL each) of three cultures were combined and centrifuged (AccuSpin400 bench top centrifuge, Fisher Scientific, Pittsburgh, PA, USA) at 2,500 *g* for 30 min. The pellet was suspended in 30 mL of 0.2% buffered peptone water (Becton Dickinson, Sparks, MD, USA), vortexed and centrifuged again under same conditions. A washing step with 0.2% buffered peptone water was repeated three times to remove residual media. After the final wash, *L. monocytogenes* pellet was suspended in 0.2% buffered peptone water to prepare a cell suspension with approximately 5 log CFU/mL for inoculation in subsequent experiments. Tryptic Soy Agar with Yeast Extract (TSAYE; Becton Dickinson, Sparks, MD, USA) was used for determination of minimum inhibitory concentration (MIC) of nisin and for demonstrating the antilisterial activity of the films. For enumeration of *L. monocytogenes* during microbial challenge studies, Tryptic Soy Agar, PALCAM agar (Becton Dickinson, Sparks, MD, USA) and TSA-PALCAM overlay plates (Kang and Fung 1999) were used. Overlay plates were prepared with PALCAM agar on the bottom and TSA on the top. These plates were incubated for 24 h at 37 °C and enumerated.

Antilisterial activity of nisin on microbiological growth media: A stock solution of 1.5 mg nisin/mL (activity of 1.2 x 10^8 IU/g) in deionized water was prepared (Padgett *et al.,* 2000). The stock solution was diluted (1:1 to 1:40) to 0.75 to 0.03 mg nisin/mL. Agar-spot-on-lawn method was used to determine the sensitivity of strains towards nisin (Dimitrieva-Moats and Ünlü, 2011). Briefly, TSAYE spread plates were inoculated with 10^8-10^9 CFU/mL of *L. monocytogenes* to produce a lawn. These plates were spotted with 10 µL of the prepared nisin dilutions, incubated for 24 h at 37 °C and observed for zones of inhibition.

Antilisterial activity of gelatin films containing nisin on microbiological growth media: Gelatin films were prepared at 10 different concentrations of nisin (0, 0.125, 0.187, 0.25, 0.50, 0.75, 1.0, 1.25, 3.75, and 6.25 mg nisin/film). This translates to nisin concentrations of 0, 0.6, 0.9, 1.0, 2.0, 3.0, 5.0, 6.0, 18, and 31 µg/cm^2 of film. Disks with a diameter of 0.7 cm were made from the films using a cork borer. These film discs were placed on TSAYE plates with a lawn of *L. monocytogenes* (Zivanovic *et al.,* 2005) generated using both spread and pour-plating techniques. The plates were incubated for 24 h at 37°C and the diameter of the zone of inhibition was measured.

Microbiological challenge studies with trout: Fresh rainbow trout (*Oncorhynchus mykiss*) was purchased from a local grocery store in Pullman, WA, USA. Fillets were sliced into 25±0.5 g pieces, placed in sterile Petri plates and air dried in biological safety hood (recommended for BSL-2 practices) for 10 min. The sliced fillet samples were challenged with 1.85 to 2.03 log CFU *L. monocytogenes*/g fillet. The level of inoculation was determined based on preliminary experiments with fresh fish. An inoculum of 100 µL was spotted at 20-25 locations on the surface of fish fillet and spread with a sterile hockey stick. Samples were allowed to air dry for 30 min in a biological safety hood. Two modes of inoculations were used. Half of the samples

were inoculated first, dried and then covered with films containing 18 µg nisin/cm^2 (designated as inoculate and coat: I+C). The other half were coated with film (18 µg nisin/cm^2) first, inoculated and dried (designated as coat and inoculate: C+I). Petri plates containing inoculated fish were placed in UV-sterilized Ziploc® bags and were stored at 4 and 10 °C for up to one month. The 10 °C storage was selected to determine the effect of moderate temperature abuse. Preliminary studies (data not shown) indicate that 30 days (at 4 °C) was a week past the limit of shelf-life for this food product. Microbiological analysis was performed on the 0, 5, 10, 20 and 30th day. Untreated trout fillets and those covered with films (no nisin) served as controls.

Enumeration of *L. monocytogenes:* At each time interval, trout samples were placed in sterile stomacher bags (Fisher Scientific, Pittsburgh, PA, USA) with 225 mL of 0.2% buffered peptone water. Samples were homogenized using a stomacher (400 Circulator, Seward, London, UK) for 2 min at 230 RPM. After homogenization, 1 mL of aliquot from the sample was serially diluted with 9 mL of sterile buffered peptone water and plated on TSA (non-selective medium), PALCAM (selective medium) and TSA-PALCAM overlay plates. Colonies on plates were enumerated after incubation at 37 °C for 24 h.

Statistical analysis: Inhibition zone tests (n=3) were conducted with duplicate analyses per test. Microbial challenge experiments were performed in duplicate (n=2) with duplicate plating at each time point. For statistical analysis of microbial challenge studies, completely randomized design was used. Data were analyzed using one-way analysis of variance (ANOVA) procedure using Minitab (Minitab 14, Minitab Inc., PA, USA). When the effect was significant (P < 0.05), the mean separation was determined using Tukey's pair wise comparison. Tukey's pair-wise comparison with a family error of α = 0.05 was used to determine significance of the means.

RESULTS AND DISCUSSION

Nisin activity: When agar-spot-on-lawn technique was used, the lowest concentration of nisin stock that showed clear zone of inhibition was 0.04 mg/mL. Since higher nisin concentration would be needed to show antimicrobial effect due to diffusion limitations in food systems, films with a range of nisin concentration (0.6 to 31 µg nisin/cm^2) were prepared. The diameter of inhibition zones were not significantly different (P > 0.05) from each other over the range of 0.6 to 5 µg nisin/cm^2 (Table 1).

However, zones of inhibition were significantly different (P < 0.05) for films with 6, 18, and 31 µg nisin/cm^2 each of which showed a clear zone of inhibition with measurements of 0.88±0.1, 1.24±0.06 and 1.41±0.06 cm, respectively. Zones of inhibition were more obvious in the spread-plate technique than the pour-plate technique especially at lower concentrations of nisin (Fig. 1).

Table 1. Zones of inhibition on a lawn of *L. monocytogenes* in the presence of trout gelatin films with different concentrations of nisin.

	Amount of nisin in film (µg/cm^2)									
	0.0	0.6	0.9	1.0	2.0	3.0	5.0	6.0	18.0	31.0
Diameter of zone of inhibition (cm)	No zone	0.62 ± 0.12	0.63 ± 0.04	0.69 ± 0.08	0.69 ± 0.05	0.68 ± 0.04	0.71 ± 0.01	0.88 ± 0.01^a	1.24 ± 0.06^b	1.41 ± 0.06^c

Values represent means ± standard error of means (n=3). Means followed by different letters (a, b, c) are significantly different (P < 0.05) and means with no letters are not significantly different (P > 0.05). Means were calculated from 6 replicates from 3 independent experiments.

It is likely that the bacterial cell density was higher in pour plates compared to spread plates. Also, *L. monocytogenes* grew on and under the surface of agar in pour plates. The intermediate concentration of 18 µg nisin/cm^2 was used for subsequent experiments.

Microbiological challenge studies on trout

Growth of *L. monocytogenes* on inoculated-uncovered trout: *L. monocytogenes* was not detected in fresh trout as purchased. For the inoculated-uncovered trout control stored at 4 °C, counts of *L. monocytogenes* increased from 2±0.1 to 7.43±0.1 log CFU/g by day 30 (Fig. 2a).

At 10 °C, counts increased to 7.56±0.27 log CFU/g by day 20 and decreased to 6.05±0.1 CFU/g by day 30 of storage (Fig. 2b) possibly due to the presence of competitive microorganisms.

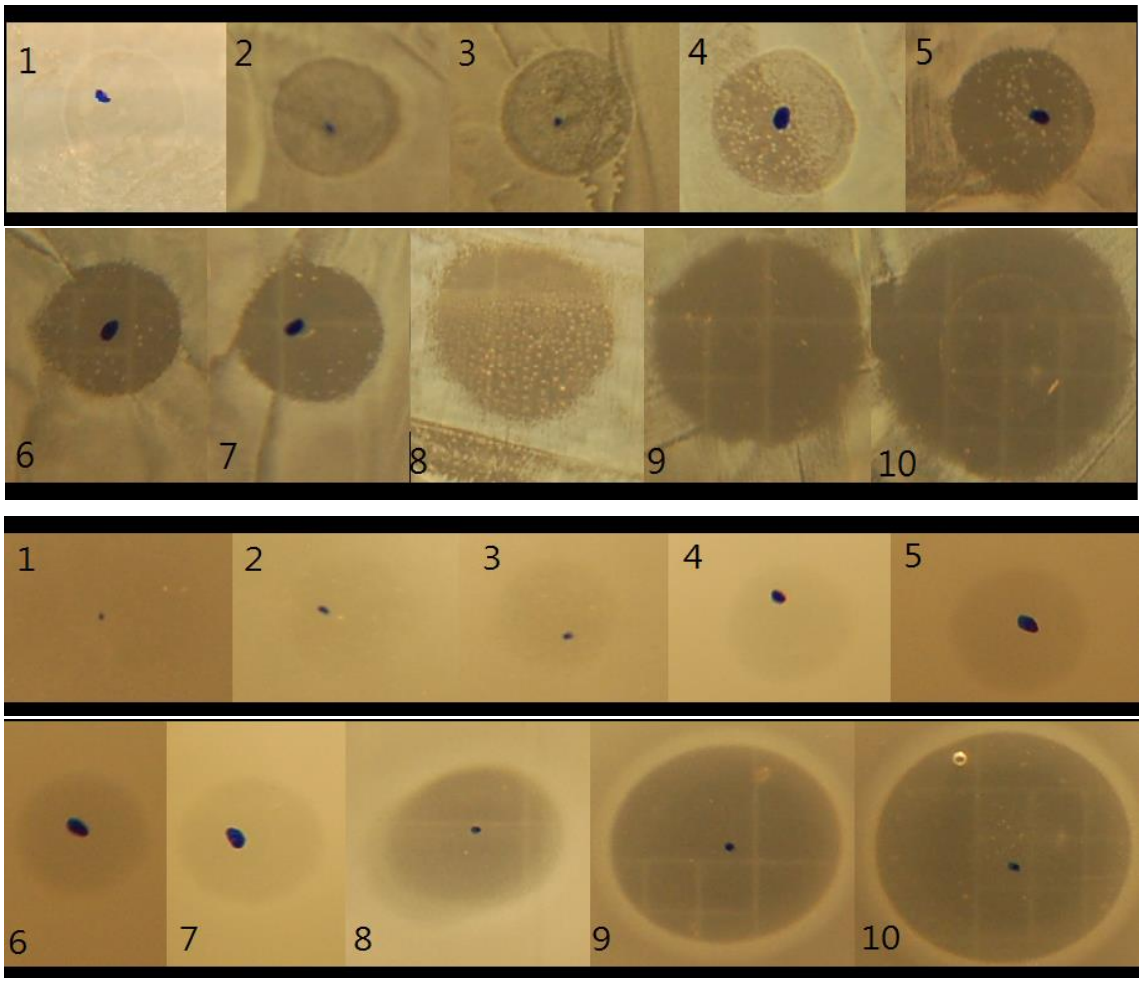

Fig 1: Zones of inhibition around trout gelatin film discs with different concentrations of nisin using spread plating (a) and pour plating technique (b) for plating L. monocytogenes cells (n=3). Numbers 1-10 correspond to nisin concentrations ranging from 0 to 31 µg nisin/cm^2.

Antilisterial activity of films with nisin: Growth of *L. monocytogenes* on trout covered with nisin-containing films was below the detection limit (< 0.3 log CFU/g) at 4 °C storage (Fig. 2a). On the I+C and C+I control films (no nisin), *L. monocytogenes* growth increased to 5.37±1.0 and 4.72±0.1 log CFU/g, respectively, by the end of storage time (Fig. 2a). This shows that films with nisin effectively inhibited growth of *L. monocytogenes* at 4°C.

Nisin-containing films inhibited growth of *L. monocytogenes* for five days at 10 °C with cell numbers being approx. 3 log CFU/g at day 10, 4 log CFU/g at day 20 and 5 log CFU/g by day 30 (Fig. 2b). Control films provided some reduction compared to uncovered trout by 1 to 2 log at each time point and by day 30 the cell numbers for I+C and C+I reached 6.04±0.8 and 5.3±0.25 log CFU/g, respectively. This might be due to the lack of access of bacterial cells to the surface of trout fillet because of the film. Cell numbers for nisin-containing and non-nisin-containing films were significantly different. These results indicate that temperature control during storage is critical and that antimicrobial treatments can be much less effective with a relatively small change in storage temperature. A storage temperature of 10 °C was chosen in our study because 20-25% home refrigerators in the US are between 7 to 10 °C (FDA, 2009). Trout held at 10 °C had a short shelf life and spoilage became apparent at around day 7 (data not shown).

In general, listeria counts were higher in I+C samples coated with control film (4 and 10 °C) and nisin film (4 °C) compared to C+I samples. With I+C mode of inoculation, bacterial cells would be in direct contact with the surface of trout fillet ensuring abundant nutrient availability. In addition, *L. monocytogenes* might have been protected from the antimicrobial effect due to the rough surface of fish fillet (Min *et al.,* 2005) reducing contact of nisin with bacterial cells. Data shown in Figure 2a, 2b are for recovery of *L. monocytogenes* on TSA-

PALCAM overlay plates. There was a trend for *L. monocytogenes* counts to be higher on TSA-PALCAM overlay plates compared to PALCAM plates at all-time points in this storage study. High cell recovery on overlay plates was reported compared to selective media (Kang and Fung, 1999). In addition, soluble matter (approx. 85%) of films might have affected the antimicrobial activity of films.

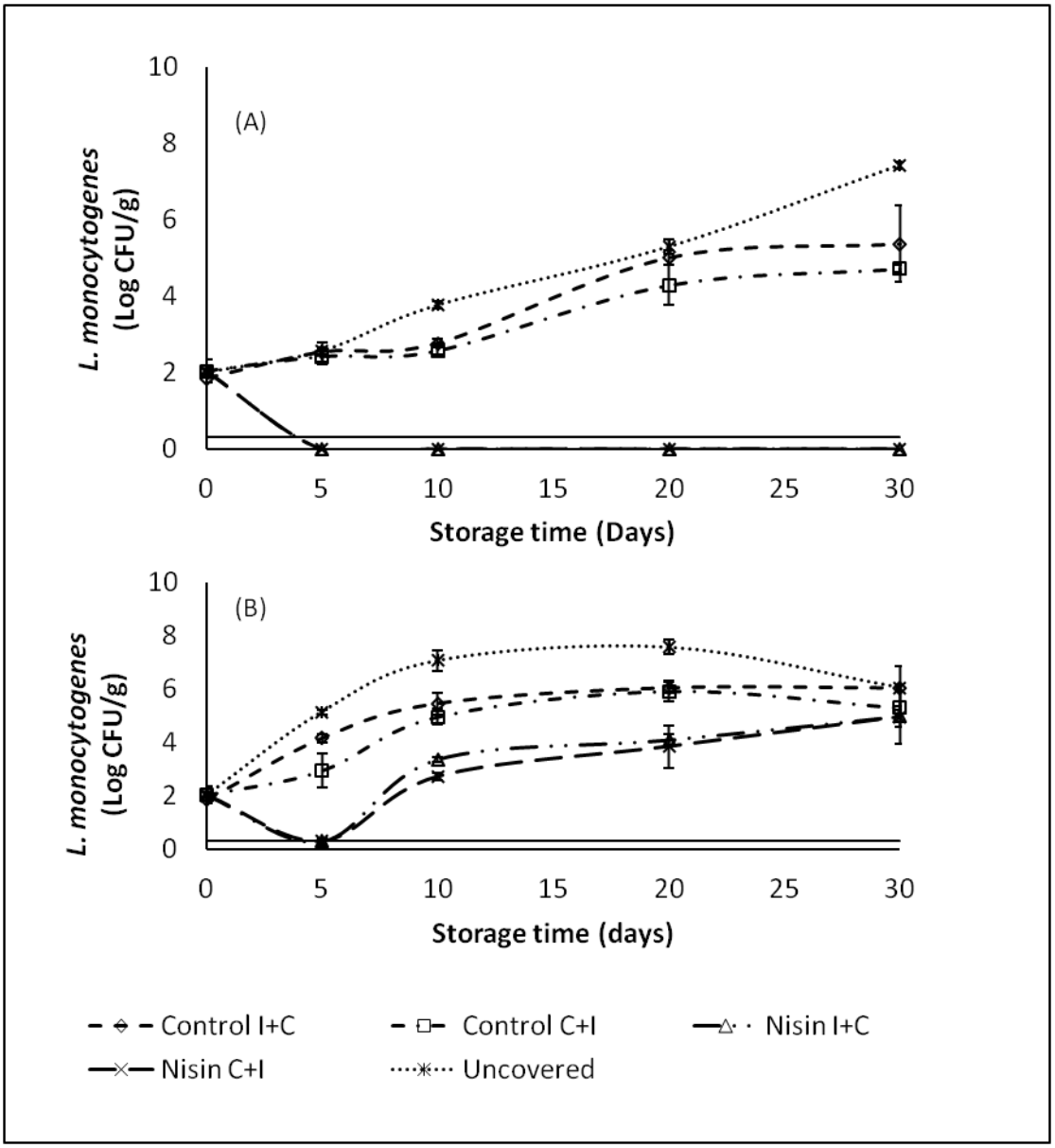

Fig 2. Counts of L. monocytogenes on trout fillets during storage at 4 °C (a) and 10 °C (b). Treatments include: uncovered, control film (no nisin) and nisin film (18 µg nisin/cm^2) covered samples. Values are represented by means ± standard error of means (n=2). Detection limit is 0.3 log CFU/g. The two modes of inoculation are denoted as C+I (Coat and Inoculate) and I+C (Inoculate and Coat).

Total aerobic plate counts: There was no significant difference (P > 0.05) in the number of total aerobic bacteria between treatments (Figure 3a, 3b). Counts were between 8 to 9 log CFU/g by the end of storage period at both temperatures. Fish spoilage under refrigeration conditions is caused by various microbiota, including LAB, *Photobacterium phosphoreum* (Budsburg*et al.,* 2003) and the members of the family *Enterobacteriaceae*. LAB can grow rapidly under refrigeration (Civera *et al.,* 1995; Leroi *et al.,* 1998; Hansen and Huss 1998) and certain LAB are tolerant to nisin (Montville and Winkowski, 1997). The LAB are likely among the spoilage microbes observed in this study (Shin *et al.,* 2008); Nisin itself is not effective

against Gram-negative bacteria. Off odors resulting from spoilage were detected prominently in samples stored at 10 °C beginning at day 7 indicative of the presence of organic acid production and proteolytic decomposition.

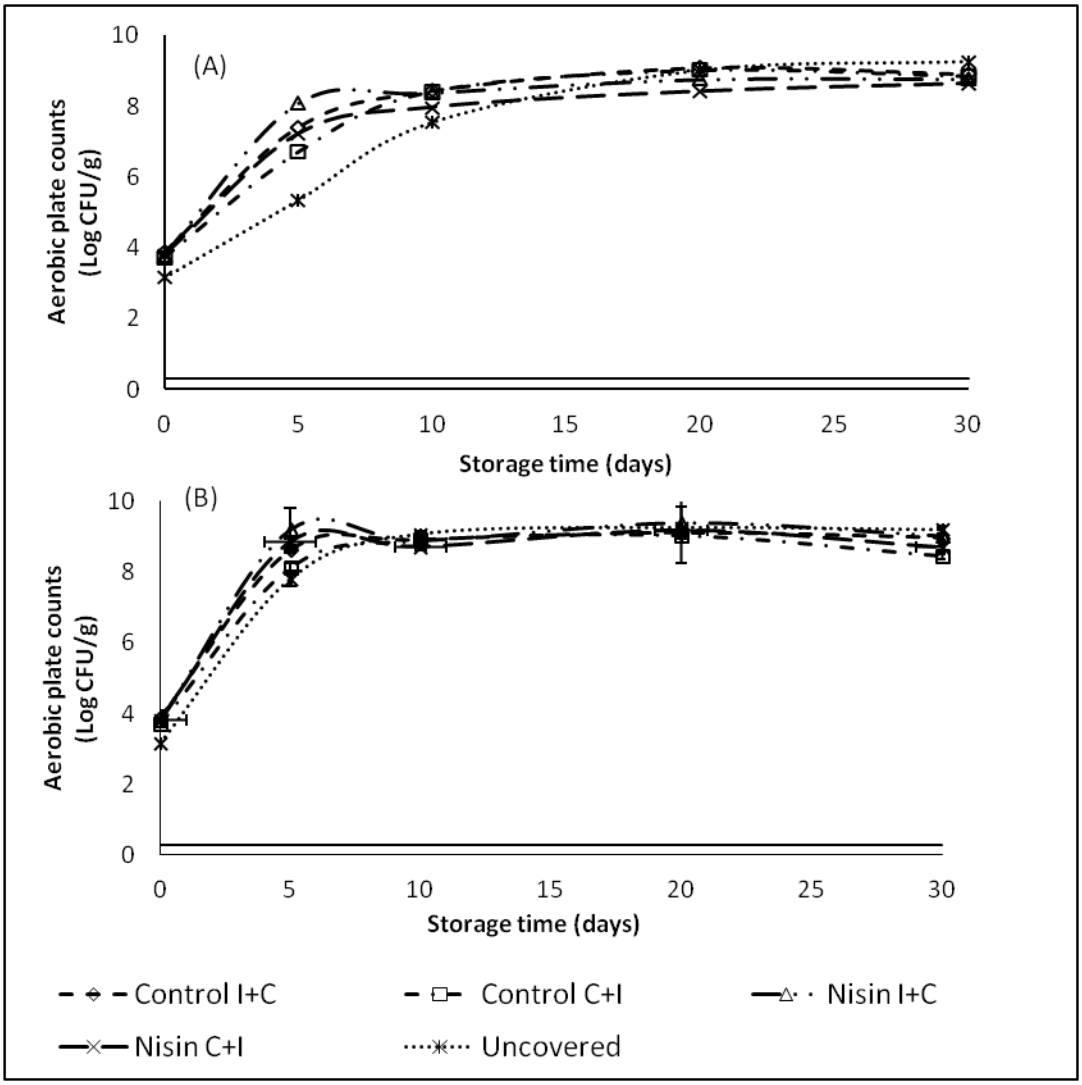

Fig 3. Aerobic plate counts on trout fillets during storage at 4 °C (a) and 10 °C (b). Treatments include: uncovered, control film (no nisin) and nisin film (18 µg nisin/cm^2) covered samples. Values are represented by means ± standard error of means (n=2). The two modes of inoculation are denoted as C+I (Coat and Inoculate) and I+C (Inoculate and Coat).

CONCLUSION

L. monocytogenes growth on trout fillets stored at 4 °C could be controlled by the application of trout gelatin films containing nisin (18 µg nisin/cm^2). The effectiveness of nisin-containing films depended on storage time, temperature and initial microbial load. Aerobic microbiota was not reduced with film treatment. Our future work will focus on improving the physical properties of fish gelatin films incorporated with nisin and other natural antimicrobials. Various microorganisms can be inhibited using edible films incorporated with different antimicrobials. However, attention must be paid to the interactions between antimicrobial agents as they may increase or decrease antimicrobial activity. Since food safety is a combination of several factors like temperature and water activity, use of antimicrobial edible films would be an additional hurdle to ensure food safety.

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