Thesis for the Degree of Master of Fisheries Science

Inhibition of bacterial growth (*Listeria monocytogenes & Staphylococcus aureus)* by Nisin Z, NaCl and their combination during fish storage at refrigerated temperature

by

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KOICA-PKNU International Graduate Program of Fisheries Science

Graduate School of Global Fisheries

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Nisin Z 와 NaCl 그리고 두 물질의 혼합에 의한 냉장 보관중인 어류에서 *Listeria monocytogenes* 와 *Staphylococcus aureus* 의 성장저해

Advisor: Prof. In-Soo Kong

by

Md Khalekuzzaman Sarker

A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of Fisheries Science

in KOICA-PKNU International Graduate Program of Fisheries Science,

Graduate School of Global Fisheries

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February 22, 2019

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Inhibition of bacterial growth (*Listeria monocytogenes & Staphylococcus aureus)* by Nisin Z, NaCl and their combination during fish storage at refrigerated temperature

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Abstract

To identify and quantify the effects of nisin Z and its combinations with different percentages of NaCl on the storage and shelflife of olive flounder (*Paralichthys olivaceus*) through antimicrobial activities against *Listeria monocytogenes* and *Staphylococcus aureus*. Nisin Z was isolated and purified from our laboratory identified *Lactococcus lactis* subsp. *Lactis* I2. The combinations of treatments are as follows: nisin Z, nisin Z + 3, 5, & 7% NaCl, and 0.02 M HCl (- control). In agar disk diffusion method, individual nisin Z and NaCl presented very little antimicrobial activities. However, antimicrobial effects of the combinations of two antimicrobials were

significantly higher ($P <$ 0.05) than control and individual components. Moreover, formation of clear zone by nisin Z with 3, 5, and 7% NaCl were significantly higher than control, whereas nisin Z + 7% NaCl demonstrated the maximum clear zone (9-11 mm) against these two foodborne pathogens. Minced olive flounder inoculated with log 5.57 and 4.94 CFU/g of *L. monocytogenes* and *S. aureus* respectively and treated with nisin Z + 7% NaCl reduced the bacterial load below log 3 and 2 respectively. After inoculating the bacteria in minced fish and treated with nisin Z and NaCl, the pH value of all treatments shows significantly different at the end of 12 days. But combined effect of nisin Z with 7% NaCl showed the pH value significantly lower ($P < 0.05$) compare to control and other treatments. The results revealed that these good combinations of nisin Z and NaCl play a vigorous antimicrobial role to increase the fish storage time in addition to shelflife of refrigerated fish products.

Keywords: nisin *Z*; NaCl; *Lactococcus lactis*; *Listeria monocytogenes*; *Staphylococcus aureus*.

Introduction

Fish and fisheries products can be easily contaminated with pathogenic microbes, if it is not properly harvested, washed, handled and stored. In modern era people prefer fish and fisheries products rather than meat, because doctors suggests that fish is better than meat to keep sound health. As a result demands are mounting on quality and safety assurance from harvesting, processing, and shelf-life extension during storage to the consumer hands. Microbial load is one of the main causes for quality deterioration and reducing the shelf-life of fish and fisheries products. In order to extend the shelf-life of these products during storage, normally different types of synthetic/chemical food preservatives are incorporated as antimicrobial agents. However, increasing demands of natural food preservatives and residual effects of generally used additives has raised question to the consumer's health safety (Bono et al., 2012; Larsson et al., 2006). Now a days to extend the shelf-life of fish and fisheries products during storage with incorporation of natural preservatives draw attention to the scientific community as well (Bazargani-Gilani et al., 2015; Economou et al., 2009).

In modern world maximum people of developed countries have changed feeding behaviours through consuming ready food because of shortage in time for cooking (CDC,

2010; Carpentier & Cerf, 2011). Throughout the world food borne diseases are detected as most public health concerning issue and serious health risk to consumers (Khan et al., 2015). It is possible to get food borne sickness when chilled dairy products like milk, raw meat, fish and vegetables, and ready-to-eat food are directly consumed without cooking (White, et al., 2002; Gandhi & Chikindas, 2007; Liu, 2008). During long-term refrigerated storage the psychrophilic pathogen like *Listeria monocytogenes* and *Staphylococcus aureus* can be grown at a high level (Rocourt et al., 2003; Gimenez and Dalgaard, 2004; Neetoo et al., 2008) and responsible to cause listeriosis (McLauchlin, 1996). These food borne pathogens cause severe problems in human like stomach pain, vomiting, meningitis and abortion (Gandhi & Chikindas, 2007). Food items can also be contaminated by infected cuts, surface contact, wrong handling and sanitation. In addition, wide range of optimum temperature these bacteria able to survive and multiply very quickly to produce toxin to cause diseases in normal temperature. To save the food industry and protection of health threats by food borne pathogens must be controlled with natural substances (Magalhaes et al., 2013).

The antimicrobial effect of nisin Z and sodium chloride (NaCl) are well recognized and both possess no detrimental or residual effects on human health. Nisin Z possessed antimicrobial peptides and generally synthesized by *Lactococcus lactis* subsp. *lactis* strains and it has wide applications in the food industry. To inhibit the growth and multiplication of gram-positive spoilage and food borne pathogenic bacteria, nisin is reported and

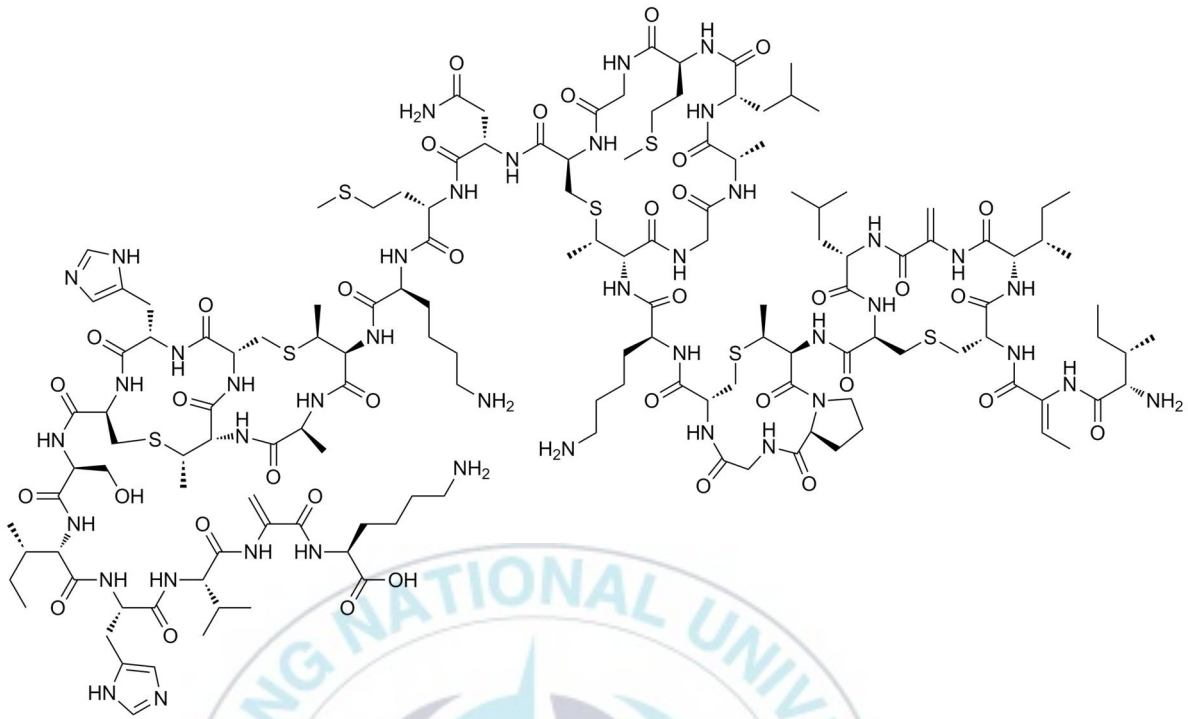

Fig.1. Chemical structure of nisin

recognized natural antimicrobial substances in various food products like milk, fish, meat, processed foods and especially ready foods (Neetoo et al., 2008). NaCl is commonly used as food preservative and a huge amounts of NaCl are used in many processing industries. The mode of action of NaCl are dehydration whereas pathogenic bacteria need moisture condition for their growth and multiplications. The bacterial growth depend the availability of unbound water, the amount of unbound water of a food indicates the susceptibility of bacterial growth. Sodium and chloride ions with water molecules disrupt the water utilization activity by the microbial population (Fennema, 1996; Potter and Hotchkiss, 1995). When salted food comes to contact with the bacteria draw out the moisture and cause the killing of bacteria which saves the food from spoilage.

Recently combination of two or more antimicrobial agents are frequently used to store the food items (Schlyter et al., 1993). Combining antimicrobial agents enhanced antimicrobial action of specific compound against pathogenic or spoilage bacteria. Two or more antimicrobial agents showed broader spectrum activity against the pathogenic bacteria than the individual compounds (Schlyter et al., 1993; Neetoo, et al., 2008). Many researchers have shown that nisin have antimicrobial activity against food borne pathogenic bacteria. Also told that combination of nisin and another compound such as several plant compounds, different essential oils etc. have stronger antimicrobial activity (Hsieh et at., 2011; Solomakos et al., 2008; Turgis et al., 2012; Yoon et al., 2011). Previously combination of nisin and organic acids or salts (Samelis, et al., 2005), nisin, lysozyme, EDTA nanoparticles,

and/or ZnO nanoparticles (Morsy, et al., 2018), nisin and trypsin (Pan, et al., 2018), sodium alginate and nisin (Raeisi, et al., 2016), nisin, reuterin, and the lactoperoxidase (Arqués, et al., 2008), nisin and plant essential oils (Abdollahzadeh, et al., 2014), nisin and thymol (Ettayebi, et al., 2000) were studied for fish or food product storage and showed significant reduction in pathogenic bacterial load. However, combination of nisin Z and NaCl has not yet studied against *S. aureus and L. monocytogenes* in minced fish during storage.

The aim of this study was to evaluate the ability of purified nisin Z, NaCl alone or in combination with different concentration levels to inhibit gram-positive pathogens *L. monocytogenes* and *S. aureus* in minced fish during refrigerated storage. The second phase was to identify and quantify the freshness of minced fish through different biochemical tests after adding biological preservatives.

Materials and Methods

2. Materials and Methods

2.1 Bacterial strains

Listeria monocytogenes and *Staphylococcus aureus* stock was collected from our laboratory. These stocks were preserved at -70 °C. Both strains were cultured through 12 hr at 37 °C on Brain Heart Infusion (BHI) broth and agar media. To get the optical density (OD) approximately 1.0/ml the broths were measured. These bacteria were used as indicator to check the antimicrobial activity of the bacteriocin. To avoid the contamination these stock was prepared continuously. The stock was prepared in BHI broth containing bacteria with 50% glycerol or Dimethyl sulfoxide (DMSO).

2.2 Nisin purification

Lactococcus lactis subsp. *lactis* stock was collected from our laboratory and cultured through 16 hr at 37 °C in De Man, Rogosa and Sharpe (MRS) broth for producing bacteriocin. Then 1% cell culture in MRS broth at 37 °C and kept overnight. The culture was centrifuged at 10000 rpm for 10 min at 4 °C to harvest the cell-free-supernatant. The

supernatant was collected and used 0.45 µm syringe filter for filtration. Added 10% PEG 4000 and 15% Na_2SO_4 in the supernatant and stirring 2 hrs. After mixing well it kept normal condition in 15 min for two clear phases. Samples were carefully withdrawn from each phase for removal of Na_2SO_4 layer. Added 2 volumes of 100% EtOH and kept overnight at 4 °C. Then the sample was centrifuged at 7000 rpm for 10 min at 4 °C and supernatant discarded. Added 1 volume of 70% EtOH and centrifuged at 7000 rpm for 10 min at 4 °C and supernatant discarded. Finally, the samples were vacuum dry at 42 °C. The partially purified nisin Z was stored at 4 °C before used.

2.3 NaCl solutions

NaCl was purchased from a local market and prepared in 3, 5, and 7% NaCl solutions. All solutions were prepared with sterilized water. This NaCl solution was kept in 4 °C storage.

2.4 Fish sample preparation

Olive flounder, *Paralichthys olivaceus* fish flesh (boneless) were bought from mega mart in Busan. The fish meat was minced by using blender (Shinil Multi mixer SMX-757CM, Seoul, Republic of Korea). The minced fish were weight 25 gm in each pack and kept into zipper system bag. To avoid contamination the samples stored in -20 ^{0}C.

2.5 pH measurement

The samples were prepared for pH measurement. One gm minced fish sample was mixed with 10 ml distilled water and homogenized thoroughly. Then the treatments were prepared- two bacteria, nisin-Z, different percentage of NaCl and also control. The pH was measured by digital pH meter (Mettler-Toledo 51343101, MP 220, InLab Surface pH electrode) in all samples for 12 days after one day interval.

2.6 Fish sample treatment preparation

Minced fish samples were inoculated with 5.57 and 4.94 log (CFU/g) of *L. monocytogens* and *S. aureus* respectively. Then 3, 5, and 7% NaCl with purified nisin Z at 1000 IU and their combinations were used per 25 gm fish. These treated samples were kept in 4 °C ± 1 °C for 12 days and observed CFU/g everyday.

2.7 Enumeration of bacteria

For microbial analysis, 2 gm of minced fish were homogenized with 18 ml phosphate buffer. Serial dilutions were made and counts of *L. monocytogenes* and *S. aureus* were determined by spreading 0.1 ml of serial dilutions on BHI agar plate. Plates were incubated at 37 °C for 24 hrs. The number of *L. monocytogenes* and *S. aureus* per gm of fish meat was reported as log CFU/g (Hegde, et. al., 2007).

2.8 Antimicrobial assay: Agar disk-diffusion method

The antimicrobial activity of the NaCl and nisin Z was tested against *L. monocytogenes* and *S. aureus* using the agar disk-diffusion technique. This procedure the bacteria were cultured in BHI broth and by spreading the agar plates when the OD value in approximately 1.0/ml. Then different concentration of NaCl, nisin Z and their combined solutions were given on agar plate in the desired amount and kept into 37 °C. To determine the antimicrobial activity, inhibits of germination and growth of the bacteria were observed. Then the diameters of clear zone were measured in mm and recorded for analysis. Experiments were carried out in triplicate.

2.9 Time-kill test (time-kill curve)

This method was the most appropriate for determining the bactericidal effect. This test was performed in broth culture medium using four tubes containing a bacterial suspension of optical density approximately 1.0/ml. The first, second and third tubes contain 3, 5, and 7% NaCl with nisin Z and fourth tube contain only nisin Z. Then the tubes kept in 37 °C and recorded the OD value after two hrs interval. The incubation is done under suitable conditions for varied time intervals (0, 2, 4, 6, 8, 10 and 12 hrs). After observing and

recorded the OD value of that solution the percentage of dead cells were calculated by the agar plate counting methods.

2.10 Statistical analysis

Data will be analysed of variance for means of comparison and significant differences will be calculated according to Duncan's test. Data will be reported as means ± standard deviation of the means. Differences at $P < 0.05$ will be considered statistically significant. SPSS Version 20 software will be used to perform the statistical analysis.

Results

3. Results

3.1 Purification of nisin Z

To determine the antimicrobial activity and used for fish and fisheries products storage or to increase the shelf-life of fisheries products commercial nisin was available. Nisin have more powerful activity against gram positive bacteria and performs two killing mechanisms. Firstly nisin inhibits cell wall synthesis and secondly nisin permeabilized the target membrane. The bindings of nisin and NaCl induced membrane integration of nisin resulting the formation of a pore. The highly specific interaction with these combination reflected to the concentrations of nisin that have sufficient ability to permeabilize the membrane of the target cells. This experiment nisin Z was produced from *Lactococcus lactis* subsp. *lactis* and purified this procedure.

Partially Purified Bacteriocin (PPB) from Lab

↓

I2 culture at 37 °C, for 16 hrs

↓

Cell down (6000~12000 rpm, 10 min)

↓

Pellet discard

↓

0.2 μm syringe filtration (750 cell free supernatant+100g PEG, 150g Na_2SO_4)

↓

10% PEG, 15% Na_2SO_4 Precipitated (2 hrs stirring)

↓

Removal of Na_2SO_4 layer

↓

2 volume EtOH add

↓

Centrifuge (6000 rpm, 10 min)

↓

Supernatant discard

↓

70% EtOH 1 volume

↓

Centrifuge (6000 rpm, 10 min)

↓

Supernatent discard

↓

Dry 42 °C

Fig.2. Schematic diagram of nisin Z production and purification

3.2 Antimicrobial activity of nisin Z and NaCl

The antimicrobial effect of nisin Z at 1000 IU combinations with 3, 5, and 7% NaCl against *L. monocytogenes* and *S. aureus* by agar disk-diffusion and time kill test method were shown in Fig-3, 4 and Table-1, 2 respectively. At 4 °C of *L. monocytogenes* and *S. aureus* the clear zone of agar disk diffusion method was increased in combined treatment groups compare to only nisin Z and control group (Fig-3, 4). Moreover, clear zone formation by nisin Z with 3, 5, and 7% NaCl were significantly higher than control, whereas nisin Z + 7% NaCl demonstrated the maximum clear zone that was 9 and 11 mm against two foodborne pathogens *L. monocytogenes* and *S. aureus* respectively. However the treatment of only nisin Z presented the clear zone around 4 mm of these two food borne pathogens.

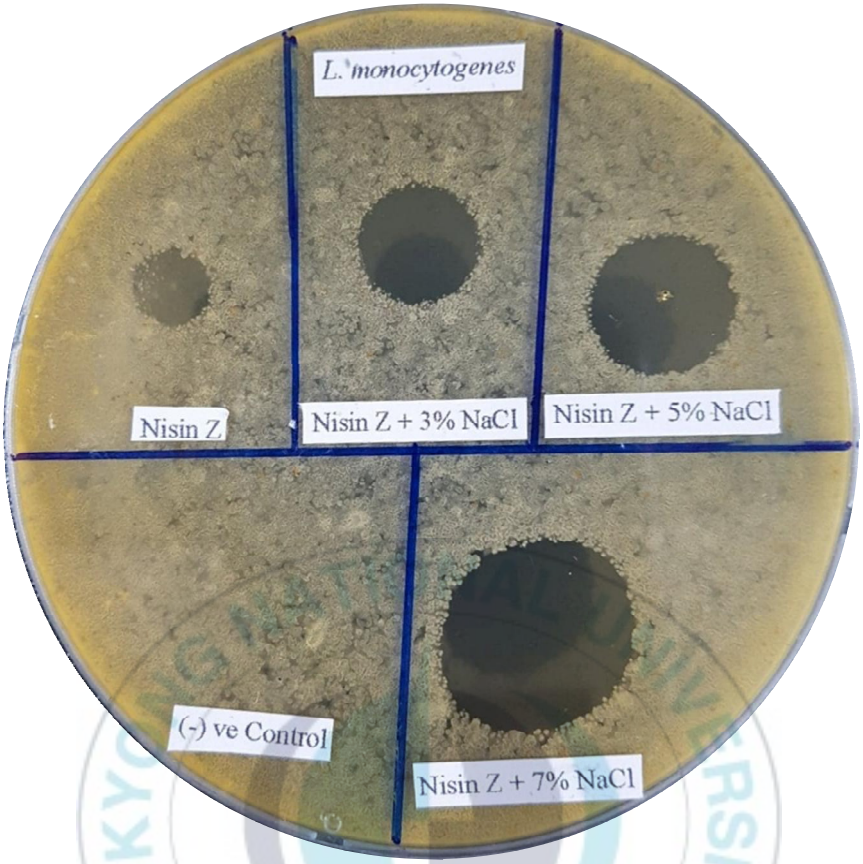

Fig.3. Agar disk diffusion method: Clear zone of nisin Z, NaCl (3, 5, and 7%) and their combination effect on *L. monocytogenes*

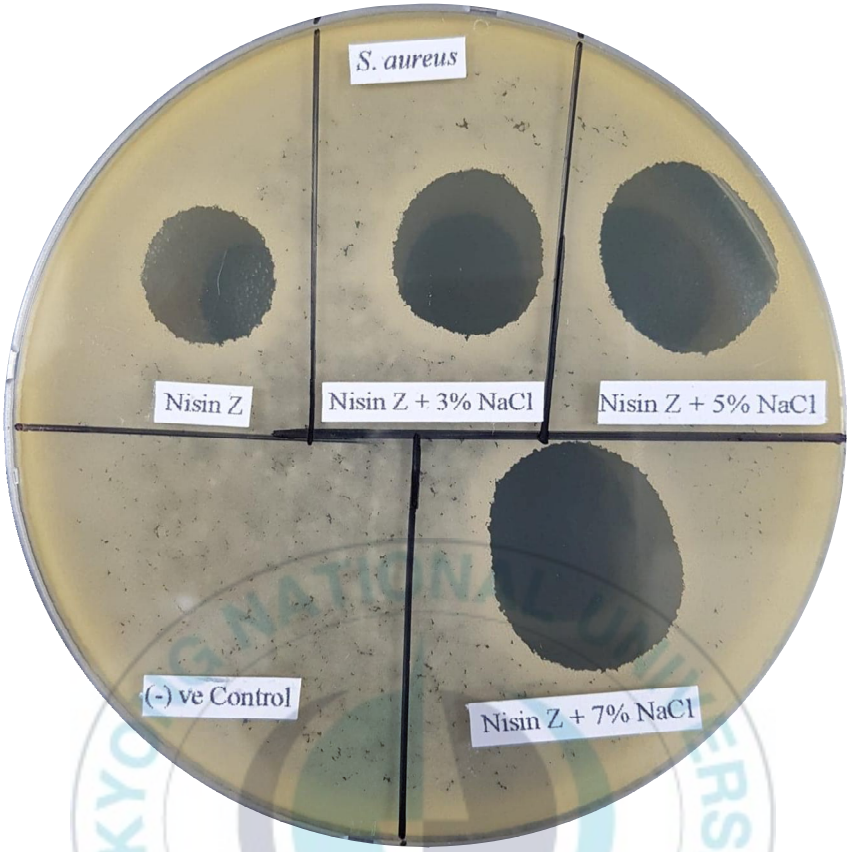

Fig.4. Agar disk diffusion method: Clear zone of nisin Z, NaCl (3, 5, and 7%) and their combination effect on *S. aureus*

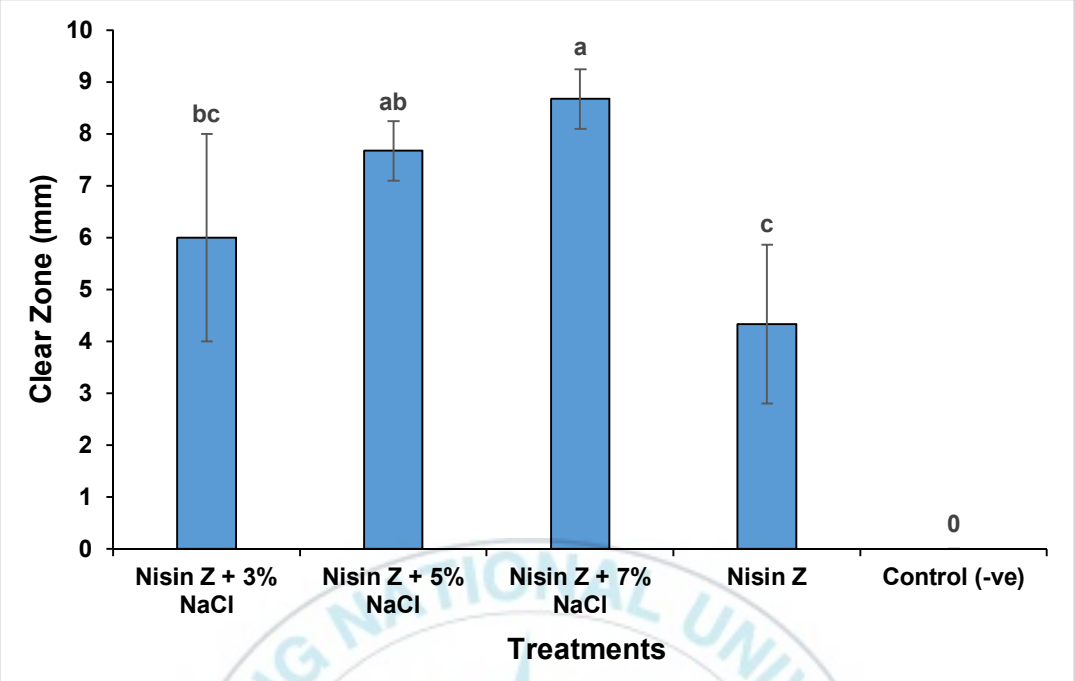

Fig.5. Clear zone of nisin Z, NaCl (3, 5, and 7%) and their combination effect on *L. monocytogenes*. Vertical bars represents the SD of means

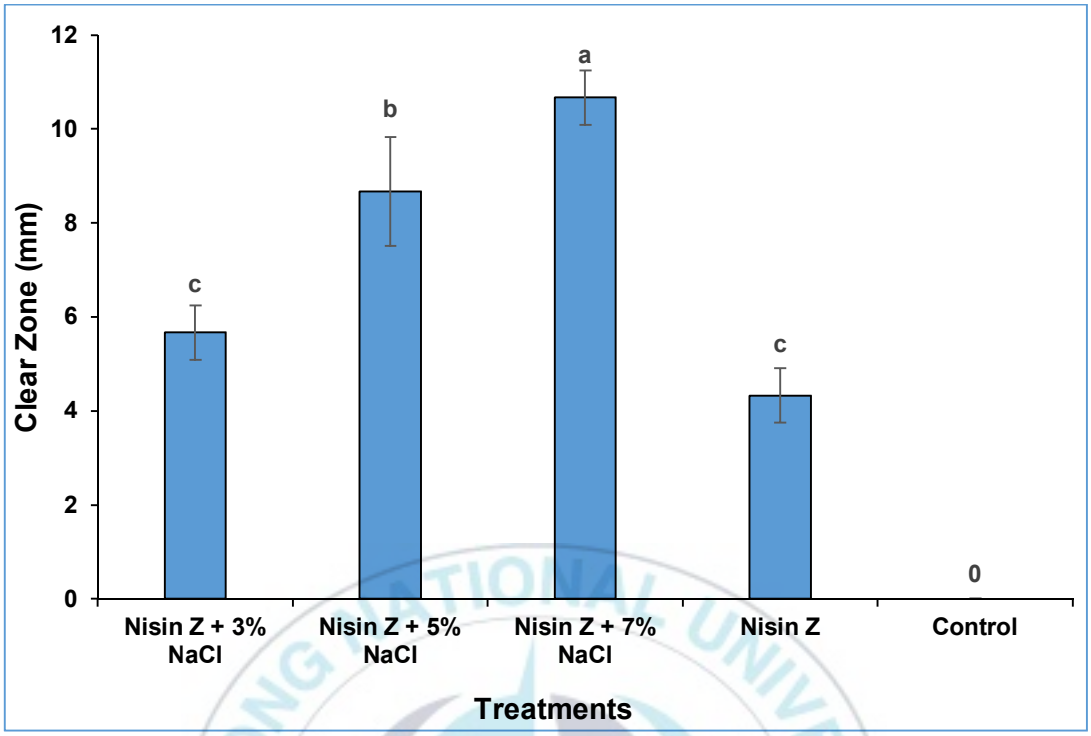

Fig.6. Clear zone of nisin Z, NaCl (3, 5, and 7%) and their combination effect on *S. aureus*. Vertical bars represents the SD of means

Results of inhibitory activity of nisin Z (1000 IU) with combination of 3, 5, and 7% NaCl against *L. monocytogenes* were displayed in Table-1. The initial bacterial load was 6.46 log (CFU/g). Nisin Z and combination of NaCl were shown in significantly ($P < 0.05$) different activity compare to control after 6 hrs. The result of only nisin Z and combination 3% NaCl did not show any significant ($P > 0.05$) activity at the end of 6 hrs. The combination of 3 and 5% NaCl with nisin Z were shown the significantly different ($P < 0.05$) activity at the end of 6 hrs but they did not show any significantly different activity compare to each other at the end of 10 hrs and the value was around 4.7 and 4.5 log (CFU/g) respectively. But the combination of nisin Z and 7% NaCl shown the outstanding activity and the bacterial load was 3.5 log (CFU/g) whereas the control was near about 7 log (CFU/g) at the end of 12 hrs.

The inhibitory activity of nisin Z (1000 IU) and combination of 3, 5, and 7% NaCl against *S. aureus* were presented in Table-2. The initial bacterial load was 7.63 log CFU/g. Nisin Z and combination of NaCl shows the significantly ($P < 0.05$) different activity compare to control after 2 hrs. The result of only nisin Z and combination of 3% NaCl did not show any significant ($P > 0.05$) activity at the end of 8 hrs. After 4 hrs the combination of 3 and 5% NaCl with nisin Z were shown significantly different ($P < 0.05$) activity and at the end of 12 hrs the value was around 4.5 and 4 log (CFU/g) respectively. But the combination of nisin Z and 7% NaCl were exposed the higher activity and the bacterial load was around 4 log (CFU/g) whereas the control was near about 8 log (CFU/g) at the end of 12 hrs. So the

4 °C storage temperature 7% NaCl and nisin Z was presented the amazing result compare to other treatments.

The antimicrobial effect of NaCl and nisin Z against *L. monocytogenes* during storage on minced olive flounder at 4 °C for 12 days were shown in Fig-7. The minced fish inoculated with bacteria 5.57 log (CFU/g) and treated to nisin Z, NaCl and their combination. Nisin Z with 7% NaCl were shown significantly ($P < 0.05$) lower bacterial load compare to control and other treatments. Whereas all the treatments shown the significantly ($P < 0.05$) decrease the bacterial load compare to control. At the end of 12 days the combination of nisin Z with 3 and 5% NaCl were shown more significantly ($P < 0.05$) decrease the bacterial load and the value was around 5.5 and 4 log (CFU/g) respectively. Although only nisin Z and combination with 3% NaCl were shown the significant activity and the value was 5.6 and 5.5 log (CFU/g) but they did not show any significantly different result compare to each other upto 12 days storage of the minced fish.

Table 1. Effects on combination of nisin Z (1000 IU) and NaCl (3, 5 & 7%) on *L. monocytogenes* at 4 °C for 12 hrs[1]

Treatment	Storage time (hr)						
	0	2	4	6	8	10	12
Control	6.46 ± 0.00	6.53 ± 0.12^{a}	6.58 ± 0.22^{a}	6.66 ± 0.13^{a}	6.72 ± 0.14^{a}	6.8 ± 0.15^{a}	6.82 ± 0.09^{a}
Nisin Z	6.46 ± 0.00	6.24 ± 0.11^{abc}	6.24 ± 0.09^{ab}	6.25 ± 0.24^{b}	6.08 ± 0.13^{b}	5.77 ± 0.10^{b}	5.74 ± 0.11^{b}
Nisin Z + 3% NaCl	6.46 ± 0.00	6.12 ± 0.16^{abc}	6.12 ± 0.20^{abc}	6.05 ± 0.29^{bc}	5.10 ± 0.40^{c}	4.78 ± 0.27^{c}	4.74 ± 0.23^{c}
Nisin Z + 5% NaCl	6.46 ± 0.00	5.97 ± 0.24^{bc}	5.93 ± 0.40^{bc}	5.80 ± 0.13^{cd}	4.85 ± 0.22^{cd}	4.51 ± 0.24^{c}	4.17 ± 0.23^{cd}
Nisin Z + 7% NaCl	6.46 ± 0.00	5.81 ± 0.34^{c}	5.70 ± 0.42^{bc}	5.46 ± 0.18^{de}	4.49 ± 0.23^{d}	3.84 ± 0.28^{d}	3.58 ± 0.63^{e}

[1]Values are mean (log CFU/g) ± SD of three replicates. Values with different superscript letters within the same column in the table are significantly different ($P < 0.05$). The same superscript letters in same column indicates no significant differences ($P > 0.05$).

Table 2. Effects on combination of nisin Z (1000 IU) and NaCl (3, 5 & 7%) on *S. aureus* at 4 °C for 12 hrs[1]

Treatment	Storage time (hr)						
	0	2	4	6	8	10	12
Control	7.63 ± 0.00	7.73 ± 0.07^{a}	7.80 ± 0.08a	7.86 ± 0.09^{a}	7.79 ± 0.07^{a}	7.88 ± 0.08^{a}	7.90 ± 0.08^{a}
Nisin Z	7.63 ± 0.00	7.01 ± 0.23^{b}	7.80 ± 0.08^{a}	6.11 ± 0.22^{c}	5.97 ± 0.19^{b}	5.71 ± 0.15^{b}	5.02 ± 0.15^{b}
Nisin Z + 3% NaCl	7.63 ± 0.00	7.09 ± 0.17^{b}	7.0 ± 0.17^{b}	6.10 ± 0.14^{c}	5.93 ± 0.11^{b}	5.14 ± 0.53^{c}	4.52 ± 0.13^{c}
Nisin Z + 5% NaCl	7.63 ± 0.00	6.98 ± 0.17^{b}	6.65 ± 0.28^{bc}	5.83 ± 0.13^{d}	5.67 ± 0.07^{c}	4.78 ± 0.33^{cd}	4.28 ± 0.18^{cd}
Nisin Z + 7% NaCl	7.63 ± 0.00	6.96 ± 0.20^{b}	6.32 ± 0.39^{cd}	5.76 ± 0.09^{d}	5.51 ± 0.15^{cd}	4.60 ± 0.12d	4.14 ± 0.14^{d}

[1]Values are mean (log CFU/g) ± SD of three replicates. Values with different superscript letters within the same column in the table are significantly different ($P < 0.05$). The same superscript letters in same column indicates no significant differences ($P > 0.05$).

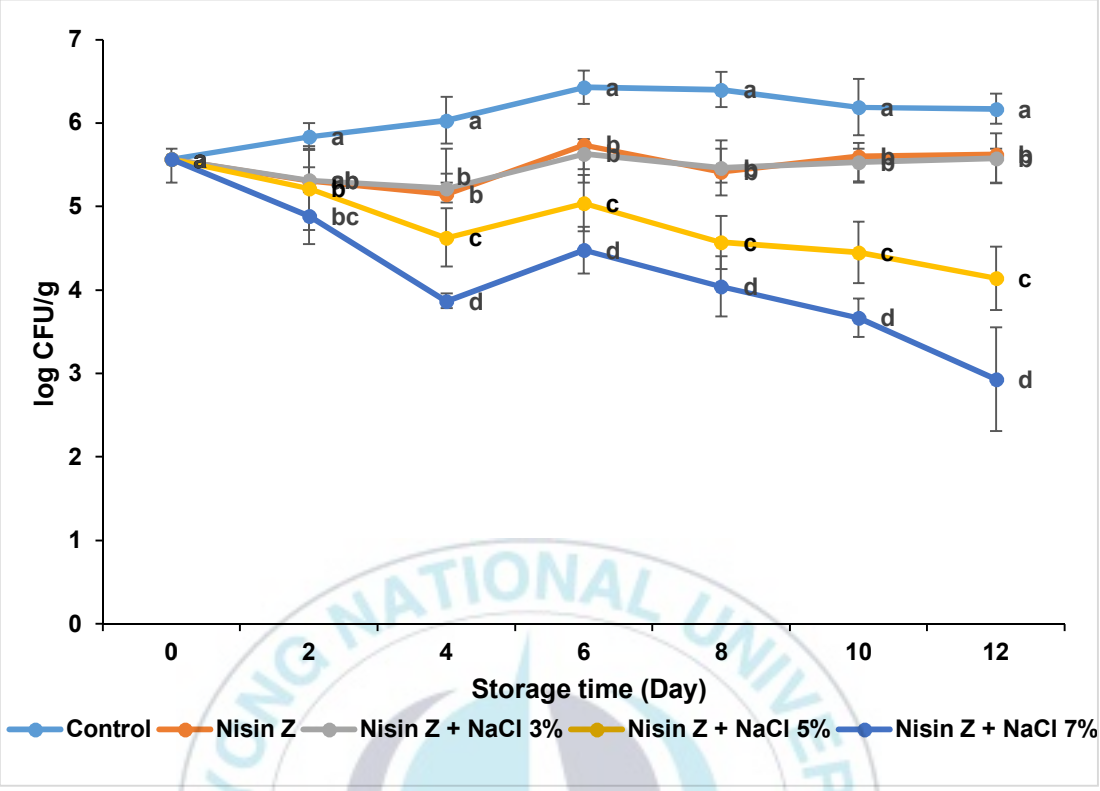

Fig.7. Effects of nisin Z, NaCl and their combination on *L. monocytogenes* in minced olive flounder during storage at 4 °C for 12 days. Vertical bars represents the SD of means

The antimicrobial effect of NaCl and nisin Z against *S. aureus* during storage on minced olive flounder at 4 °C for 12 days were displayed in Fig-8. The minced fish inoculated with bacteria 4.94 log (CFU/g) and treated to nisin Z, NaCl and their combination. Nisin Z with 7% NaCl were exhibited significantly ($P < 0.05$) lower bacterial load compare to control and other treatments and the value was 1.8 log (CFU/g) at the end of 12 days. Whereas all the treatments shown significantly ($P < 0.05$) decrease the bacterial load compare to control. At day 4 and 6 there were no significantly difference of the treatments of nisin Z with 3 and 5% NaCl in each other but these treatments were shown significantly different ($P < 0.05$) after 8 days. At the end of 12 days the combination of nisin Z with 3 and 5% NaCl were shown more significantly ($P < 0.05$) decrease the bacterial load and the value was 4.2 and 2.5 log (CFU/g) respectively. But only nisin Z and combination with 3% NaCl did not show any significant result upto 10 days storage of the minced fish compare to each other.

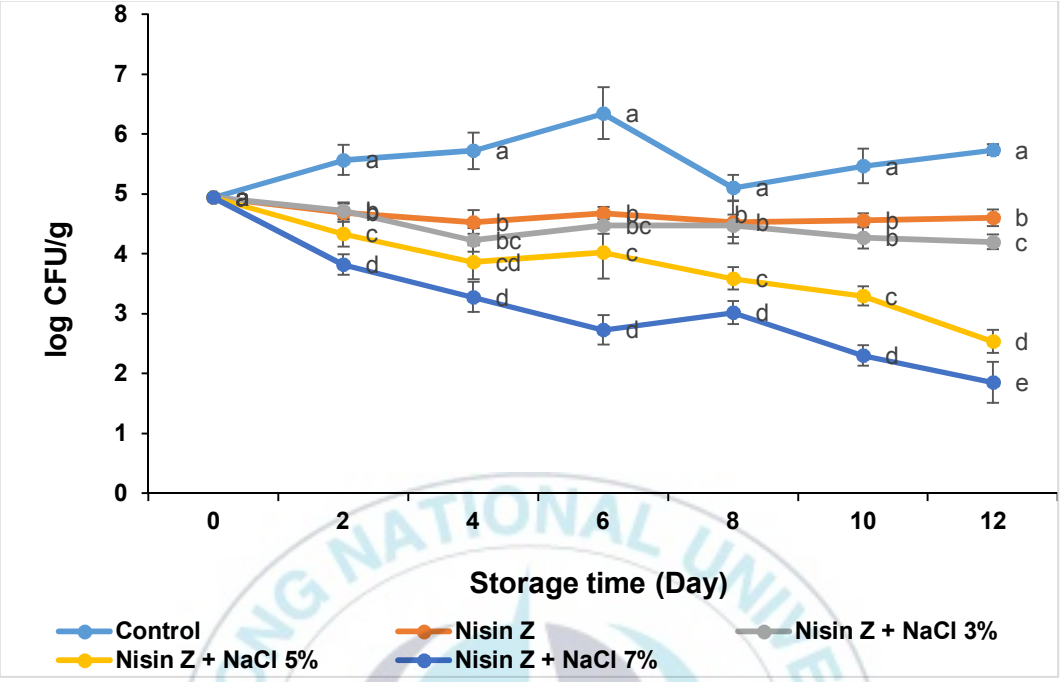

Fig.8. Effects of nisin Z, NaCl and their combination on *S. aureus* in minced olive flounder during storage at 4 ℃ for 12 days. Vertical bars represents the SD of means

3.3 Analysis of pH value

Fish freshness of the fish and fisheries products were the major or fundamental parts to the consumers. Different methods or techniques used for freshness test of fish and fisheries products. Freshness of fish depends on the various factors and the most important key was presence of microbial load. Determination of pH value was one of the indicator of freshness test of fish and fisheries products. Changes in pH value during storage on minced olive flounder at 4 ℃ for 12 days were shown in Fig-9 and 10. The initial pH of live fish was around 7.0 but the value of pH was initially decreased during storage period to increase the shelf-life of fish.

The pH value of minced olive flounder treated with NaCl and nisin Z against *L. monocytogenes* during storage at 4 ℃ for 12 days were presented in Fig-9. The minced fish inoculated with bacteria 6.50 log (CFU/g) and treated to nisin Z, NaCl and their combination. The initial pH value was 6.3. All the treatments were shown the value decreasing after initial storage and the value was increasing with the late storage.

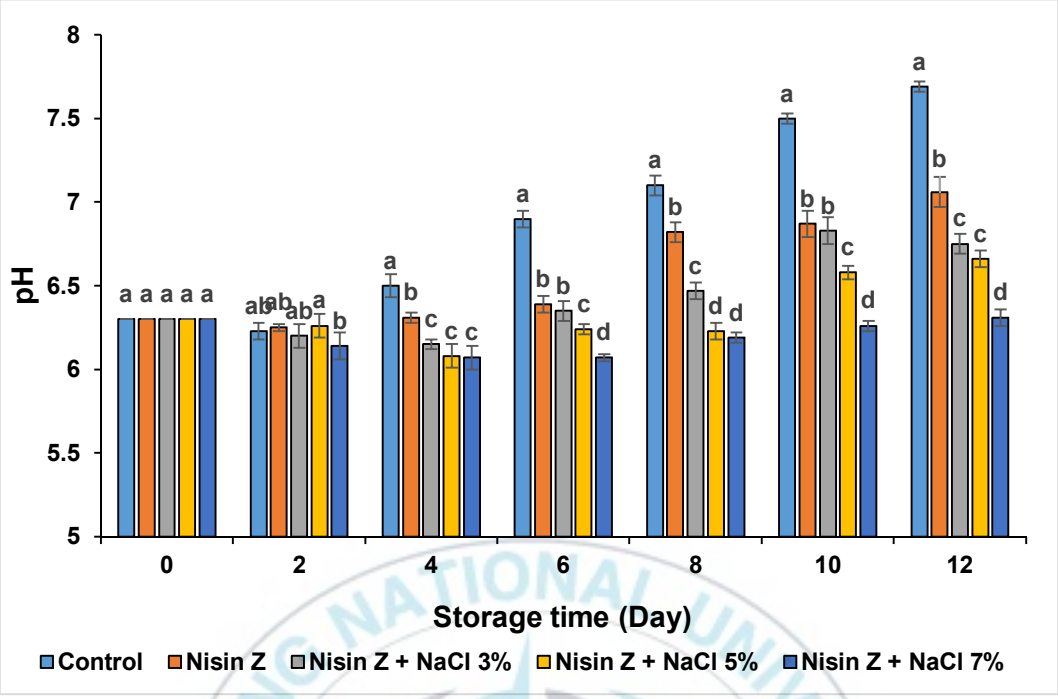

Fig.9. Changes in pH value of minced olive flounder against *L. monocytogenes* during storage at 4 °C for 12 days. Vertical bars represents the SD of means. Different superscript letters indicate a significant ($P < 0.05$) difference between different treatment groups on the same day. The same superscript letters in same day indicates no significant differences ($P > 0.05$)

Nisin Z with 7% NaCl were displayed the significantly ($P < 0.05$) lower value compare to control and other treatments at the end of 12 days. Whereas all the treatments shown the significantly ($P < 0.05$) lower value compare to control. The lower value indicated the inhibition of bacterial spoilage of fish that was the acceptance quality of fish.

The pH value of minced olive flounder treated with NaCl and nisin Z against *S. aureus* during storage at 4 °C for 12 days were shown in Fig-10. The minced fish inoculated with bacteria 5.96 log (CFU/g) and treated to nisin Z, NaCl and their combination. The initial pH value was 6.1. All the treatments shown the significantly ($P < 0.05$) lower value compare to control. The value of all treatments shown decreasing after initial storage and the value was increasing with the late storage time. Nisin Z with 7% NaCl shown the significantly ($P < 0.05$) lower value compare to control and other treatments and shown the pH value started to increase and reaching the value 6.10 after 6 days of fish storage and at the end of the trial the value was 6.29. However, at the end of 12 days the pH value of control was shown in near about 7.5 that moves the alkaline pH which was favour for microbial growth. The lower value indicated the inhibition of bacterial spoilage to the fish that was the better quality of fish.

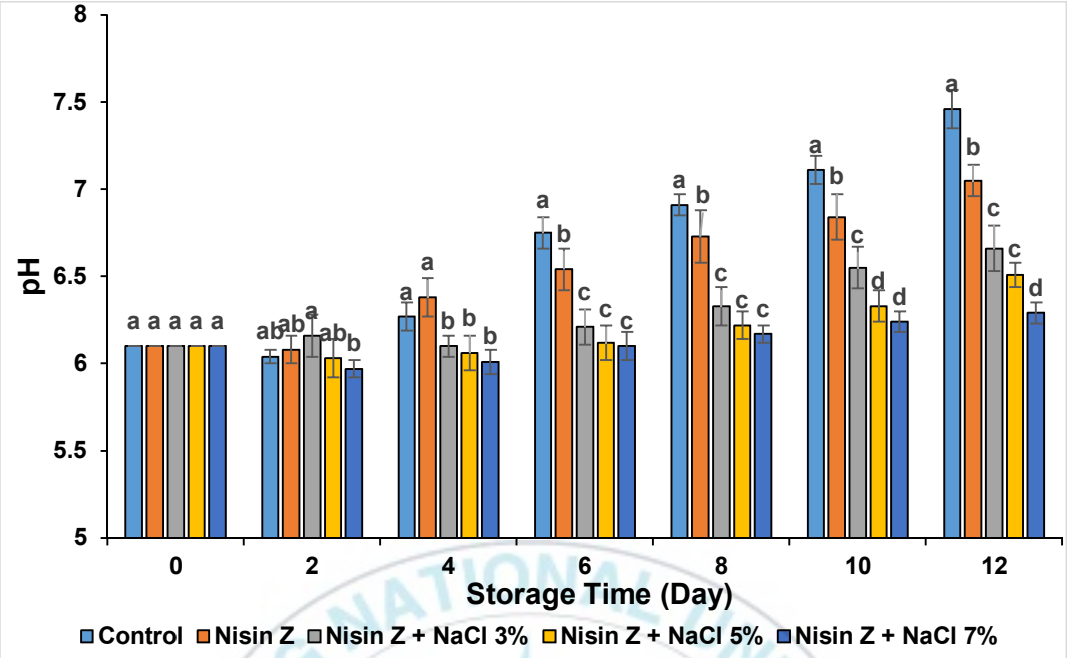

Fig.10. Changes in pH value of minced olive flounder against *S. aureus* during storage at 4 °C for 12 days. Vertical bars represents the SD of means. Different superscript letters indicate a significant ($P < 0.05$) difference between different treatment groups on the same day. The same superscript letters in same day indicates no significant differences ($P > 0.05$)

Discussion

In this study, the antimicrobial activities of single nisin Z, NaCl, and their combinations (nisin Z + NaCl) were assessed against *L. monocytogenes* and *S. aureus* through the formation of inhabitation/clear zone diameter. These two mentioned food borne pathogens are able to grow in the refrigerated conditions and also cause fish spoilage following breakdown of amino acid linkage and production of odours. Nisin Z performs very effective activities to delay the growth of microbes as well as slows down the fish spoilage. Nisin Z contains some amino acids or antimicrobial peptides that are most effective for controlling the growth of spore formers bacteria especially *L. monocytogenes*, *S. aureus,* and also lactic acid bacteria (Mansour and Milliere, 2001).

According to our results of primary evaluation minced olive flounder treated with nisin Z and NaCl was acceptable and treated substances showed the significantly better performances compared to the control. When nisin Z reach to the bacterial cell membrane initial interactions between each other caused by hydrophobic action between the amino acid residues of nisin Z and fatty acids of phospholipids in the cell membrane (Sahl and Bierbaum 1998). Moreover, the antimicrobial activity of the treated substances depends on its quality, quantity and also nature of bacteria that were tested for the experiment (Bakkali et al., 2008; Burt, 2004; and Rather et al., 2012). The mesophilic microorganisms which

grow in refrigerated temperature and caused food spoilage. Nisin were used to delay the mesophilic pathogens growth in processed beef storage (Carlin et al., 2000). The treatments of NaCl and nisin Z used in single or combination against the pathogenic bacteria also showed significant inhibition compared to control (Pawar et al., 2000). Report by Samelis et al. (2005) exhibited that nisin Z combination with other antimicrobial substances possessed significantly higher activities compared to individual treatment.

Microbial load play the vital role for the fish and fisheries product quality and the antimicrobial substances are commonly used to inhibit the growth and activities of microbes to extend the shelf-life (Rahman et al., 2013). The inhibitory effects depicted by nisin Z against gram positive as *L. monocytogenes* and *S. aureus* in this study were very similar to Gharsallaoui, et al., (2016). Combination of nisin Z and NaCl showed higher activities against *L. monocytogenes* than single nisin Z (Pawar et al., 2000; Hammou et al., (2010), NaCl have a little antimicrobial activity but when it mixed with nisin, NaCl act as an activity enhancer (Thomas and Wimpenny 1996; Parente et al., 1998) supports the present findings of this study. *L. monocytogenes* and *S. aureus* have important characteristics as food borne pathogens that were positively controlled by nisin Z and NaCl. Now a day's consumer demand natural preservatives and antibacterial substances or compound should be the natural substances.

These two natural substances have shown the strong antimicrobial activity against the pathogens for fish and fisheries products preservations at low or refrigerated temperature and not possesses any residual effect for human health (Gould, 1992).

Previous studies have reported that nisin were collected from *Lactococcus lactis* used massively as natural preservatives to control the pathogenic bacteria that caused fish spoilage (Kathiresan and Thiruneelakandan, 2008). The presence of microorganisms in fish and fisheries products causes quality deterioration and the number of microbial load depends on moisture content, temperature and also initial population (Carlin et al., 2000; Paik et al., 2006). Nisin Z and NaCl performed to decrease the number of microorganisms at the initial stage which was the main strategy to increase the shelf-life of fish during storage also remain attractive to the consumers. The minced olive flounder treated with only nisin Z and combination with 3% NaCl, showed a little activity against *L. monocytogenes* and *S. aureus* was similar to the results reported by Solomakos et al., (2008) in minced beef. The main cause of this weak activities against two pathogenic bacteria was lower dose of NaCl means lower stimulation nisin Z activity and also nisin Z reaction with fish muscle (Aasen et al., 2003; Stergiou et al., 2006). Increasing the concentration of NaCl with nisin the inhibition of pathogenic bacteria especially gram positive bacterial growth remained incredible. Pawar et al. (2000) reported that the concentration of nisin increased from 400 to 800 IU and 500 to 1000 IU in the storage of raw minced buffalo meat and raw beef respectively against gram positive pathogenic bacteria showed significantly higher activities than the lower concentrations. The result of this study demonstrated that combination of nisin Z (1000 IU) with 7% NaCl were displayed the strongest antimicrobial activity against the mentioned food born pathogenic bacteria during cold storage of minced

fish meat in 12 days at 4 °C. These agreements were similar to the findings of raw minced buffalo meat treated with nisin (800 IU) used against *L. monocytogenes* (Pawar et al., 2000). The combination effect of nisin Z (IU) and NaCl (3, 5, and 7%) revealed the higher antimicrobial activity than the individual use of substances in minced fish during storage at 4 °C. It was previously reported that combined effect of nisin with some plants extracts or oils or any antimicrobial substances exhibited the more activity rather than used of single substances (Ettayebi et al., 2000; Gutierrez et al., 2009; Hsieh et al., 2011; and Yoon et al., 2011).

The pH value played an enthusiastic role for the confirmation of quality and freshness of fish and fisheries products. The beginning pH value of the fish was more or less neutral but increasing with the storage period the value becomes lower because of microbial activity. The fish treated with NaCl and nisin Z against the two pathogenic bacteria found the resilient activity especially nisin Z with 7% NaCl prevent the fish spoilage because pH value was more or less neutral. This characteristic of extended shelf life was similar to sea bass (*Centropristis striata*) stored in ice (Kyrana and Lougovious 2002). These two trials the pH value of the minced fish treated with 7% NaCl and nisin Z consisted near about neutral or slightly below the neutral pH at the end of 12 days in refrigerated preservation that indicates the better quality of fish for eating. Abbas et al., (2006) affirmed that the storage temperature have played the key function of pH value and the high storage temperature reflects the shorter shelf-life of fish and decreased pH value rapidly in favor of microbes resulting fish spoilage or shorter shelf-life. The pH value of all treatments decreasing in early storage of fish but it increased with the late storage (Ko et al. 2016). The reason of decreasing the bacterial load in initial storage was formation of citric acid by glycolysis (Qiu et al., 2014) and bacterial load increasing during the late storage of fish increase ammonia (Xu et al., 2014). Nisin Z expressed the more antimicrobial activity in acidic pH compare to alkaline pH. The similar agreement of nisin activity was more stronger when the pH value 5.5 or below 6 (Buncic et al.,1995; De Martinis et al., 1997; and Ukuku et al., 1997).

Conclusion

The present study investigated the antimicrobial activity of nisin Z and NaCl against the pathogenic bacteria when the fish stored at 4 °C for 12 days. The findings of this experiment we found that nisin Z were shown the potential antimicrobial activity against *L. monocytogenes* and *S. aureus*. NaCl shown a little bit antimicrobial activity. Combining antimicrobial activity of nisin Z and NaCl was remarkable. When used the both antimicrobial compounds, nisin Z shown the activity first and NaCl performs as stimulating agent to irritate the nisin Z. As a result the combination antimicrobial effect of nisin Z and NaCl shown higher than the single agent used against the pathogenic bacteria. All treatments showed the significant ($P < 0.05$) activity to preserve the olive flounder than control. But the treatment of nisin Z only and combination with 3% NaCl did not show the satisfactory result to preservation the olive flounder. Especially, the findings of this experiment suggest that the combination of nisin Z at 1000 IU with 7% NaCl exhibited the higher activity compare to other treatments and to inhibit the bacterial growth in the late storage period. So, to inhibit the bacterial growth these two compounds have been used to preserve the fish and fisheries products during storage for better quality particularly to increase their shelf-life in broad spectrum.

Acknowledgements

All praise to Almighty Allah who gave me the ability to complete the research work and successfully writing the thesis.

It is my pleasure, gratitude and cordial thanks to my respected supervisor Professor Dr. In-Soo Kong, Department of Biotechnology, Pukyong National University, Korea. It was nearly impossible to complete my research without his valuable suggestions and passionate supervision.

I am also thankful to KOICA to give me the scholarship under KOICA-PKNU International Graduate Program of Fisheries Science. Without their assistance I would not be able to study in Pukyong National University, Korea.

I would like to extend my thanks to all of laboratory members specially, Dr. Sharmin Suraiya, Md Tawheed Hasan, Won Je Jang, Hae Dae Park,Yu Bin Choi for their valuable support and co-operation for my study period.

I would also thankful to Bangladeshi people Professor Dr. Md Aminul Islam, Md Jobidul Alam, Dr. Raju Ahmed, Md Abul Kalam Azad, Dr. Mohammad Shariful Azam, Dr. Monjurul Haq, Md Rifat Mahmud, Mohammad Habibur Rahman Mollah.

It is my privilege to thank to my wife Mst Hizbunnahar Disha, my daughter Dianat Tasfia, my son Nabhan Awsaf and specially my parents for mental support and encouragement that gave me to complete this task.

I am also grateful to Director General, all personnel of Union Project, all personnel of Natore District Office, Department of Fisheries, Bangladesh who helped me a lot for my job purpose.

Md Khalekuzzaman Sarker

References

Aasen, I., Markussen, S., Moretro, T., Katla, T., Axelsson, L., & Naterstad, K. (2003). Interactions of the bacteriocins sakacin P and nisin with food constituents. International Journal of Food Microbiology, 87, 35-43.

Abbas, K.A., S.M. Sapuan and A.S. Mothtar, (2006). Shelf life assessment of Malayasian *Pangasius sutchi* during cold storage. J Sadhana, 31:635-643.

Abdollahzadeh, E., Rezaei, M., & Hosseini, H. (2014). Antibacterial activity of plant essential oils and extracts: The role of thyme essential oil, nisin, and their combination to control *Listeria monocytogenes* inoculated in minced fish meat. Food Control, 35(1), 177-183.

Arqués, J. L., Rodríguez, E., Nuñez, M., & Medina, M. (2008). Antimicrobial activity of nisin, reuterin, and the lactoperoxidase system on *Listeria monocytogenes* and *Staphylococcus aureus* in cuajada, a semisolid dairy product manufactured in Spain. Journal of dairy science, 91(1), 70-75.

Bakkali, F., Averbeck, S., Averbeck, D., & Idaomar, M. (2008). Biological effects of essential oils-a review. Food and Chemical Toxicology, 46, 446-475.

Bazargani-Gilani, B., Aliakbarlu, J., & Tajik, H. (2015). Effect of pomegranate juice dipping and chitosan coating enriched with *Zataria multiflora* Boiss essential oil on the shelf-life of chicken meat during refrigerated storage. Innovative Food Science & Emerging Technologies, 29, 280–287.

Bono, G., Badalucco, C. V., Cusumano, S., & Palmegiano, G. B. (2012). Toward shrimp without chemical additives: A combined freezing-MAP approach. LWT – Food Science and Technology, 46(1), 274–279.

Buchanan, R.L., Damert, W.G., Whiting, R.C., Schothorst, M.V. (1997). Use of epidemiologic and food survey data to estimate a purposefully conservative dose–response relationship for Listeria monocytogenes levels and incidence of Listeriosis. Journal of Food Protection 60, 918–922.

Buncic S, Fitzgerald CM, Bell RG, Hudson JA (1995). Individual and combined listericidal effects of sodium lactate, potassium sorbate, nisin and curing salts at refrigeration temperatures. J. Food Safety 15: 247–264.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. International Journal of Food Microbiology, 94, 223-253.

Carlin, F., Guinebretière, M. H., Choma, C., Pasqualini, R., Braconnier, A., and Nguyen-The, C. (2000). Spore-forming bacteria in commercial cooked, pasteurized and chilled vegetable purees. Food Microbiol. 17, 153-165.

Carpentier, B., & Cerf, O. (2011). Persistence of Listeria monocytogenes in food industry equipment and premises. International Journal of Food Microbiology, 145, 1-8.

CDC (Center for Disease Control and Prevention). (2010). Q & A for the Food Net MMWR with data from 2009. http://www.cdc.gov/foodnet/mmwr/2010_FoodNet_MMWR_QA.pdf Accessed 26.08.11.

De Martinis ECP, Crandall AD, Mazzotta AS, Montville TJ. (1997). Influence of pH, salt and temperature on nisin resistance of *Listeria monocytogenes*. J. Food Prot. 60, 420–423.

Economou, T., Pournis, N., Ntzimani, A., & Savvaidis, I. N. (2009). Nisin–EDTA treatments and modified atmosphere packaging to increase fresh chicken meat shelf-life. Food Chemistry, 114(4), 1470–1476.

Ettayebi, K., El Yamani, J., & Rossi-Hassani, B.-D. (2000). Synergistic effects of nisin and thymol on antimicrobial activities in Listeria monocytogenes and Bacillus subtilis. FEMS Microbiology Letters, 183, 191-195.

Gandhi, M., & Chikindas, M. L. (2007). Listeria: a foodborne pathogen that knows how to survive. International Journal of Food Microbiology, 113, 1-15.

Gau HG Jr. (1992). Statistical analysis of regional yield trials. Elsevier, Amsterdam, Netherlands, 20-25.

Gharsallaoui, A., Oulahal, N., Joly, C., & Degraeve, P. (2016). Nisin as a food preservative: Part 1: Physicochemical properties, antimicrobial activity, and main uses. Critical Reviews in Food Science and Nutrition, 56(8), 1262–1274.

Gimenez, B., Dalgaard, P. (2004). Modelling and predicting the simultaneous growth of Listeria monocytogenes and spoilage micro-organisms in coldsmoked salmon. Journal of Applied Microbiology 96, 96–109.

Gould. (1992). Gould, Ecosystem approaches to food preservation, J. Appl. Bacteriol. 21: 58S–68S.

Gutierrez, J., Barry-Ryan, C., & Bourke, P. (2009). Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. Food Microbiology, 26, 142-150.

Hammou FB, Skail SN, Idaomar M, Abrini J. (2010) Combinations of nisin with salt (NaCl) to control Listeria monocytogenes on sheep natural sausage casings at 6°C. African J Biotechnol 9:1190-1195.

Hsieh, Y. H., Yan, M., Liu, J. G., & Hwang, J. C. (2011). The synergistic effect of nisin and garlic shoot juice against Listeria spp. in soymilk. Journal of the Taiwan Institute of Chemical Engineers, 42, 576-579.

Kathiresan K, Thiruneelakandan G. (2008) Prospects of lactic acid bacteria of marine origin. Indian J Biotechnol 7:170-177.

Khan, A., Vu, K. D., Riedl, B., & Lacroix, M. (2015). Optimization of the antimicrobial activity of nisin, Na-EDTA and pH against gram-negative and gram-positive bacteria. LWT-Food Science and Technology, 61(1), 124-129.

Kim JB, KIM SK and Suh KH. (2003). Removal of suspended solids and nitrification by floating bead filter in recirculating aquaculture system. Kor J Fish Aquat Sci 36, 163-169.

Ko W, Yang S, Chang C and Hsieh C. (2016). Effects of adjustable parallel high voltage electrostatic field on the freshness of tilapia (*Orechromis niloticus*) during refrigeration. LWT – Food Sci Technol 66:151–157.

Kutty MN. (1996). Some studies on the respiratory quotient in goldfish and rainbow trout. Ph.D. Thesis, University of Toronto, Toronto, Canada.

Kyrana, V.R. and V.P. Lougovious. (2002). Sensory, chemical and microbiological assessment of farm raised European sea-bass (*Dicentrarchus labrax*) stored in melting ice. Int. J. Food Sci. Technol., 37: 319-328.

Larsson, S. C., Orsini, N., & Wolk, A. (2006). Processed meat consumption and stomach cancer risk: A meta-analysis. Journal of the National Cancer Institute, 98(15), 1078–1087.

Liu, D. (2008). Epidemiology. In D. Liu (Ed.), Handbook of *Listeria monocytogenes* (pp. 27-59).

Magalhaes, L., & Nitschke, M. (2013). Antimicrobial activity of rhamnolipids against Listeria monocytogenes and their synergistic interaction with nisin. Food Control, 29(1), 138-142.

Maita T, Aoki H, Tanaka S, Shimizu Y, Yamagata Y and Watanabe T. (1995). Abstract, Ann Meet. Jap Soc Sci Fish 1-36.

Mansour, M., & Milliere, J.B. (2001). An inhibitory synergistic effects of a nisin-monolaurin combination on *Bacillus sp*. Vegetative cells in milk. Food microbiology, 18, 87-94.

McLauchlin, J. (1996). The relationship between Listeria and listeriosis. Food Control, 7, 187-193.

MOMAF. (2007). Statistic Database for Fisheries Production. Retrieved from http://fs.fips.go.kr/main.jsp on July 14.

Morsy, M. K., Elsabagh, R., & Trinetta, V. (2018). Evaluation of novel synergistic antimicrobial activity of nisin, lysozyme, EDTA nanoparticles, and/or ZnO nanoparticles to control foodborne pathogens on minced beef. Food Control, 92, 249-254.

Neetoo, H., Ye, M., & Chen, H. (2008). Potential antimicrobials to control *Listeria monocytogenes* in vacuum-packaged cold-smoked salmon pate and fillets. International journal of food microbiology, 123(3), 220-227.

Paik, H. D., Kim, H. J., Nam, K. J., Kim, C. J., Lee, S. E., and Lee, D. S. (2006). Effect of nisin on the storage of sous vide processed Korean seasoned beef. Food Control 17, 994-1000.

Pan, D., Zhang, D., Hao, L., Lin, S., Kang, Q., Liu, X., Lu, L., & Lu, J. (2018). Protective effects of soybean protein and egg white protein on the antibacterial activity of nisin in the presence of trypsin. Food chemistry, *239*, 196-200.

Pawar, D., Malik, S., Bhilegaonkar, K., & Barbuddhe, S. (2000). Effect of nisin and its combination with sodium chloride on the survival of *Listeria monocytogenes* added to raw buffalo meat mince. Meat Science, 56, 215-219.

Purbayanto A, Akiyama S, Arimoto T and Sondita MFA. (2000). Capture process of sweeping trammel net with special reference on operation method and catch pattern. In: Proceedings of the 3rd JSPS International Seminar on Fisheries Science in Tropical Areas. Arimoto T and Haluan J, eds. Tokyo University of Fisheries, Tokyo, Japan, 98-103.

Qiu XJ, Chen SJ, Liu GM and Yang QM. (2014).Quality enhancement in the Japanese sea bass (*Lateolabrax japonicas*) fillets stored at 4 ^{0}C by chitosan coating incorporated with citric acid or licorice extract. Food Chem, 162:156–160.

Rahman, S. M. E., Wang, J., & Oh, D.-H. (2013). Synergistic effect of low concentration electrolyzed water and calcium lactate to ensure microbial safety, shelf life and sensory quality of fresh pork. Food Control, 30(1), 176–183.

Raeisi, M., Tabaraei, A., Hashemi, M., & Behnampour, N. (2016). Effect of sodium alginate coating incorporated with nisin, *Cinnamomum zeylanicum*, and rosemary essential oils on microbial quality of chicken meat and fate of *Listeria monocytogenes* during refrigeration. International journal of food microbiology, 238, 139-145.

Randall DJ, Brauner CJ and Neuman JF. (1996). Water chemistry at the gill surfaces of fish and the uptake of xenobiotics. In: Toxicology and Aquatic Pollution. Taylor EW, ed. Cambridge University Press, London, U.K., 20-25.

Rather, M. A., Dar, B. A., Dar, M. Y., Wani, B. A., Shah, W. A., Bhat, B. A., et al. (2012). Chemical composition, antioxidant and antibacterial activities of the leaf essential oil of *Juglans regia* L. and its constituents. Phytomedicine, 19, 1185-1190.

Rocourt, J., BenEmbarek, P., Toyofuku, H., Schlundt, J., (2003). Quantitative risk assessment of *Listeria monocytogenes* in ready-to-eat foods: the FAO. FEMS Immunology and Medical Microbiology 35, 263–267.

Rombough PJ. 1988. Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. In: Fish Physiology. Hoar WS and Randall DJ, eds. Academy Press, New York, U.S.A., 20-25.

Sahl HG, Bierbaum G. (1998). Lantibiotics: Biosynthesis and biological activities of uniquely modified peptides from Gram positive bacteria, Ann. Rev. Microbiol. 52: 41–79.

Samelis J, Bedie GK, Sofos JN, Belk KE, Scanga JA, Smith GC. (2005). Combinations of nisin with organic acids or salts to control *L. monocytogenes* on sliced pork Bologna stored at 4 ^{0}C in vacuum packages. Lebensmittel-Wissenschaft. 38: 21–28.

Schlyter, J.H., Glass, K.A., Loeffelholz, J., Degnan, A.J., Luchansky, J.B., (1993). The effects of diacetate with nitrite, lactate, or pediocin on the viability of *Listeria monocytogenes* in turkey slurries. International Journal of Food Microbiology 19, 271–281.

Solomakos, N., Govaris, A., Koidis, P., & Botsoglou, N. (2008). The antimicrobial effect of thyme essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. Food Microbiology, 25, 120-127.

Stergiou, V. A., Thomas, L. V., & Adams, M. R. (2006). Interactions of nisin with glutathione in a model protein system and meat. Journal of Food Protection, 69, 951-956.

Turgis, M., Vu, K. D., Dupont, C., & Lacroix, M. (2012). Combined antimicrobial effect of essential oils and bacteriocins against food borne pathogens and food spoilage bacteria. Food Research International, 48, 696-702.

Ukuku DO, Shelef LA (1997). Sensitivity of six strains of *Listeria monocytogenes* to nisin. J. Food Prot. 60: 867–869.

White, D. G., Zhao, S., Simjee, S., Wagner, D. D., & Mc Dermott, P. F. (2002). Antimicrobial resistance of food borne pathogens. Microbes and Infection, 4, 405-412.

Yoon, J. I., Bajpai, V. K., & Kang, S. C. (2011). Synergistic effect of nisin and cone essential oil of *Metasequoia glyptostroboides* Miki ex Hu against *Listeria monocytogenes* in milk samples. Food and Chemical Toxicology, 49, 109-114.