International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:4, No:5, 2010

Inhibitory Effect of Lactic Acid and Nisin on Bacterial Spoilage of Chilled Shrimp

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Abstract**—** Lactic acid alone and its combined application with nisin were evaluated for reducing population of naturally occurring microorganisms on chilled shrimp. Fresh shrimps were dipped in 0, 1.0% and 2.0% (v/v) lactic acid alone and their combined application with 0.04 (g/L/kg) nisin solution for 10 min. Total plate counts of aerobic bacteria (TPCs), Psychrotrophic counts, population of *Pseudomonas* spp., H_2S producing bacteria and Lactic acid bacteria (LAB) on shrimps were determined during storage at 4 °C. The results indicated that total plate counts were 2.91 and 2.63 log CFU/g higher on untreated shrimps after 7 and 14 days of storage, respectively, than on shrimps treated with 2.0% lactic acid combined with 0.04 (g/L/kg) nisin. Both concentrations of lactic acid indicated significant reduction on *Pseudomonas* counts during storage, while 2.0% lactic acid combined with nisin indicated the highest reduction. In addition, H_2S producing bacteria were more sensitive to high concentration of lactic acid combined with nisin during storage.

Keywords**—**Shrimp, lactic acid, nisin, spoilage bacteria

I. Introduction

ORGANIC acids seem to be an alternative decontaminant in fish preservation, like the treatments of meat and poultry products with lactic acid. Unfortunately, nisin, a bacteriocin produced by *Lactococcus lactis*, inhibiting many Gram-positive food-borne pathogens like *Listeria* and *Clostridium* [1], could not be used alone for fish products because the initial spoilage flora primarily consists of Gram-negative (especially *Pseudomonas*) bacteria. However, several studies have indicated the effect of nisin on Gram-negative bacteria in the case of its combined use with other compounds such as organic acid, ethylenediaminetetraacetic acid (EDTA) and ethyl alcohol by increasing the rate of nisin penetration into Gram-negative cell walls [2], [3], [4]. Moreover, nisin and high hydrostatic pressure alone or combined applications with lysozyme, lactoferrin and lactoferricin in foods including egg and poultry, appeared as another meat protection alternative [5], [6]. The application of nisin as a food preservative has been studied extensively [7], [8]. Based upon its target microorganisms, nisin application falls into one of three categories: (1) prevent spoilage by sporeforming bacteria, (2) prevent spoilage by lactic acid bacteria and related microorganisms or (3) kill or inhibit gram- positive pathogenic bacteria, e.g., *Bacillus cereus*, *C. botulinum* or *L. monocytogenes* [9].

The aim of this study was to evaluate the applicability of lactic acid and nisin alone and their combination to control population of naturally occurring microorganisms on chilled shrimp during storage at refrigerator temperature.

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II. Materials and Methods

A. Sample Preparation and Treatment Application

Freshly caught shrimps (*Penaeus merguiensis*) were purchased directly from Penang, Malaysia. They were transferred immediately to the laboratory in polystyrene boxes with an appropriate quantity of crushed ice. Within1 hour of arrival, they were divided into six groups as each contains 1 kg of shrimp (as 0.5-kg sample and 0.5-kg replicate). The sample to be analyzed for zero time (initial microbial load) was taken. For alone application shrimps were dipped into 1.0 and 2.0% lactic acid (PURAC, Singapore) and 0.04 mg/L/kg nisin (Sigma, USA) separately for 10 min at 25 °C. The nisin solution was prepared by adding 0.04-g nisin into 1-L distilled water at pH 5.2. For the combined applications of nisin and lactic acid, shrimps were dipped for 10 min in nisin solutions which was prepared the same way and then transferred into the 1.0 and 2% lactic acid solution, respectively. Shrimps dipped into distilled water were used as control treatments. All samples were kept in duplicate sterile plastic bags at 4 °C. At predetermined periods, samples were randomly removed from the storage bag. Each sampling time, three shrimps from each group were randomly selected and taken to all analysis.

B. Microbiological Sampling and Analysis

Twenty-five grams of shrimp samples were aseptically removed from the package and homogenized for 30s with a Lab blender in 225 ml sterile 0.1% peptone water (Merck, Germany). 1 ml of primary 1/10 suspension was then withdrawn and further decimal serial dilutions were prepared from this homogenate in the same chilled sterile diluents. The appropriate dilutions were subsequently used for enumeration and differentiation of microorganisms and particular microbial

genera in the samples, at each of the pre-determined time intervals, during refrigerated storage.

1) *Aerobic Plate Count*
Total plate counts of aerobic bacteria (TPCs) were determined by inoculating 1 ml of the sample homogenate, at selected dilutions, onto duplicate sterile plates of plate count agar (PCA; Merck) using "pour-plate" method, then the plates were incubated for 48 hrs at 25 °C [10]. The plates showing colony numbers of 25 to 250 were then selected for counting with a colony counter. Results were expressed as CFU/g.
2) *Psychrotrophic count*
Psycrotrophic bacteria (PTCs) were enumerated in a similar method to that for TPC except that plates were incubated at 4 °C for 10 days.
3) *Pseudomonas count*
Pseudomonas were counted on Pseudomonas Agar Base (CM 0559; Oxoid, UK) supplemented with cetrimide, fucidin, and cephaloridine (C-F-C) supplements (SR 0103E; Oxoid) providing a selective isolation medium for *Pseudomonas* spp. Colonies were counted after 48 hrs incubation at 25 °C.
4) *Hydrogen sulfide-producing bacteria*
H_2S-producing organisms were enumerated on Triple sugar iron agar (Merck, Germany) by spread method. The plates were incubated at 25 °C for 48 hrs [11]. On this medium, these bacteria are recovered as black colonies due to precipitation of ferrous sulfide. Twelve selected black colonies were isolated, pure cultured, and then characterized by morphology, Gram reaction, motility test, catalase and oxidase production.
5) *Lactic acid bacterial count*
For determination of lactic acid bacteria (LAB), diluted samples were plated on de Man, Rogosa, and Sharpe (MRS) agar (Oxiod, UK) and incubated at 30 °C for 3–5 days in anaerobic jars with GasPak bags for the generation of an anaerobic medium.

C. Statistical analysis

Statistical analysis was performed using SPSS 15.0 (SPSS Inc.) 2006 Windows XP. Means were compared with an analysis of variance (ANOVA) followed by Duncan test to determine among means at $P \leq 0.05$ level.

III. Results and Discussion

A. Total plate counts (TPCs)

The TPC counts in shrimps treated with different solutions on day one of storage ranged from the highest numbers for control samples (5.08 log CFU/g) to lowest numbers for 2% lactic acid combined with nisin treated samples (2.39 log CFU/g). The reductions of TPC for nisin (N), 1% lactic acid (1% LA), 1% lactic acid combined with nisin (1% LA+N), 2% lactic acid (2% LA) and 2% lactic acid combined with nisin (2% LA+N) were 0.28, 1.22, 2.62, 2.53, and 2.69 log CFU/g on day 1 respectively, whereas, it was 0.64, 1.64, 2.07, 2.17, and 2.52 log CFU/g respectively on day 14 of storage (Fig. 1). This indicated that dipping of the shrimp in the different treatment solutions resulted in drastic reduction of TPCs on day 1. However, TPCs in shrimps for all of the different treatments were still around 4 log CFU/g, while that of control attained a count of 6.58, which is in close proximity to the maximal recommended limit of 7 log CFU/g for TPC in raw fish product [12].

On the other hand the results indicated that nisin alone were less effective on TPCs growth when compared with lactic acid (1-2% v/v) alone. However, after 7 days of storage there was a synergy effect especially for higher concentration of lactic acid when used in combination with nisin ($P \leq 0.05$). On the day 14, TPCs for 2% LA+N treated shrimps was kept around 6.30 log CFU/g whereas the control had reached 8.92 log CFU/g. Therefore, a reduction of 2.63 log CFU/g in TPC was obtained with respect to control samples at this point of storage. Thus, it can be clearly expressed that nisin should be applied as combined with lactic acid application, rather than its alone application. This greater inhibition of 2% LA+N may be due to the lactic acid's increasing effect of nisin's penetration into also Gram-negative bacteria, by decomposing the Gram-negative cell wall prior to nisin application [13], [14].

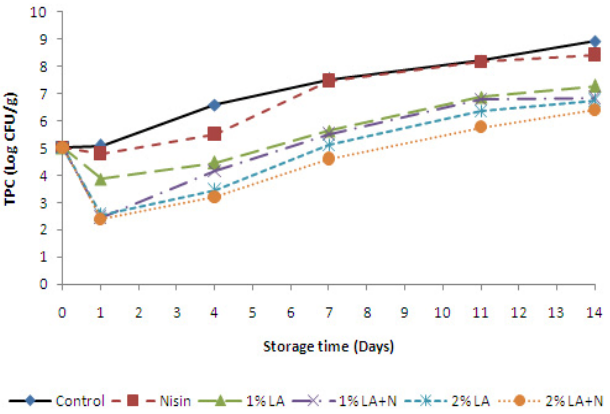

Fig. 1 Effect of lactic acid and nisin treatments on total aerobic plate count (TPC) of shrimp during storage at 4 °C

B. Psychrotophic counts

The Gram-negative psychrotrophic bacteria are the major group of microorganisms responsible for spoilage of aerobically stored fresh fish products at chilled temperatures [15]-[11]. In this study, the psychrotophic counts of shrimp were ranged from not-detectable value (negligible; log CFU/g <2), in 2%LA+N-dipped samples, to 3.64 log CFU/g in control samples on day 1. (Fig. 2) Additionally, the growth pattern of psychrotophic showed same behavior as that of TPC, with control also being the highest at day 14 (8.95 log CFU/g), followed by samples treated with Nisin (8.74 log CFU/g), and 1% LA (7.61 log CFU/g), while lower count (6.59 log CFU/g) was detected in samples treated with 2% LA+N. Nisin alone indicate lowest effect on psychrotophic growth when compared with other treatments, while 2% LA

and/or 2% LA+N treatments indicate the most effect on psychrotophic growth. The psychrotrophic population, however, were relatively higher than TPCs, which was attributed to the refrigerated storage condition. It should be noted that refrigeration will restrict the growth of mesophiles, generally a major component of the initial microflora, and allow psychrotrophic microorganisms to grow during storage period and eventually dominate the microflora.

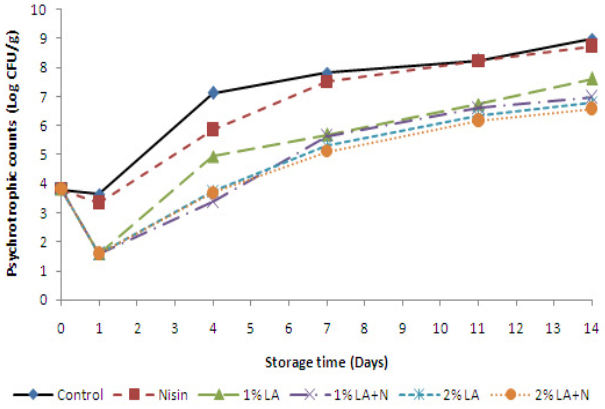

Fig. 2 Effect of lactic acid and nisin treatments on Psychrotrophic counts of shrimp during storage at 4 °C

C. *Pseudomonas counts*

The initial *Pseudomonas* counts in this study were ranged from not detectable (2≥log CFU/g) in 2% LA+N treated samples to 2.87 in control samples by the day1. *Pseudomonas* count for 1% LA+N, 2% LA, and 2% LA+N were kept around 6.90, 6.35, and 6.13 log CFU/g (which is in the limits of consumption risk), whereas the control had reached 7.99 log CFU/g at the end of the 14th day. (Fig. 3) More inhibition, which was made by LA+N combination, indicates a synergistic effect of lactic acid (epically in higher concentration) on the effectiveness of nisin, when compared to applications of lactic acid and nisin alone, which caused less inhibition on *Pseudomonas* spp. as the initial spoilage Gram-negative flora of shrimp.

Under aerobic iced storage, the flora is composed almost exclusively of *Pseudomonas* spp. and *S. putrifcirn* [16], [11]. This is true for all fish and shellfish whether caught or harvested in temperate or sub-tropical and tropical waters [17], [18]. Furthermore, *Pseudomonas fragi* was reported as the major spoilage organism in tropical shrimps [19].

Counts of *Pseudomonas* spp. in the early of storage period are slightly lower than the TPCs and psychrotophic counts (Fig. 3), indicating the importance of these species in the spoilage of fresh shrimp examined. This could be interest since microbial spoilage usually does not take part initially during the storage. Indeed, the microbial population of fish products stored aerobically under chilling condition consists almost exclusively of *Pseudomonas* spp. and H_2S-producing bacteria (presumably, *Shewanella putrefaciens*) [15].

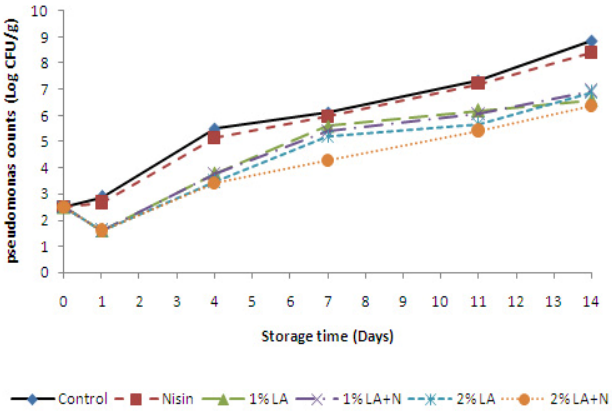

Fig. 3 Effect of lactic acid and nisin treatments on *Pseudomonas* spp. counts of shrimp during storage at 4 °C

D. H_2S *producing counts*

The initial H_2S producing microorganisms were below the detection limit (2≥ log CFU/g) and they start to growth and colonize after 1 day of storage. (Fig. 4) The species is sensitive to high concentration of lactic acid combined with nisin during storage ($P \leq 0.05$). Nisin and 1% lactic acid alone was found in high number of this group of microorganisms. The development of specific spoilage bacteria in a fish ecosystem is a result of both environmental conditions and microbial competition. Therefore, the low count of H_2S-producing bacteria might be the result of inhibition by *Pseudomonas* spp. . Actually, it was reported that *Pseudomonas* spp. can inhibit the growth of H_2S-producing bacteria (including *S. putrefaciens*) because of the ability of the former to produce siderophores, and this interaction can be the major factor governing the development of spoilage flora [20].

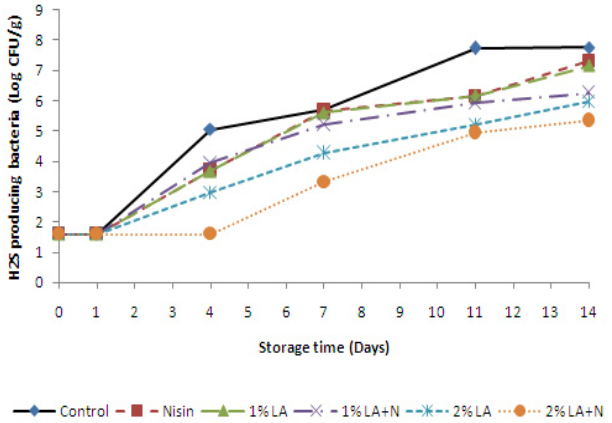

Fig. 4 Effect of lactic acid and nisin treatments on H_2S producing bacteria of shrimp during storage at 4 °C

E. *Lactic acid bacteria counts*

The initial counts of LAB were below the detection limit (2≥ log CFU/g). The count of LAB was lower than the other bacterial counts determined in this study at the time of

spoilage. (Fig 5) A final count of 5.41 was reached in control samples at the end of storage period (day 14), whereas samples treated with nisin alone application did not attained significant reduction (P>0.05) in LAB count compared to other treatments. On the day 14, a significant reduction (P≤0.05) in LAB counts was attained in 1% LA and 2% LA-treated samples when compared with the control (4.94 and 4.88 versus 5.41, respectively), but these applications showed less effect when compared with their combinations with nisin to inhibit LAB growth. Consequently, among all the above treatments the most effective one is 2% LA+N. Therefore, it can be clearly expressed that solutions of nisin and LA alone, were less effective on LAB growth when compared with their combined application (Fig. 5).

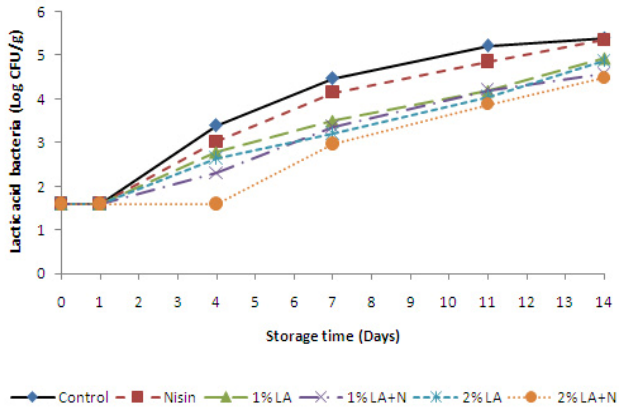

Fig. 5 Effect of lactic acid and nisin treatments on Lactic acid bacteria (LAB) counts of shrimp during storage at 4 °C

The low LAB count in this study was expected since lactic acid bacteria tend to grow slowly at refrigeration temperatures and are under aerobic condition generally out-competed by pseudomonas [21]. In contrast, the contribution of LAB as the major spoiling microorganisms had been reported in fresh vacuum-packed Atlantic salmon portions stored at 4 °C [22].

It should be noted that the antimicrobial properties of lactic acid are attributed to the undissociated lactic acid molecule and to a reduction of pH below the level at which the growth of many bacteria is inhibited (5). Undissociated weak acids possess the ability to cross membranes of micro microorganisms, become dissociated inside the cell and acidify the cell interior. It has been suggested also that nisin disrupts membrane activity via pore formation and may have additional effects on electron transfer chain components [23]. Organic acids and their salts can potentiate the activity of bacteriocins greatly, whereas acidification enhances the antibacterial activity of both organic acids and bacteriocins [24]-[25]. The increase in net charge of bacteriocins at low pH might facilitate translocation of bacteriocin molecules through the cell wall. The solubility of bacteriocins may also increase at lower pH, facilitating diffusion of bacteriocin molecules. Moreover, the outcome of nisin activity within a food system depends on numerous factors. Nature of the food, other preservative hurdles such as heat treatments, low water activity, modified atmosphere, low temperature, and pH enhanced activity [26], [27], [28], [29]. For instance, Nisin works better in liquid or homogenous foods compared to solid or heterogeneous products because the bacteriocin can be better and more evenly distributed throughout the food matrix of the former. There are some considerations for nisin that need to be concerned e.g. certain food additives should be avoided in foods preserved with nisin. For example, nisin is degraded in the presence of sodium metabisulphite that used as an antioxidant, bleach, antimicrobial, and also in shrimp processing to prevent of melanosis or black spot [30].

IV. Conclusion

This study successfully indicated that the combined application of lactic acid and nisin would be preferred in reducing total plate count of aerobic bacteria (TPCs) and extending the shelf life of shrimps, rather than their alone application. The growth pattern of psychrotrophic bacteria showed same behavior as that of TPC. The psychrotrophic population, however, were relatively higher than TPCs, which was attributed to the refrigerated storage condition of the shrimps. In addition, lactic acid (especially in higher concentration) combined with nisin, indicates a synergistic effect in inhibition of *Pseudomonas* spp. as the initial spoilage flora of shrimp. Similarly, H_2S producing microorganisms are more sensitive to high concentration of lactic acid combined with nisin during storage.

Acknowledgment

We would like to thank the Malaysian Ministry of Science, Technology and Innovation (MOSTI) and PURAC Asian Pacific Pte. Ltd. Singapore for their financial research grant that was provided for this study.

References

[1] Ogden, K. And Tubb, R.S., 1985. Inhibition of Beer-Spoilage Lactic Acid Bacteria by Nisin. J. Inst. Brew. 91, 390–392.
[2] Stevens, K.A., Sheldon, B.W., Klapes, N.A. and Klaenhammer, T.R., 1991. Nisin Treatment for Inactivation of *Salmonella* Species and Other Gram-Negative Bacteria. Appl. Environ. Microbiol. 57(12), 3613– 3614.
[3] Sheffet, S.M., Sheldon, B.W., and Klaenhammer, T.R., 1995. Efficiency of Optimized Nisin-based Treatments to Inhibit *Salmonella typhimurium* and Extend Shelf life of Broiler Carcasses. J. Food Prot. 58:1077-1082.
[4] Taoukis, P.S., Koutsoumanis, K. and Nychas, G.J.E., 1999. Modelling for Shell Life Control of Chilled Fish. Int. J. Food Microbiol. 53(1), 21–31.
[5] Ponce, E., P.L.A., R., Sendra, E., Guamis, B. and Mor-Mur, M., 1998. Combined Effect of Nisin and High Hydrostatic Pressure on Destruction of *Listeria Innocua* and *Escherichia coli* in Liquid Whole Egg. Int. J. Food Microbiol. 43(1–2), 15–19.
[6] Yuste, J., M. Mor-Mur, M. Capellas, B. Guamis, and R. P.L.A., 1998. Microbiological Quality of Mechanically Recovered Poultry Meat Treated With High Hydrostatic Pressure and Nisin. Food Microbiol. 15, 407-414.
[7] Hurst, A. and Hoover, D.G., 1993. Nisin. In Davidson, P.M. and Branen, A.L. (Eds). Antimicrobials In Foods. Marcel Dekker, New York: 367.
[8] Cleveland J., Montville T.J., Nes I.F., Chikindas M.L., 2001. Bacteriocins: Safe, Natural Antimicrobials for Food Preservation. Int. J. Food Microbiol. 71:1-20.
[9] Thomas, L.V., Clarkson M.R. and Delves-Broughton, J., 2000. "Nisin", in Naidu A.S., Natural Food Antimicrobial Systems, CRC Press, Boca Raton, Florida.

[10] APHA "American Public Health Association", 1984. Compendium of Methods for the Microbiological Examination of Foods. 2nd ed. American Public Health Association, Washington, DC.
[11] Gram, L., Trolle, G. and Huss, H.H., 1987. Detection of Specific Spoilage Bacteria from Fish Stored at Low (0 °C) and High (20 °C) Temperatures, Int. J. Food Microbial. 4, 65-72.
[12] ICMSF "International Commission on Microbiological Specifications for Foods", 1986. Microorganisms in foods. 2-Sampling for microbiological analysis: principles and specific applications. University of Toronto Press, Toronto.
[13] Helander. 2000. Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting The Outer Membrane. Appl. Environ. Microbiol. 66, 2001-2005.
[14] Samelis, J., Bedie, G.K., Sofos, J.N., Belk, K.E., Scanga, J.A. and Smith, G.C., 2005. Combinations of Nisin with Organic Acids or Salts to Control *Listeria monocytogenes* on Sliced Pork Bologna Stored at 4°C in Vacuum Packages. LWT Food Sci. Technol. 38, 21–28.
[15] Gram, L. and Huss, H.H., 1996. Microbiological Spoilage of Fish and Fish Products. Int. J. Food Microbiol. 33, 121-137.
[16] Levin, R.E., 1968. Detection and Incidence of Specific Species of Spoilage Bacteria on Fish. I. Methodology. Appl. Microbiol. 16: 1734-1737.
[17] Gram, L., Neergaard, W.C. and Huss, H.H., 1990. The Bacteriology of Fresh Spoiling Lake Victorian Nile Perch (*Lates niloticus*). Int. J. Food Microbiol. 10(3–4), 303–316.
[18] Shamshad, S.I., Kher-Un-Nisa, Riar, M., Zuberi, R. and Qadri. R.B., 1990. Shelf Life of Shrimp *(Penaeus Merguiensis)* Stored at Different Temperatures. J. Food Sci. 55, 1201-1205, 1242.
[19] Chinivasagam, H.N., Bremner, A.H., Thrower, S.J. and Nottingham, S.M., 1996. Spoilage Pattern of Five Species of Australian Prawns: Deterioration Is Influenced By Environment of Capture and Mode of Storage. J. Aquat. Food Prod. Technol. 5, 25–50.
[20] Gram, L., Melchiorsen, J., 1996. Interaction between Fish Spoilage Bacteria *Pseudomonas* spp. and *S. putrefaciens* in Fish Extracts and on Fish Tissue. Journal of Applied Bacteriology. 80:589–595.
[21] Huis In't Veld JH.J., 1996. Microbial and Biochemical Spoilage of Foods: An Overview. Int. J. Food Microbiol. 33:1–18.
[22] Rasmussen S.K.J, Ross T, Olley J, Mcmeekin T., 2002. A Process Risk Model for the Shelf Life of Atlantic Salmon Fillets. Int. J. Food Microbiol. 73:47–60.
[23] De Vuyst, L. & Vandamme, E.J., 1995. Nisin, A Lantibiotic Produced by *Lactococcus Lactis* subsp. Lactis: Properties, Biosynthesis, Fermentation and Applications. In: Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications (Edited By L. De Vuyst & E.J. Vandamme). pp. 151–221. London: Blackie Academic and Professional.
[24] Jack R.W., Tagg J.R., Ray B., 1995. Bacteriocins of Gram-Positive Bacteria. Microbiol. Rev. 59:171-200.
[25] Stiles M. E., 1996. Biopreservation by Lactic Acid Bacteria. Antonie Van Leeuwenhoek. 70:331–45.
[26]Rogers A.M. and Montville T.J., 1994. Quantification of Factors Which Influence Nisin's Inhibition of *Clostridium Botulinum* 56a in a Model Food System. J. Food Sci. 59:663–8.
[27] Thomas, L.V., Wimpenny, J.W.T., 1996. Investigation of The Effect of Combined Variations in Temperature, pH, and NaCl Concentration on Nisin Inhibition of *Listeria Monocytogenes* and *Staphylococcus aureus*. Applied and Environmental Microbiology 62, 2006–2012.
[28] Blom H, Katla T, Hagen B.F., Axelsson L. 1997. A Model Assay to Demonstrate How Intrinsic Factors Affect Diffusion of Bacteriocins. Int. J. Food Microbiol. 38:103-9.
[29] Szabo E.A., Cahill M.E., 1998. The Combined Effects of Modified Atmosphere, Temperature, Nisin and Alta(Tm) 2341 On The Growth of *Listeria Monocytogenes*. Int J Food Microbiol 43:21-31.
[30] Delves-Broughton, J., Blackburn, P., Evans, R.J. and Hugenholtz, J., 1996. Applications of the Bacteriocin, Nisin, *Antonie Van Leeuwenhoek* 69, 193–202.