Synergistic Antimicrobial Effect of Chitosan with Nisin and Sodium Lactate, Sodium Diacetate, or Potassium Sorbate as Edible Coatings against *Listeria monocytogenes* and Bacterial Flora of Cooked Tuna Loins

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ABSTRACT— ***Listeria monocytogenes is a gram positive, psychrotrophic, facultative anaerobic bacterium and it is the etiological agent of listeriosis, a severe foodborne disease of major public health concern. There is rising concern about the presence of L. monocytogenes in tuna products. The objective of this study was to evaluate the efficacy of chitosan-based edible coatings incorporating nisin (Nis) alone or in combination with one of three Generally Recognized as Safe (GRAS) antimicrobials, sodium lactate (SL), sodium diacetate (SD) and potassium sorbate (PS) against L. monocytogenes on cooked tuna loins. Loins cut into discs were inoculated with L. monocytogenes to a final population density of ca. 3.4 log cfu/g and then coated with a layer of chitosan incorporating Nis (500 IU/g) singly or in binary combination with SL (1.2% w/w), SD (0.125% w/w) or PS (0.15% w/w). Binary combination of (Nis + SL) was only moderately effective, reducing the population of L. monocytogenes by a maximum of 1.5 log cfu/g. After four weeks of refrigerated storage, L. monocytogenes grew on samples coated with (Nis + SL) reaching a mean population density of 4.1 log cfu/g, which was not significantly different from the control (P > 0.05). In conclusion, this coating formulation failed to demonstrate any sustained inhibitory effect against both L. monocytogenes and background microflora on cooked tuna loins over an extended refrigerated storage period.***

Keywords— chitosan, *Listeria monocytogenes*, tuna, antimicrobials, nisin

1. INTRODUCTION

The global consumption of fish and derived fish products has greatly increased during recent decades, due to a number of distinct factors [1]. Healthy eating trends have given rise to an increased demand for fish and seafood products, which have many health benefits [2]. Fish is an important source of protein in the local diet and the per capita consumption of fish in Mauritius stands at 20 kg (representing one quarter of animal protein intake) [3]. Mauritians often consume skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*) and big eye tuna (*Thunnus obesus*) in various dishes where the tuna meat have been, either poached, fried, stewed or grilled [4]. Tuna meat is particularly popular in the Mauritian diet due to its low cost, ready availability, high quality, high protein composition, culinary versatility and ease of preparation [5].

Although seafood is a major global food commodity and is an important part of a healthy diet, seafood consumption is not risk-free [6]. In fact, seafood is one of four food categories with the highest risk responsible for large numbers of foodborne illnesses and outbreaks [7]. Seafood also has a long history of being contaminated with *Listeria monocytogenes* [8-11] and is responsible for causing foodborne illnesses [12]. The incidence of food-related listeriosis has increased dramatically in the last few years, where *L. monocytogenes* has been listed in the top five highest-ranking pathogens with respect to the total cost of foodborne illness in the United States in terms of loss of income by the affected individual, cost of health care, loss of productivity due to absenteeism, costs of investigations of an outbreak, loss of income due to closure of businesses, consumer litigations, or losses of product sales when consumers avoid particular products [13].

Previous outbreaks of listeriosis have also been linked to a variety of ready-to-eat fishery products such as cold smoked salmon [14] and trout [15]. The public health importance of listeriosis is not always recognized, particularly since listeriosis is a relatively rare and sporadic disease compared with other common foodborne illnesses such as salmonellosis or botulism [16]. However, because of its high case fatality rate, listeriosis ranks among the most frequent causes of death due to foodborne illness: second after salmonellosis [11]. In addition to salmon and trout, *L. monocytogenes* has also been isolated from a variety of tuna products. A survey of minced tuna collected from retail

stores in Japan between 2002 and 2003 revealed that *L. monocytogenes* was present in 14.3% of the raw material [8]. In addition, the incidence of *L. monocytogenes* in ready-to-eat (RTE) minced tuna was 5.7% [12, 16]. Since RTE food are normally consumed without cooking, RTE seafood products such as cooked tuna represent a high risk of causing foodborne illness if they are contaminated with foodborne pathogens and not handled properly during storage, preparation and serving [17]. Guillier *et al.* [9] demonstrated the unabashed growth of two *L. monocytogenes* strains in tuna rillettes, corroborating the need for hurdles to control microbial growth and activity in these ready-to-eat types of seafood.

Natural antimicrobials have promising applications in many fishery products. Sodium lactate (SL) is a GRAS additive that is widely used to enhance flavor, control microbial growth, and increase shelf life of fish products [18, 19]. The use of lactates as antimicrobial agents is primarily due to their ability to reduce pH and water activity. Currently, the addition of SL is allowed at 4.8% for the decontamination of seafood products [20]. Sodium diacetate (SD) and Potassium sorbate (PS), derivatives of acetic and sorbic acid respectively, are used to achieve an antimicrobial effect in baked goods, fats and oils, gravies and sauces, snack foods, meat products, and soups and soup mixes, as well as to flavor these foods [20]. They are also GRAS substances recommended for use at levels not exceeding 0.25 and 0.3% respectively [20]. Nisin is a bacteriocin with significant activity against Gram-positive bacteria and has been approved for use in a wide range of food products [21]. Chitosan is a natural biopolymer derived from deacetylation of chitin, a major component of the shells of crustaceans [22]. Antibacterial activity of chitosan depends on the degree of deacetylation, molecular weight, temperature, pH of the medium and other components presence [23]. Combining antimicrobial agents can result in synergistic effects and a wider antimicrobial spectrum, allowing the use of lower concentrations of individual compounds.

Edible coatings incorporating antimicrobials allow the controlled diffusion and gradual release of embedded antimicrobials onto the food surface [24] and could be an effective means to control the growth of pathogenic and spoilage contaminants [25]. Antimicrobial coatings certainly have overriding advantages over dipping or spraying of foods with antimicrobial solutions, by controlling the release and diffusion of the incorporated bacteriostatic agents into the food matrix [24]. Overall, coatings are thought to help maintain the necessary antimicrobial concentration for a longer period of time on the food surface where contamination is most likely to occur [24, 26]. Neetoo *et al.* [27] found that alginate coatings supplemented with 2.4% SL and 0.25% SD significantly delayed the growth of *L. monocytogenes* in CSS during a 30-day storage at 4°C. Moreover, Ye *et al.* [28] reported that chitosan-coated film containing sodium lactate or potassium sorbate produced a higher inhibition of *Listeria monocytogenes* than chitosan films alone on cold smoked salmon or ham steaks, respectively. Growth of *L. monocytogenes* on the surface of smoked salmon was inhibited by whey-protein films incorporating a lactoperoxidase system [29]. Thus, a multiple-hurdle approach relying on the combination of nisin with other antimicrobials, such as chemical preservatives, is desirable.

Refrigeration and (blast) freezing are the most common industrial means to ensure the quality and longevity of cooked tuna loin products [30]. However, the efficiencies of these processes on inhibiting or retarding the growth of foodborne pathogen such as *L. monocytogenes* in seafood products are limited [30]. Previous outbreaks of listeriosis due to contamination of ready-to-eat seafood indicate that *L. monocytogenes* has the ability to survive at refrigeration and freezing temperatures and cause human infection when the product is consumed [31]. Although there is no official statistics on the incidence of listeriosis in Mauritius, owing to absence of a mandatory notification system, previous investigations have demonstrated the consistent isolation of *L. monocytogenes* from cooked tuna loins [Personal Communication]. Despite considerable efforts to improve process hygiene and sanitation procedures, the complete elimination of *L. monocytogenes* from the processing environments in which cooked tuna is graded and packaged, is currently considered to be practically very difficult, if not impossible [Personal Communication]. Thus, inhibitors that can control the growth of *L. monocytogenes* without adversely affecting taste are needed to prevent *L. monocytogenes* growth for the expected shelf-life of cooked tuna loin products.

The overall objective of this study was thus to evaluate the efficacy of chitosan-based edible coatings incorporating GRAS natural antimicrobials, nisin (N) and sodium lactate (SL), sodium diacetate (SD) or poatssium sorbate (PS) on inhibiting the growth of *L. monocytogenes* and background microbiota on cooked tuna loins.

2. MATERIALS

2.1. Effect of Chitosan-Based Edible Coatings on Controlling the Growth of L. monocytogenes on Cooked Tuna Loins during Refrigerated Storage

2.1.1. Preparation of chitosan-based edible coatings

Ten grams of low molecular weight chitosan (Sigma) were dissolved in 500 ml of 1% (w/v) acetic acid (Sigma) and stirred overnight at room temperature. Fifty ml of the chitosan stock solution was dispensed into five 250 ml-conical flasks. 1.25 g of nisin (Sigma) was added to the chitosan base singly or in binary combination with 30 g of 60% sodium lactate syrup (Sigma), 3.13 g of sodium diacetate (Sigma), or 3.75 g of potassium sorbate.

2.1.2 L. monocytogenes *inoculum preparation*

L. monocytogenes strain ATCC 19115 was used in this study. The strain was maintained on Plate Count Agar (Hi-Media, Mumbai, India) agar plates at 4°C. For growth, a single colony of *L. monocytogenes* was inoculated into a tube of Nutrient Broth (Hi-Media) and incubated at 35°C for 24 h. The culture was then transferred to 10 ml of fresh Nutrient Broth and incubated for 24 h at 35°C to reach an estimated cell density of 10^9cfu/ml. The culture was then serially diluted in 0.1% peptone water (Hi-Media, Mumbai, India) to cell densities of ca. 10^5 cfu/ml, which served as the inoculum.

2.1.3 Samples preparation

Slices of tuna loins were punched aseptically into 5-cm diameter round pieces weighing ca. 25 g. The tuna loin discs were inoculated with 125 µL of the diluted culture of *L. monocytogenes* and left undisturbed for 5 min to allow the inoculum to seep in [32]. The discs were then flipped and inoculated on the other side to achieve an estimated cell density of 3 log cfu/g. After inoculation, the samples were staged in the refrigerator at 4°C for 15 min to allow bacterial attachment [32]. Samples were then coated with a total of 500 µL of the chitosan coating solutions (250 µL on each side) with a drying time of 10 min between each coating application to achieve final concentrations of 500 IU/g, 0.125% w/w, 0.15% w/w and 1.2% w/w for Nis, SD, PS and SL, respectively. The samples were then inserted into vacuum-pouches made of high resistance, high-barrier, multi-layer, co-extruded shrink plastic material with a nominal thickness of 90 µm and maximum oxygen and carbon dioxide transmission rates of 50 and 100 cm^3/m^2/24 hr under standard conditions (Cryovac® SealedAir, North Carolina, USA), vacuum packaged and stored at 4°C for 4 weeks. The populations of *L. monocytogenes* were determined weekly for a period of 4 weeks.

2.1.4 Microbiological analysis

For determination of *L. monocytogenes* counts, pouches of vacuum-packaged samples were opened aseptically and the tuna loin samples were transferred to a sterile stomacher bag and homogenized for 2 min with 225 ml of 0.1% peptone water. Ten-fold serial dilutions were made using 0.1% peptone water. Counts of *L. monocytogenes* were determined by an overlay method [33]. Briefly, the serial dilutions were spread-plated on solidified Plate Count Agar (PCA) and the plates were incubated at 35°C for 3 h. Approximately 7 ml of Oxford agar (Oxoid, UK) tempered at 45°C was overlaid on the PCA plates. The plates were incubated at 35°C and small grey colonies with black haloes on the plates were counted after 48 h [34].

2.2 Effect of chitosan-based edible coatings on controlling the growth of spoilage aerobic and anaerobic bacteria on cooked tuna loins during refrigerated storage

Un-inoculated tuna loin discs prepared as described above were also subjected to the same treatment. All the samples were then vacuum-packaged and stored at 4°C for up to 4 weeks. Un-inoculated samples were analyzed for aerobic and anaerobic counts every week. Anaerobic bacterial counts were determined on Anaerobic Agar (Hi-Media, Mumbai, India) incubated with Anaerobic Gas Packs (BD) for 3 days at 35°C. Aerobic bacterial counts were determined by plating on Plate Count Agar (Oxoid, UK) and incubated aerobically at 35°C for 2 days.

2.3. Statistical analysis

All experiments were conducted in three independent trials. Where appropriate, statistical analyses were conducted using Minitab® Release 17 (Minitab Inc, USA). A single factor analysis of variance (ANOVA) and Tukey's one-way multiple comparisons were conducted to determine differences in bacterial population. Significant differences were considered at the 95% confidence level ($P < 0.05$).

3. RESULTS & DISCUSSION

3.1 *Effect of chitosan-based edible coatings incorporating nisin to inhibit the growth of* L. monocytogenes *on cooked tuna loins*

Figure 1 shows the effect of chitosan-based edible coatings containing nisin alone or in combination with other antimicrobials on the growth of *L. monocytogenes* on cooked tuna loins. The mean initial concentration of *L. monocytogenes* on inoculated tuna loin samples was 3.4 log cfu/g. In the control sample, *L. monocytogenes* grew to 7.3 log cfu/g after 4 weeks of storage at refrigerated temperature (4°C). The 'chitosan-only' and 'Chitosan + Nis' coatings did not appreciably slow down the growth of *L. monocytogenes*, which reached a mean of 6.3-6.4 log cfu/g at the end of the storage period. In fact, the population of *L. monocytogenes* in treated samples with the 'chitosan-only' coating and the 'chitosan + Nis' coatings were not significantly different from those of the control sample ($P > 0.05$). On the other hand, the binary antimicrobial treatments (Nis + SL, Nis + SD and Nis + PS) halted growth of the pathogen over the first week and significantly reduced the initial counts by an average of 1.0-1.5 log cfu/g after two weeks following application ($P < 0.05$). The combination of Nis + SL was most effective, reducing the population of *L. monocytogenes* by a maximum of 1.5 log cfu/g relative to the initial inoculum level. After the four weeks of refrigerated storage, *L.*

monocytogenes grew on cooked tuna samples coated with Nis + SL, albeit slowly, reaching 4.1 log cfu/g which was 3.2 log cfu/g significantly lower relative to the control (7.3 log cfu/g) ($P < 0.05$).

3.2 *Effect of chitosan-based edible coatings incorporating nisin to inhibit the growth of aerobic and anaerobic spoilage microflora on tuna loins*

Figures 2 and 3 depict the growth of spoilage aerobic and anaerobic bacteria on un-inoculated cooked tuna loin discs, respectively. Spoilage aerobic and anaerobic bacteria grew very rapidly, increasing by 6.2 log cfu/g and 7.5 log cfu/g after 4 weeks of storage. Antimicrobial coatings slowed down their growth to varying degrees. Chitosan coatings incorporating Nis, singly or in combinations exhibited a bacteriostatic effect with virtually no increase in aerobic and anaerobic bacterial density over the first week. Although the spoilage flora continued to increase over the subsequent weeks, the bacterial populations were consistently lower than the other untreated controls throughout the storage period studied. Of all the antimicrobial treatments applied, the most effective were chitosan coatings incorporating Nis + SL, Nis + SD and Nis + PS, which resulted in final populations of aerobes and anaerobes that were 2.0-2.2 and 1.4-1.7 log cfu/g lower than the controls, respectively although the differences were not statistically significant ($P > 0.05$). Taken together, background aerobic and anaerobic microorganisms proliferated rapidly in untreated tuna loins and resulted in spoilage of the product within < 3 weeks, assuming a population of 7 log cfu/g as the spoilage limit. On the other hand, binary antimicrobial treatments were able to delay the growth of aerobic and anaerobic spoilage bacteria, thereby extending the shelf-life of cooked tuna loins from 3 to > 4 weeks.

4. DISCUSSION

There has been increasing concern about the presence of *L. monocytogenes* in tuna products [12, 16] resulting in product recalls or foodborne disease outbreaks. The inherent antilisterial effect of chitosan has already been demonstrated elsewhere [35]. Roberts and Greenwood [36] compared the antimicrobial activity of chitosan edible film, dissolved in lactic acid or acetic acid against *L. monocytogenes* on RTE roast beef. The author revealed that the solvent acetic acid used in the making of the chitosan coating was more effective in reducing *L. monocytogenes* counts than lactic acid-based coating. Roberts and Greenwood [36] also compared the antimicrobial activity of low and high molecular weight chitosan on roast beef and concluded that there was an improvement in the microbial quality of the roast beef when treated with chitosan coating and also that higher molecular weight polymers had slightly lower antibacterial properties. Farajzadeh *et al.* [37] investigated the effect of chitosan-gelatin coating on the quality of shrimp (*Litopenaeus vannamei*) under refrigerated condition and observed that the chitosan-gelatin coating exhibited significant antibacterial activity by delaying the growth of total and psychrotrophic bacteria with inhibition greater than 3 log cycles at the end of storage. Moreover, the author demonstrated that chitosan-gelatin coating significantly improved texture and color properties of shrimp compared with uncoated samples and extended the shelf-life of the product by 6 days.

Contrary to findings reported by Farajzadeh *et al.* [37] and Roberts and Greenwood [36], we failed to demonstrate any appreciable and sustained inhibitory effect of the acetic acid-based chitosan coating against both *L. monocytogenes* and background microflora on cooked tuna loins. It is worth mentioning that the 'chitosan-only' coating was weakly bacteriostatic during the first week of storage by reducing the population of *L. monocytogenes* and aerobic flora by 1.0-1.3 log cfu/g relative to the control, although they grew more rapidly over the subsequent weeks. Devlighere *et al.* [23] and Coma *et al.* [38] also investigated the antimicrobial effect of low molecular weight chitosan, against *L. monocytogenes* and both authors found that *L. monocytogenes* was only weakly susceptible to chitosan. Since chitosan did not demonstrate any appreciable inhibitory activity, other food-grade antimicrobials were included in the chitosan coating. Ma *et al.* [39] studied the effectiveness of chitosan coatings supplemented with lauric arginate (LAE), cinnamon oil (CO) and EDTA in controlling the growth of foodborne pathogens *Salmonella*, *E. coli* O157:H7 and *Listeria monocytogenes*. Chitosan coating with 0.1% LAE, 0.1% EDTA, and 1% CO was the most effective and brought about a > 3 log CFU/cm^2 reduction of *Escherichia coli* O157:H7 and *Listeria monocytogenes* immediately after coating and reduced *Salmonella enterica* to below the detection limit during a 14-day storage. Total molds and yeasts also were reduced to the detection limit by the coating.

In our study, nisin was incorporated in the chitosan base singly or in binary combinations with other salts of organic acid. Nisin alone showed a slight degree of antilisterial activity however, when combined with additional antimicrobials, the formulations considerably delayed growth of *L. monocytogenes*, aerobic and anaerobic spoilage flora. Indeed, the antilisterial activity of nisin used in combination with chitosan has been demonstrated previously. In addition, the activity of nisin alone or in combination with salts of organic acids including SL, SD and/or PS embedded in other edible coatings was also shown to be variably effective on turkey [40], cold smoked salmon [27], ham steaks [28] and a meat model system [41]. Several authors have reported that antimicrobials and antimicrobial coatings had minimal adverse sensorial impact on the overall consumer acceptance of coated products. Zhu *et al.* [42] demonstrated that SL addition to ready-to-eat products brought about minimal impact on the sensory quality. We thus anticipate that the application of chitosan incorporating organic salts onto cooked tuna loins would present minimal sensory concern. Additional research is however required in the future to evaluate the effects of these combinations on the sensory quality and physicochemical characteristics of cooked tuna loins.

5. CONCLUSION

In this study, the bacteriostatic effect of chitosan-based edible coatings incorporating GRAS antimicrobials against *Listeria monocytogenes* was investigated on refrigerated cooked tuna loins. Different chitosan coating formulations were tested but failed to demonstrate any sustained inhibitory effect against both *L. monocytogenes* and background microflora on cooked tuna loins. The coating incorporating Nis +SL reduced the population of *L. monocytogenes* by 1.5 log cfu/g relative to the control, although they grew more rapidly over the subsequent weeks. In addition, acetic-acid based chitosan coating incorporating Nis + SL was able to extend the shelf-life of the product from 3 to > 4 weeks assuming the onset of spoilage occurs at 7 log cfu/g. Findings of this research highlight the usefulness of antimicrobial edible coatings as a minimal processing technology to enhance the microbiological safety and quality of cooked tuna products.

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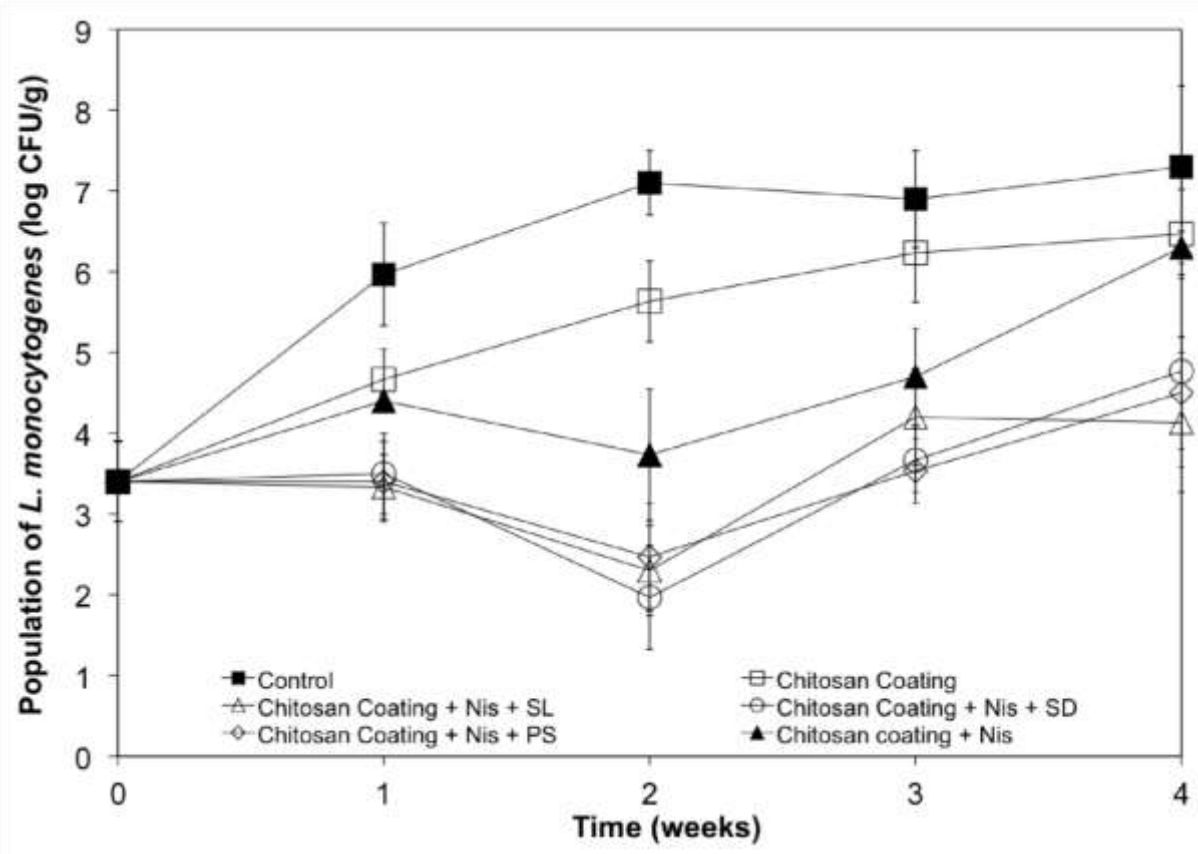

Figure 1: Effect of chitosan-based edible coatings incorporating GRAS antimicrobials on the growth of *L. monocytogenes* on vaccum-packaged cooked tuna loin stored at 4°C

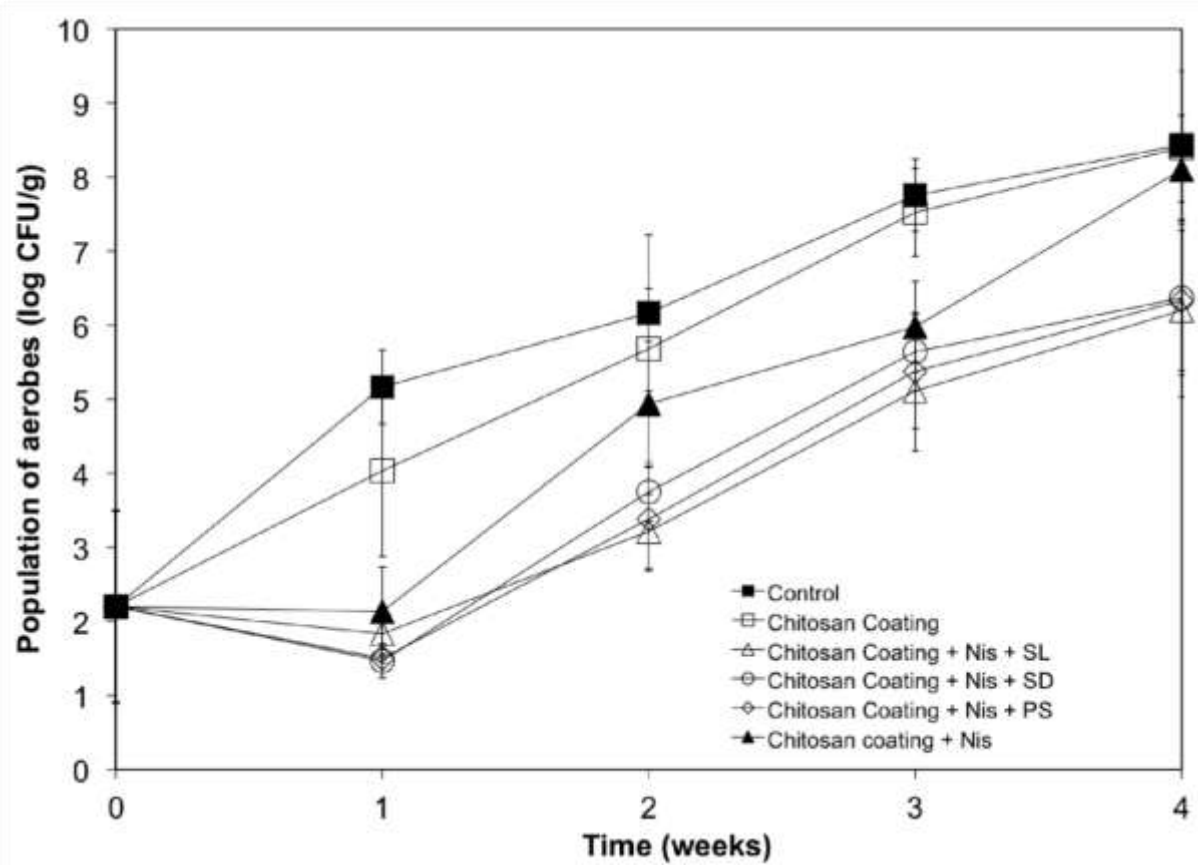

Figure 2: Effect of chitosan-based edible coatings incorporating GRAS antimicrobials on the growth of aerobic spoilage bacteria on vaccum-packaged cooked tuna loin stored at 4°C

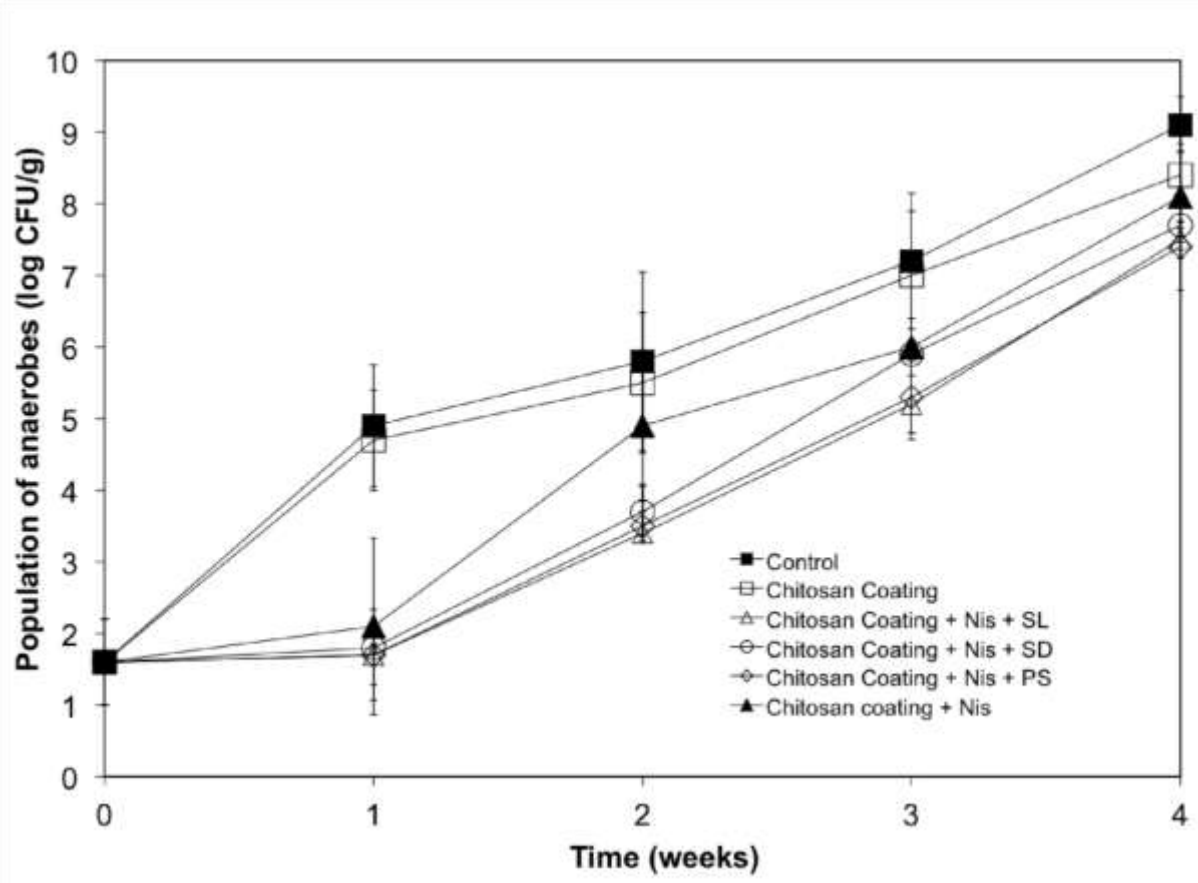

Figure 3: Effect of chitosan-based edible coatings incorportaing GRAS antimicrobials on the growth of anaerobic spoilage bacteria on vaccum-packaged cooked tuna loins stored at 4°C