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Inhibitory effect and cell damage on bacterial flora of fish caused by chitosan, nisin and sodium lactate

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ABSTRACT

The effect of the combined use of chitosan, nisin and sodium lactate on the growth of *Listeria innocua*, *Shewanella putrefaciens* and psychrophilic bacteria isolated from fish was investigated in broth by means of minimum inhibitory concentrations (MIC). Furthermore, the sites of cell-injury caused by mentioned antimicrobials and their combinations on *L. innocua* and *S. putrefaciens* were studied. MIC of antimicrobial mixtures were evaluated by Berembaum design and check board method. Antimicrobials' sites of injury were investigated by the evaluation of cell constituents' release, cell surface hydrophobicity and differential scanning calorimetry. Results depended on antimicrobial used; several combinations inhibited the growth of *L. innocua* and *S. putrefaciens* and all combinations inhibited psychrophilic bacteria. Besides, some mixtures showed synergistic effects. All the mixtures affected ribosomes and DNA of the studied bacteria. Regarding cellular envelope, antimicrobials acted according to the structural characteristics of target microorganisms. Cell damage was higher when antimicrobials were combined, which could explain the observed synergistic effects. This study demonstrates and justifies the synergistic action of chitosan, nisin and sodium lactate on the inhibition of microorganisms related to fish spoilage and remarks the promissory use of the synergic combination of antimicrobials for fish preservation.

1. Introduction

Generally, fish meat is sterile while it is alive. However, a large number of bacteria are found on the outer surface, scales, gills and intestine [1]. Microbial flora of fish is dominated by Gram negative and psychrophilic bacteria, belonging to the genera *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Flavobacterium* and *Shewanella*. During the capture and handling of fish, the muscle is colonized by these microorganisms. Microbial contamination causes fish deterioration and leads to the end of its shelf-life when reaches levels between 10^7 and 10^9 CFU/g [2].

Listeria monocytogenes represents one of the most important foodborne pathogens, since once it enters to an animal or human host, it can cause severe problems [3]. *L. monocytogenes* is a facultative anaerobic, Gram positive bacterium, which is able to survive to a wide spectrum of adverse conditions, such as acidic pH, low temperatures and high concentrations of sodium chloride. Moreover, *L. monocytogenes* could form biofilms which increase the resistance of bacteria against environmental stresses [4]. Consequently, the growth of this microorganism is difficult to control in foods, especially in ready to eat ones [5].

Shewanella putrefaciens, a Gram negative, psychrotrophic bacterium, is one of the principal microorganisms responsible for fish spoilage [6]. Fish flesh provides an enabling environment where *S. putrefaciens* is able to grow and to produce metabolites that cause intensive off-odors and the consequent rejection of the product [7]. This bacterium is pH sensitive and its growth rate decreases at acidic pH [2]. Furthermore, it has been reported that *S. putrefaciens* is able to attach and form biofilms on food processing surfaces [8] and that it retains pathogenic potential, since infections and bacteremia caused by this microorganism have been observed [9].

Aforementioned microorganisms are often found associated to fishery products. The use of many natural antimicrobials has been proposed to increase shelf-life of seafoods and reduce the occurrence of foodborne diseases caused by *L. monocytogenes* [10]. Chitosan, nisin and sodium lactate show different properties which make them useful in food preservation. These preservatives are

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Table 1
Modified Berembaum design.

Experiment	Antimicrobials			Microorganism					
	Chitosan	Nisin	Sodium lactate	*L. innocua*		*S. putrefaciens*		Psychrophilic bacteria	
	MIC fractions			Growth	FIC_I	Growth	FIC_I	Growth	FIC_I
1	1	0	0	–	1	–	1	–	1
2	0	1	0	–	1	–	1	–	1
3	0	0	1	–	1	–	1	–	1
4	0	1/3	1/3	–	0.66	–	0.66	–	0.66
5	1/3	0	1/3	–	0.66	–	0.66	–	0.66
6	1/3	1/3	0	–	0.66	–	0.66	–	0.66
7	1/3	1/3	1/3	–	1	–	1	–	1
8	1/6	1/6	1/6	+		+		–	0.5
9	1/6	1/6	1/3	+		+		–	0.66
10	1/3	1/6	1/6	–	0.66	–	0.66	–	0.66
11	1/6	1/3	1/6	–	0.66	–	0.66	–	0.66
12	1/12	1/12	1/3	+		+		–	0.5
13	1/3	1/12	1/12	+		–	0.5	–	0.5
14	1/12	1/3	1/12	–	0.5	–	0.5	–	0.5
Control	–	–	–	+		+		+	

obtained from natural sources and are all generally recognized as safe compounds [11,12]. It has been reported that all of them are able to inhibit the growth of *Listeria innocua* and *S. putrefaciens*, and that the combined use of chitosan with nisin or sodium lactate may cause synergistic effects [13]. In order to optimize their application, it might be useful to explore their effect when combined in tertiary mixtures, and also, their sites of injury on bacterial flora of fish. Regarding the last purpose, the evaluation of cell constituents' release, cell surface hydrophobicity and differential scanning calorimetry (DSC) thermograms may provide worthwhile information. Cell constituents' release is considered as an indicative value of cell membrane damage. Cell constituents are capable of absorbing at 260 nm [14]. Therefore, an increase in absorbance is linked with the increase of intracellular constituents' loss, as a result of the activity of antimicrobials or other stress factors on cell membrane [15]. Moreover, hydrophobicity of bacterial cell surface is related to the structure and composition of bacteria membrane, as a consequence a change in hydrophobicity implies that membrane structure has been altered [16]. Differential scanning calorimetry can be used to characterize the thermal transitions of bacterial components. When microorganisms are heated, endothermic peaks are observed, which correspond to the denaturation of cell components [17,18]. Changes on thermograms obtained indicate that cellular components have been affected.

The aim of this study was to investigate the effect of the combined use of chitosan, nisin and sodium lactate on the growth of *L. innocua* (a surrogate bacterium for *L. monocytogenes*), *S. putrefaciens* and psychrophilic bacteria isolated from fish in laboratory media. On a second stage, the sites of injury of mentioned antimicrobials and binary or tertiary combinations of them on *L. innocua* and *S. putrefaciens* were identified.

2. Materials and methods

2.1. Bacterial strains, culture conditions and antimicrobial agents

L. innocua was used in this study because of its close genetic relationship with *L. monocytogenes* and as a consequence its similar response to stress factors [19]. *L. innocua* 6a ATCC 33090, *S. putrefaciens* ATCC 8071 and psychrophilic bacteria isolated from vacuum-packed grouper (*Epinephelus marginatus*) filets stored at 6 °C for 14 days, were stored at −30 °C in Mueller Hinton broth (Biokar Diagnostics, Beauvais, France) plus 10% glycerol (Sintorgan S.A., Buenos Aires, Argentina) and 10% skim milk. Before being used, they were grown twice in Mueller Hinton broth at 30 °C for 18 h. Psychrophilic bacteria were isolated from grouper filets, as previously described [20].

Chitosan (Sigma, USA) -deacetylation degree 85%-, nisin (added in the form of Nisaplin, Danisco A/S DK, Denmark) and sodium lactate (Parafarm, Buenos Aires, Argentina) were used in this study. Antimicrobials solutions were prepared according to Schelegueda, Gliemmo and Campos [13]. Solution pH values were adjusted to 5.50 using 10% w/w citric acid (Parafarm, Buenos Aires, Argentina) or 0.4 mol/l NaOH (Parafarm, Buenos Aires, Argentina).

2.2. Antimicrobials interactions

The evaluation of the existence of interaction among antimicrobials at pH 5.50 was performed by the microdilution method described by Schelegueda et al. [13] using as indicator strains of *L. innocua* and *S. putrefaciens*. Furthermore, the study was conducted using psychrophilic bacteria isolated from fish in order to illustrate the effect of antimicrobial mixtures on indigenous bacteria commonly found in refrigerated fishery products. Tested concentrations were selected using modified Berembaum design [21] (Table 1), which includes minimum inhibitory concentrations (MIC) of each antimicrobial and combinations of sub-inhibitory concentrations. The MIC of each antimicrobial had been determined in previous studies: 96 μg/g chitosan, 1183 IU/ml nisin and 18,000 μg/g sodium lactate against *L. innocua*; 125 μg/g chitosan, 1075 IU/ml nisin and 19,600 μg/g sodium lactate against *S. putrefaciens* [13]; and 100 μg/g chitosan, 1075 IU/ml nisin and 18,000 μg/g sodium lactate against psychrophilic flora of fish. Microplates were incubated at 30 °C for 24 h. At the beginning and at the end of incubation, after a slight stirring, the absorbance of each microplate well was measured at 600 nm, using a microplate reader commanded by the program Gen5 Data Analysis Software (Reader Control and Data Analysis Software, BioTek Instruments, ELx808, USA). The absorbance of the negative controls was used as blank. Inhibition was considered when a variation of less than 0.1 in the absorbance value was observed. Antimicrobials MIC were used to graph the isobolograms and to calculate fractional inhibitory concentrations (FIC) which express the value of the MIC of an antimicrobial when is combined ($MIC_{A\text{-}BC}$ or $MIC_{B\text{-}AC}$ or $MIC_{C\text{-}AB}$) divided by the MIC of this antimicrobial used alone (MIC_A or MIC_B or MIC_c). Then, the FIC index $[FIC_I = (MIC_{A\text{-}BC}/MIC_A) + (MIC_{B\text{-}AC}/MIC_B) + (MIC_{C\text{-}AB}/MIC_C)]$, determines the type of interaction among the antimicrobials. A FIC index value near to 1 indicates an additive effect; if it is less than 1, it indicates synergism; and if it is greater than 1, the interaction is antagonist [22].

Table 2
System composition for cell injury determination.

System	Composition		
	Chitosan (μg/g)	Nisin (IU/ml)	Sodium lactate (μg/g)
Control	–	–	–
Chitosan	200	–	–
Nisin	–	2,000	–
Sodium lactate	–	–	36,000
Chitosan-nisin	200	2,000	–
Chitosan-sodium lactate	200	–	36,000

After MIC evaluation, minimum bactericidal concentrations (MBC) were determined according to Schelegueda et al. [13].

2.3. Identification of sites of injury

The identification of sites of injury promoted by the use of chitosan, nisin and sodium lactate were performed only on *L. innocua* and *S. putrefaciens* because of the need to work with inbred strains for the correct interpretation of the results.

2.3.1. Cell constituents' release

L. innocua and *S. putrefaciens* were grown in Mueller Hinton broth for 18 h at 30 °C in order to obtain an inoculum of 10^9 CFU/mL. To separate the growth medium, aliquots of each culture were centrifuged (8000 × *g*, for 15 min at 4 °C). Then, each bacterium was washed and resuspended in Ringer's solution (Biokar Diagnostics, Beauvais, France) at pH 5.50. Antimicrobials were added according to Table 2. Used concentrations were twice the previously reported MIC [13]. Systems were stored at 30 °C for 24 h under agitation. Samples were taken at the beginning and after 3 and 24 h of incubation. Bacteria were pelleted twice by centrifugation (8000 × *g*, for 15 min at 4 °C) and cell-free supernatants were obtained. Their absorbances were measured at 260 nm by means of a spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Uninoculated systems were used as blanks.

2.3.2. Cell surface hydrophobicity

Systems were prepared according to Section 2.3.1 and incubated at 30 °C for 3 h (Table 2). After incubation, cell surface hydrophobicity was obtained according to the method described by Gliemmo et al. [23].

It must be stressed that this assay was not carried out in systems containing nisin since phases could not been separated because of nisin emulsifier action. This activity had been previously reported and it is linked with its amphiphilic properties [24].

2.3.3. Differential scanning calorimetry

Differential scanning calorimetry determinations were performed according to Schelegueda et al. [25]. Briefly, pellets obtained from cell constituents' release assay after 24 h of incubation were washed twice with sterile distilled water and centrifuged again. Then, an aliquot of 5–15 mg was transferred into stainless steel DSC crucibles. Pellets water contents were determined drying the samples at 105 °C as 80–85% on wet basis. An empty crucible was used as reference. The latter and the samples were heated in the calorimeter (Q100, TA Instruments Waters, USA) at 3 °C/min from 5 to 150 °C.

2.4. Statistical analyses

Microdilution method assays were carried out by six fold, obtaining the same results for each trial. Data obtained from the study of cell constituents' release were analyzed by two-way repeated measures ANOVA, while data obtained from cell surface hydrophobicity determination were analyzed by one-way ANOVA. Both of them were followed by Tukey's multiple comparison tests. Experiments were conducted by six fold. Data were processed using the statistical program Statgraphics (Statgraphics Plus for

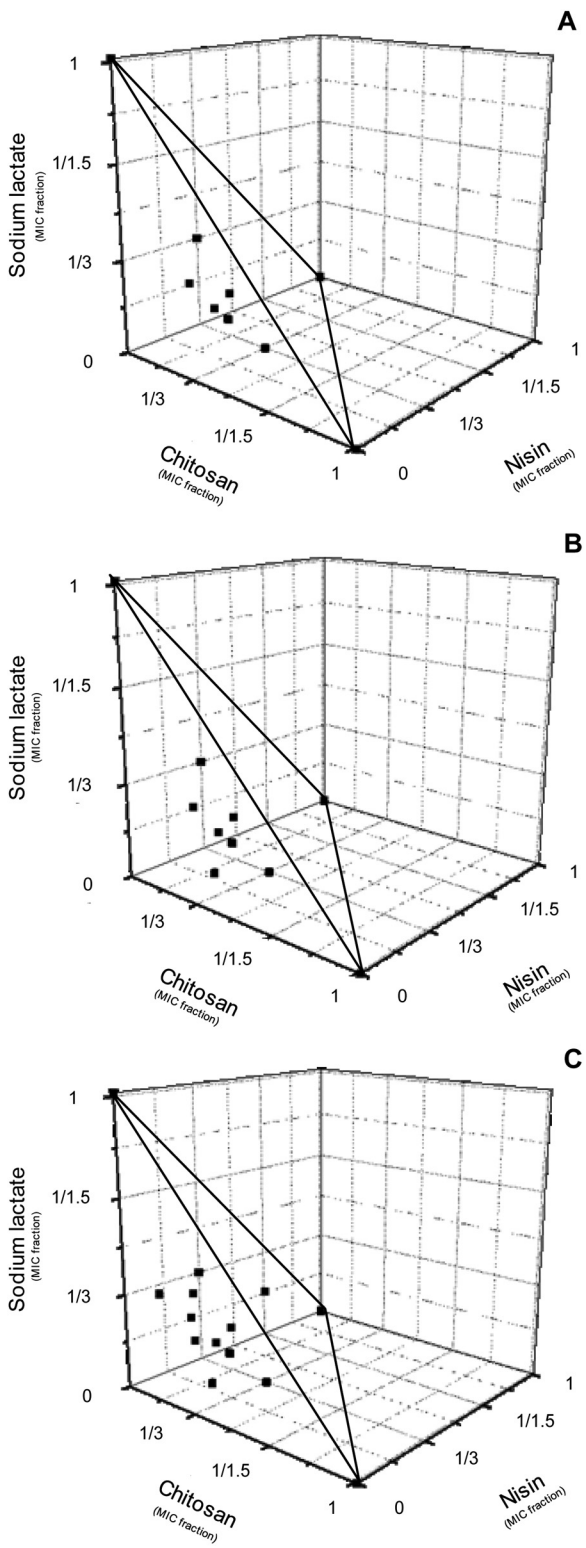

Fig. 1. Isobolograms of chitosan, nisin and sodium lactate MIC against *L. innocua* (panel A), *S. putrefaciens* (panel B) and psychrophilic bacteria isolated from fish (panel C). Dots represent the composition of antimicrobial mixtures that exhibited a synergistic effect.

Windows, version 5.1, 2001, Manugistics, Inc., Rockville, Maryland, USA). The significance level was 0.05%. Differential scanning calorimetry analyses were conducted in duplicate. Same results were obtained in each replicate.

3. Results

3.1. Antimicrobials interactions

L. innocua growth was inhibited by some of the antimicrobials combinations tested (Table 1; Fig. 1, panel A). The use of 1/3 of chitosan or nisin MIC, in combination with 1/6 of other two antimicrobials inhibited the growth of *L. innocua*. However, 1/3 of sodium lactate MIC combined with 1/6 of chitosan and nisin MIC did not inhibit *L. innocua* growth. These results suggest that inhibitory action of sodium lactate on *L. innocua* is less effective than the action of chitosan or nisin. The greatest synergistic effect was achieved by 1/3 of nisin MIC in combination with 1/12 of chitosan and sodium lactate MIC. Similar trends were observed for *S. putrefaciens* (Table 1; Fig. 1, panel B). The addition of 1/3 of chitosan MIC combined with 1/12 of nisin and sodium lactate MIC also inhibited the growth of the latter bacterium. All combinations tested were able to inhibit the development of psychrophilic flora isolated from fish (Table 1; Fig. 1, panel C). The greatest synergistic effects were found when 1/6 of each antimicrobial MIC were combined or using 1/3 of one antimicrobial MIC in combination with 1/12 of the two remaining MIC. It must be noted that the use of 1/3 of sodium lactate MIC combined with 1/12 of chitosan and nisin MIC was enough to cause the highest inhibition, suggesting that psychrophilic flora isolated from fish is more sensitive to the studied antimicrobials than the collection strains evaluated. Despite the inhibitory effect of different mixtures of antimicrobials, no bactericidal effect was found since all bacteria were able to grow in agar free of additives (data not shown). This trend suggests that studied antimicrobials must be combined with other stress factors in order to cause bacterial inactivation.

3.2. Identification of sites of injury

3.2.1. Cell constituents' release

No differences in absorbance values of different treatments were found at the beginning of incubation. The use of nisin, alone or combined with chitosan, increased cell constituents' release of both bacteria during the incubation (Fig. 2). Regarding *L. innocua*, the highest value was observed when the mixture was used (Fig. 2, panel A); however, no differences between the effects of the bacteriocin alone or combined with chitosan were found in the case of *S. putrefaciens* (Fig. 2, panel B). When it comes to the addition of chitosan, no differences were detected between the absorbance of treated and untreated samples (data not shown). Sodium lactate, and its combination with chitosan, did not exert any effect on *L. innocua* cell constituents' release (data not shown). However, regarding *S. putrefaciens*, the treatment with sodium lactate alone produced a slight increase (Fig. 2, panel B). It must be noted that the greatest increase of the cell constituents' release was observed when the tertiary mixture was added.

3.2.2. Cell surface hydrophobicity

Hydrophobicity values of untreated bacteria (incubated in Ringer's solution) were approximately 40%. No changes were observed when *L. innocua* and *S. putrefaciens* were incubated in presence of sodium lactate (Fig. 3). However, when bacteria were treated with sodium lactate and chitosan, significant increases were found (Fig. 3). Regarding the treatment with chitosan alone, two different trends were observed. *L. innocua* showed a hydrophobicity increase similar to these produced by the mixture, while *S. putrefaciens* did not shown any change with respect to control samples (Fig. 3).

3.2.3. Differential scanning calorimetry (DSC)

Thermograms obtained by DSC were studied to identify other sites of injury on *L. innocua* and *S. putrefaciens* (Fig. 4). When it comes to *L. innocua*, three major peaks were observed when untreated samples were heated. One of them (peak A) corresponds to thermal denaturation of ribosomes. The second one (peak B), is a consequence of the melting of DNA; while the last one (peak C), is associated with cell wall components [17]. Thermograms obtained from *L. innocua* treated with antimicrobials showed changes on temperature and/or area of mentioned peaks. The temperature range and the percentage reduction of the peaks area are shown in Table 3. Although a fourth peak, associated with the outer membrane, was reported when Gram negative bacteria were studied by DSC [17], three peaks were observed on untreated *S. putrefaciens* samples. It must be noted that the third peak spanned a wider temperature range than the one obtained in the case of *L. innocua*. Changes observed in the thermograms when *S. putrefaciens* was treated with antimicrobials are summarized in Table 3.

4. Discussion

Results suggest that the combined use of the tertiary mixture of chitosan, nisin and sodium lactate produces greater synergistic effects than those obtained in the presence of binary mixtures or by each antimicrobial alone. Through the studies of cell constituents'

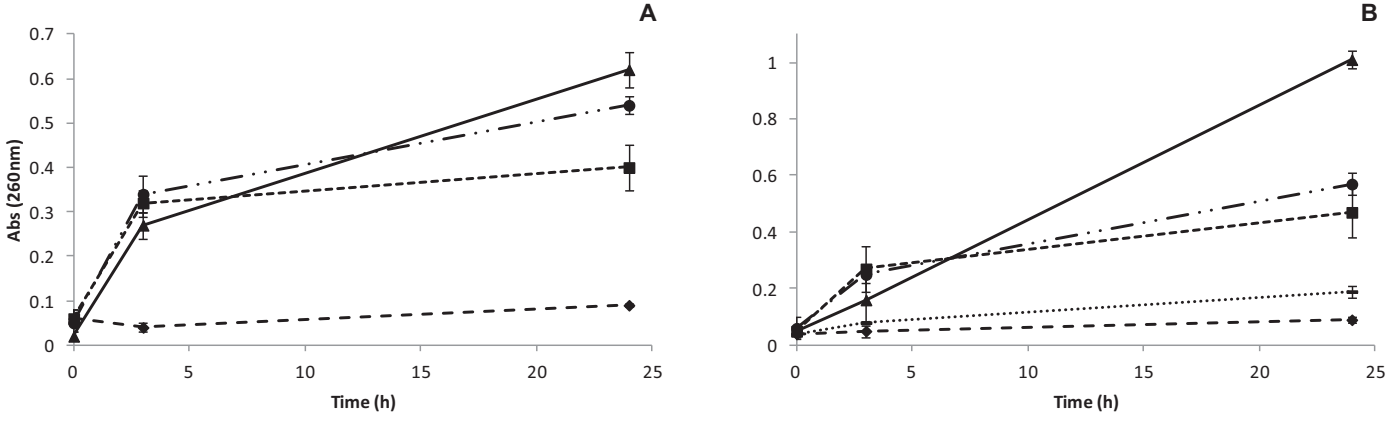

Fig. 2. Cell constituents' release of *L. innocua* (panel A) and *S. putrefaciens* (panel B). – ◆ – control, ...▬... sodium lactate, –■– nisin, —●— chitosan and nisin, —▲— chitosan, nisin and sodium lactate. Treatments not shown were not significantly different from control samples (α: 0.05%).

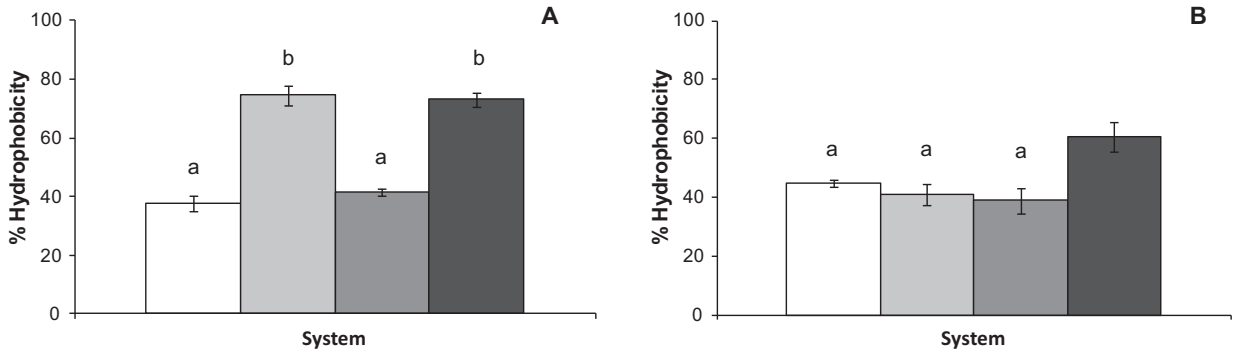

Fig. 3. Cell surface hydrophobicity of *L. innocua* (panel A) and *S. putrefaciens* (panel B). □ control, ▒ chitosan, ▒ sodium lactate, ■ chitosan and sodium lactate. Error bars represent the standard error. Columns with the same letter are not significantly different (α: 0.05%). Columns without letters are significantly different (α: 0.05%).

release, cell surface hydrophobicity and DSC it was possible to identify the sites of action of mentioned antimicrobials and to explain the interactions among them.

Chitosan, nisin and sodium lactate and the binary or tertiary combinations acted on *L. innocua* and *S. putrefaciens* according to the structural characteristics of the microorganisms.

Chitosan caused a reduction in size and temperature of the peaks corresponding to ribosomes and DNA of both bacteria. Its presence affected the cell surface hydrophobicity and reduced the apparent enthalpy of the peak associated with cell membrane of *L. innocua*, while the opposite effect was observed in *S. putrefaciens*. Results suggest that chitosan may cause damage in ribosomes and DNA, but that its action on the cell envelope depends on the characteristics of the target microorganism. It has been reported that Gram negative bacteria are less sensitive to chitosan than Gram positive bacteria [26]. Moreover, Gram negative bacteria showed an additional layer of material and a thickened cell envelope when treated with chitosan [27]. Mentioned thickening may be responsible for the increase of the peak corresponding to *S. putrefaciens* envelope. Interaction between chitosan and microbial cell membranes with the subsequent leakage of proteinaceous and other intracellular constituents due to a change in permeability has been postulated as a possible mechanism of action [28]. However, this effect was not observed. Probably, the concentration tested was not enough to damage *L. innocua* and *S. putrefaciens* cell membrane, which is consistent with the results obtained by Helander et al. [27].

As expected, nisin showed a strong action on *L. innocua* cell membrane. The ability of nisin for inhibiting Gram positive bacteria, in particularly its antilisterial action, has been widely reported [29]. It is well known that nisin forms pores and disrupt the membrane leading to leakage of intracellular material [30]. In the case of Gram negative bacteria, nisin is not effective unless the outer membrane is disrupted [29]. Citric acid used as acidulant may have caused this effect, allowing nisin to act on *S. putrefaciens*. Moreover, according to thermograms, nisin would affect intracellular components such as ribosomes and DNA.

Lactates action on microbial growth is related with the action of their undissociated form and by their ability to depress water activity [31]. Furthermore, lactates are able to disrupt Gram negative bacteria outer membrane [32]. The first is in concordance with the reduction of peaks corresponding to ribosomes and DNA observed in the thermograms; while the latter may explain the increase in the cellular constituents' release and the disappearance of the peak corresponding to cell envelope of *S. putrefaciens*.

The combined use of chitosan and nisin produced changes in the cell envelopes of both bacteria, which resulted in the highest increase of the cell constituents' release and reduction of thermograms peaks. Presence of chelating agents, such as chitosan, leaks the outer membrane of Gram negative bacteria by removing divalent cations which confer stability [29,33]. Nisin is able to act on Gram negative bacteria when the outer membrane is disrupted, which is the case of *S. putrefaciens* at pH 5.50 or in the presence of chitosan. Moreover, Cai et al. [34], observed by Fourier Transform Infrared Spectroscopy that chitosan and nisin formed complexes through electrostatic interactions between the protonated amino group of chitosan and the carboxilate ion of nisin. This

Table 3
Effects of antimicrobials on bacteria thermal transitions.

		Peak A		Peak B		Peak C	
		T (°C)	Reduction of peak area (%)	T (°C)	Reduction of peak area (%)	T (°C)	Reduction of peak area (%)
L. innocua	Control	58–72		80–91		92–99	
	Chitosan	60–70	76	72–80	74	91–97	44
	Nisin	ND		87–95	43	96–101	50
	Sodium lactate	60–78	72	79–84	94	87–93	6
	Chitosan-nisin	ND		87–96	23	ND	
	Chitosan-sodium lactate	ND		83–93	42	ND	
	Chitosan-nisin-sodium lactate	ND		ND		ND	
S. putrefaciens	Control	50–73		78–86		101–119	
	Chitosan	64–67	68	76–81	66	95–118	*
	Nisin	62–74	82	78–84	4	108–118	82
	Sodium lactate	61–77	66	83–96	87	ND	
	Chitosan-nisin	ND		71–92	37	ND	
	Chitosan-sodium lactate	ND		87–95	45	101–136	*
	Chitosan-nisin-sodium lactate	ND		ND		ND	

ND: Not detectable.
* An increase of peak area was observed.

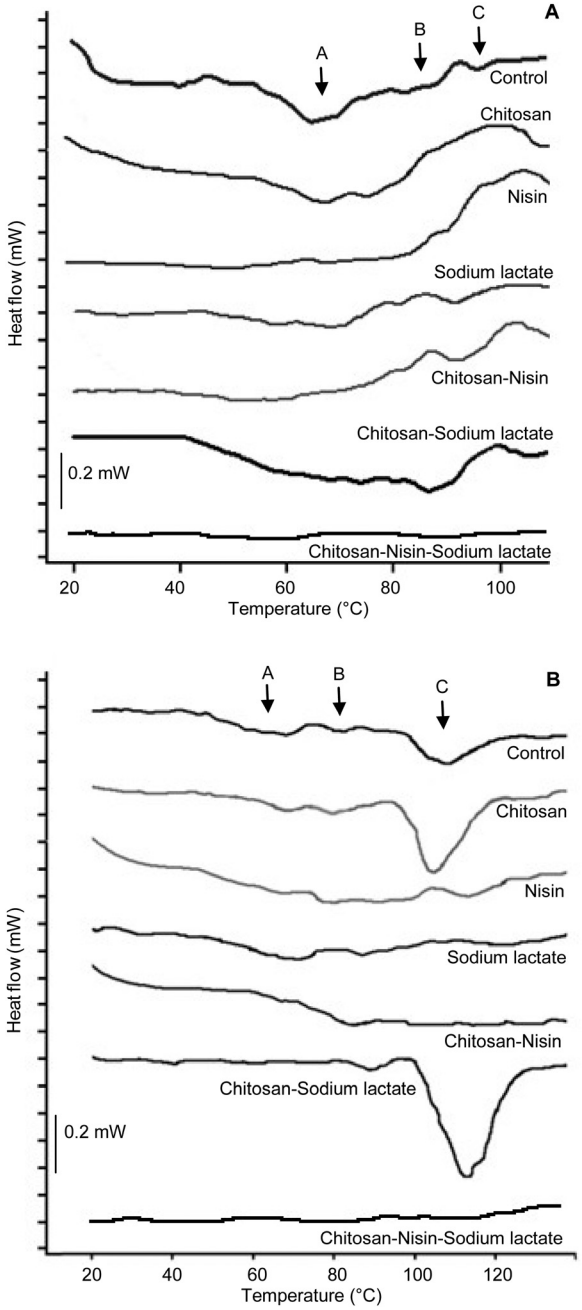

Fig. 4. Thermograms obtained by differential scanning calorimetry (DSC) of *L. innocua* (panel A) and *S. putrefaciens* (panel B).

complex had higher antimicrobial activity than each antimicrobial alone.

The mixture of chitosan and sodium lactate produced an increase of cell surface hydrophobicity of both bacteria, suggesting that antimicrobials act cooperatively. However, the increase of the cell constituents' release of *S. putrefaciens* observed when sodium lactate was used alone was disappeared by chitosan addition. It is possible that the thickening of cell envelope caused by the presence of chitosan produced masking of results related to the quantification of the loss of intracellular material. Besides, this thickening would be reflected in the increase of the peak corresponding to the cell envelope of *S. putrefaciens*. As in all other cases presented above, the mixture of chitosan and sodium lactate affected cell ribosomes and DNA, a fact that is reflected in the changes observed in thermograms.

The combined use of chitosan, nisin and sodium lactate increased the cell constituents' release of both bacteria, suggesting that the damage on the cell envelope was greater than in the presence of antimicrobials used alone or in binary combinations. Furthermore, no peaks were detected in their thermograms, indicating that the effect of the tertiary mixture on DNA of bacteria was higher than those observed in the case of binary mixtures.

5. Conclusions

This study demonstrated the synergistic action of different combinations of chitosan, nisin and sodium lactate on the inhibition of *L. innocua*, *S. putrefaciens* and psychrophilic bacteria isolated from fish. Different tertiary combinations exhibited inhibitory action. The greatest synergistic effect on *L. innocua* and *S. putrefaciens* inhibition was achieved using 1/3 of nisin MIC in combination with 1/12 of chitosan and sodium lactate MIC. The evaluation of cell constituents' release, cell surface hydrophobicity and thermograms obtained by DSC suggest that antimicrobials affected ribosomes and DNA of the studied bacteria. Regarding cellular envelope, antimicrobials acted according to the structural characteristics of the microorganisms. The action of nisin on *L. innocua* cell membrane was evident when the cell constituents' release was evaluated. Regarding to chitosan, it also seems to act on the cell surface, but in a smaller magnitude than nisin, since cell constituents' were not released but hydrophobicity was increased. Sodium lactate would pass the cell membrane and acts in the cytoplasm. When it comes to *S. putrefaciens*, nisin and sodium lactate would be able to disrupt the outer membrane, while chitosan may cause the inhibition through other mechanisms. Damage caused by the tertiary combination of antimicrobials was higher than those caused by binary combination or by each one used alone. These facts explain the synergistic effects observed. Results suggest that the mixtures of antimicrobials evaluated would be applicable for fish preservation since they were able to control the development of microorganisms related to the spoilage or safety of mentioned food. Applied studies using fish products are in progress to verify the trends obtained in aqueous systems.

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