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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].