Brazilian Journal of Microbiology (2005) 36:151-156
ISSN 1517-8382

ACTION OF NISIN AND HIGH PH ON GROWTH OF *STAPHYLOCOCCUS AUREUS* AND *SALMONELLA* SP. IN PURE CULTURE AND IN THE MEAT OF LAND CRAB (*UCIDES CORDATUS*)

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Submitted: March 16, 2004; Returned to authors for corrections: December 08, 2004; Approved: June 02, 2005

ABSTRACT

The aim of this study was to evaluate the potential of nisin and high pH to inhibit the growth of *Staphylococcus aureus* and *Salmonella* sp. in broth culture and when inoculated into meat of land crab. In pure cultures, the growth of *S. aureus* was strongly inhibited by nisin and the growth of *Salmonella* sp. was inhibited by nisin–EDTA (20 mM). The inhibition of *S. aureus* lasted for eight hours and *Salmonella* sp. growth was inhibited throughout the experiment (24 h). The high pH (pH 10.0 and 11.0 with $NaHCO_3$-NaOH buffer) was very effective for *in vitro* inhibition of *S. aureus* and *Salmonella* sp. Nisin and high pH, when applied to the contaminated meat, did not yield the same effect. Nisin was not effective in preventing growth of both pathogens in the crab meat, while pH 10.0 showed significant inhibitory effect on *Salmonella* sp. The results suggest that high pH has a potential as antibacterial agent, and may be useful in chemical preservation of crab meat.

Key words: crab meat, nisin, high pH, *Staphylococcus aureus*, *Salmonella* sp.

INTRODUCTION

The land crab (*Ucides cordatus*) is one of the principal species of crustaceans living in the Atlantic Ocean mangroves, inhabiting the large coastal area extending from the south of USA to the south of Brazil. The meat of this crab has a big market in Brazil, principally in Northeastern states, as well as in other South and Central American countries. The crab meat offered to consumers is often highly contaminated with bacteria due to inadequate processing and storage techniques (2,4,5). The microbiological status of the meat is influenced by its quality, including the contamination level of the crab living area, and its subsequent handling and processing. Preservation of fresh crab meat currently involves its freezing and maintenance in low temperatures. Use of chemicals for washing the crab meat before freezing to prevent or inhibit microbial growth would be a way to improve the product quality.

The use of nisin, the bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, is successfully used as antibacterial agent in various food products. Nisin affects several Gram-positive bacteria such as *Listeria* spp., *Staphylococcus* spp. but does not inhibit the majority of Gram-negative bacteria (1,6,11). Nisin showed to be efficient in inactivating Gram-negative bacteria when used together with chelating agents (EDTA), causing an aberration in cell membrane lipopolisaccharyde component (17).

High pH treatment has showed to kill Gram-negative (3,12,15) and some Gram-positive bacteria (8,18). The use of high pH $NaHCO_3$-NaOH buffer solutions inhibited effectively some Gram-negative bacteria causing rapid death of *Salmonella enteritidis* and *Escherichia coli*, but with less influence on the growth rate of *Listeria monocytogenes* (12,18).

In the present study we evaluated the effect of nisin and high pH on *Staphylococcus aureus* and *Salmonella* sp. in pure

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culture and inoculated in the meat of the land crab (*Ucides cordatus*).

MATERIALS AND METHODS

Bacterial strains

We used *Staphylococcus aureus* strain ETP33 (*Ucides cordatus* meat isolate) and *Salmonella* sp. STP221 (mangrove water isolate) of the collection of the Molecular Biology Department of Federal University of Paraiba, PB, Brazil.

Chemicals

Purified nisin (Sigma) was used in the present work. The nisin stock solution was prepared by dissolving 0.1 g of nisin in 10 mL of 0.02 N HCl (17). This solution was used to prepare the required nisin concentrations. The $NaHCO_3$-NaOH buffer solutions (pH 7, 9, 10, and 11) were prepared by adjusting the pH of 0.05 M $NaHCO_3$ with 10 N NaOH (12).

Effect of antibacterial agents on pure cultures

S. aureus and *Salmonella* sp. were grown in the BHI broth (Difco Laboratories, Detroit, Mich.) at 37ºC for 24 hours. An 0.1-mL aliquot of this culture was used to inoculate the BHI medium with the additives (final volume 15 mL). The BHI medium was reconstituted in distilled water and nisin was added in the following concentrations: 0, 300, 600, and 1200 µg/mL for the *S. aureus* control; and 0, 100, 300, and 600 µg/mL of nisin in combination with 20 mM of EDTA (Merck, Darmstad, Germany) for the control of *Salmonella* sp. For the high pH antibacterial activity assay the BHI medium was reconstituted in $NaHCO_3$-NaOH buffer at pH 7, 9, 10, and 11. The BHI medium prepared in distilled water without additives was the control. The cultures were incubated at 37ºC for 24 hours. All tests were done in triplicate. The growth of cultures was examined by spectrophotometric monitoring of optical density at 580 nm (O.D. 580 nm) at the following intervals: 0, 1, 2, 4, 6, 8, and 24 hours, using the respective non-inoculated medium as a blank.

Inoculation of crab meat with *S. aureus* and *Salmonella* sp.

Fresh land crab meat (*Ucides cordatus*) was obtained at the free market and frozen until used. The crab meat was autoclaved at 121ºC for 25 minutes and cooled to 4ºC in the refrigerator. The bacterial strains were inoculated in 900 mL of the BHI medium, incubated at 37ºC for 24 hours, and centrifuged (Sorvall RC5C, Wilmington, USA) for 15 minutes at 6000 rpm (4ºC). The supernatant was discarded and the cells were resuspended in 900 mL of sterile distilled water. The 900 mL of the *S. aureus* or *Salmonella* sp. cell suspension was added to 900 g of meat (inoculation level 10^7-10^8 CFU/g) and mixed using a sterile glass rod for 2 min. Each strain was used separately. The meat suspension inoculated with bacteria was then poured into a large funnel with gauze and drained into a 1-liter graduated cylinder. The meat was pressed in a sterile pestle until 900 mL of the fluid (original volume of inoculum) was recovered.

Treatment of inoculated meat with antibacterial agents and microbiological analysis

The crab meat inoculated with bacterial strains was divided into three lots of 300 g for the bacterial agents treatment. The following sterile solutions were used to wash the meat: distilled water (control), 600 µg/mL of nisin for *S. aureus*, 100 µg/mL of nisin with 20 mM of EDTA for *Salmonella* sp., and $NaHCO_3$ – NaOH buffer at pH 10. Each solution (300 mL) was added separately to individual lots of crab meat in a 600-mL beaker, mixed and kept at 4ºC for 30 min. The meat was then drained as described above. Each 300 g of treated meat sample was divided into 20-g aliquots and placed into sterile Petri dishes. The meat was stored at 4ºC and the duplicate samples were monitored for the numbers of *S. aureus* or *Salmonella* sp. before washing and after 6, 24, 48, 72, 96, and 120 hours of storage at 4ºC. The number of bacteria in the inoculated meat was determined by homogenizing 20-g sample of meat with 180 mL of 1% peptone and spread plating dilutions onto a Baird-Parker medium (Difco Laboratories, Detroit, Mich.) for *S. aureus* and an XLD medium (Oxoid, UK) for *Salmonella* sp. The plates were incubated at 35ºC for 24-48 hours. The pH of the meat during the storage of the crab meat at 4ºC was measured.

In the bioassay, the significant differences obtained in bacterial growth rate among treatments with main effects of treatment type and incubation/storage time were examined statistically by two-way ANOVA. These statistical tests were done for broth culture assay and crab meat treatment experiment with the aid of a computer program package (Statistica, version 5).

RESULTS

Broth culture assay

In untreated BHI medium the growth of *S. aureus* and *Salmonella* sp. was characterized by a short lag phase followed by the rapid growth period, entering the stationary phase after 4 hours of incubation. ANOVA revealed that the growth of both pathogens depended on the concentration of nisin/nisin + EDTA and pH level, as well as the time of incubation (Table 1). Significant inhibition ($P<0.001$) of *S. aureus* in pure culture was observed for all nisin concentrations (100 – 300 µg/mL) used, but only during 8 hours of incubation (Fig. 1). The inhibition phase was followed by a period of a rapid growth of the strain, achieving levels similar to those of the control after 24 hours. On the other hand, the nisin in concentrations ranging from 300 to 1200 µg/mL and 20 mM EDTA showed strong inhibitory effect on *Salmonella* sp., that continued over the 24 hours of the experiment (Fig. 2). Slow increase of *Salmonella* sp. cell numbers was detected in all samples; however, its level was significantly

Table 1. Results of two-way ANOVA on the *S. aureus* and *Salmonella* sp. growth in broth culture assay.

Variable	*S. aureus*				*Salmonella* sp.			
	df	MS	F	P	df	MS	F	P
Nisin[a]/ Nisin-EDTA[b]	3	1.071	16038.06	0.000*	3	0.564	3207.21	0.000*
Time	6	1.119	16762.53	0.000*	6	0.395	2249.08	0.000*
Nisin x Time	18	0.151	2264.47	0.000*	18	0.050	286.96	0.000*
pH	4	1.137	5122.34	0.000*	4	1.433	203.10	0.000*
Time	6	0.498	2246.40	0.000*	6	0.834	118.19	0.000*
pH x Time	24	0.126	567.53	0.000*	24	0.190	26.93	0.000*

[a] *S. aureus*; [b] *Salmonella* sp.;
Mean Squares (MS) and F values are indicated with degrees of freedom (df);
* Significant at 1 % level.

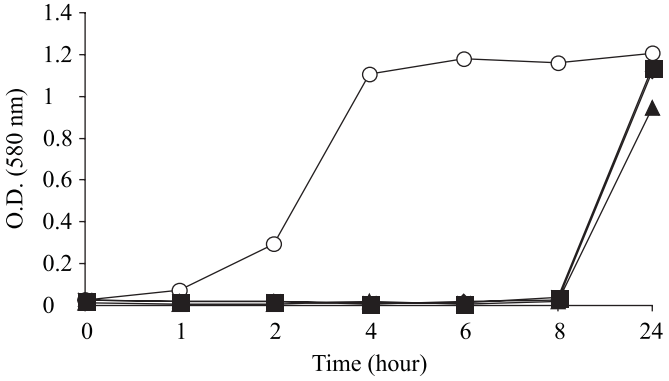

Figure 1. Growth of *Staphylococcus aureus* in the BHI broth without additives (control) (○) and supplemented with 100 µg/ml (■), 300 µg/ml (◆) and 600 µg/ml (▲) of nisin.

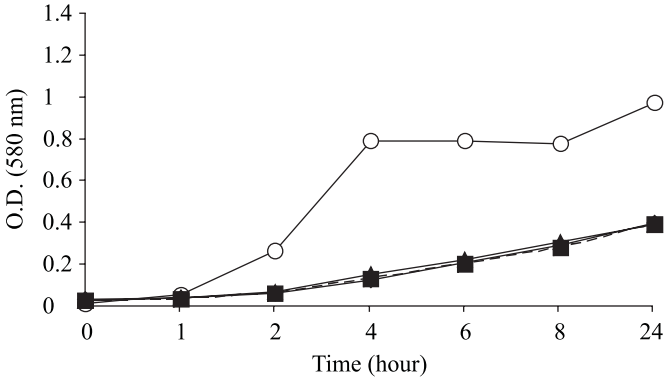

Figure 2. Growth of *Salmonella* sp. in BHI broth without additives (control) (○) and supplemented with 300 µg/ml (■), 600 µg/ml (◆) and 1200 µg/ml (▲) of nisin in combination with 20 mM of EDTA.

lower (P<0.001) in nisin treated sample when compared to the control.

The use of high pH (pH 10 and 11 of $NaHCO_3$-NaOH buffer) was shown to be very effective in the *in vitro* inhibition of *S. aureus* and *Salmonella* sp. (Fig. 3 and 4). Their growth was completely inhibited during 24 hours of incubation. The effect of pH 9 was also significant (P<0.001), reducing the cell number of both pathogens by 50-60% when compared to the control. The *S. aureus* growth was significantly reduced (P<0.01) at pH 7, while no significant differences (P>0.05) were observed between the growth of *Salmonella* sp. in the control and pH 7-treated media (Fig. 3 and 4).

Crab meat treatment

In the experiment with *S. aureus* and *Salmonella* sp. inoculated crab meat, we used 600 and 100 µg/mL of nisin wash solution, respectively and pH 10 sodium bicarbonate buffer. The choice of the nisin concentration and pH level was based on their efficiency, preventing the growth of the pure cultures used, by applying the lowest efficient inhibitory concentration.

The pH of crab meat varied from 7.0 to 9.0 in different treatments (Table 2). We did not observe any changes in pH level during 5 days storage of meat at 4ºC, with the exception of nisin + EDTA treated samples.

The decrease of the counts of *Salmonella* sp. was observed in all treated and non-treated crab meat samples during the storage, while *S. aureus* numbers increased in all samples (Fig. 5).

ANOVA showed that the treatment and its duration affected significantly the growth of *S. aureus* and *Salmonella* sp. inoculated in crab meat (Table 3). We found that nisin was not effective in preventing growth of *S. aureus* and *Salmonella* sp. in crab meat (Fig. 5). On the other hand, pH 10 $NaHCO_3$-NaOH buffer showed significant inhibitory effect (P<0.05) on *Salmonella* sp., but not on *S. aureus*, inoculated into the crab meat.

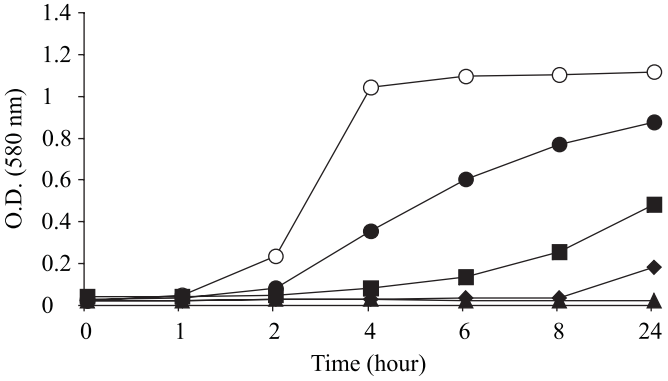

Figure 3. Growth of *Staphylococcus aureus* in BHI broth reconstituted in distilled water (control) (○) and in $NaHCO_3$-NaOH buffer at different pHs: 7.0 (●), 9.0 (■), 10.0 (◆), and 11.0 (▲).

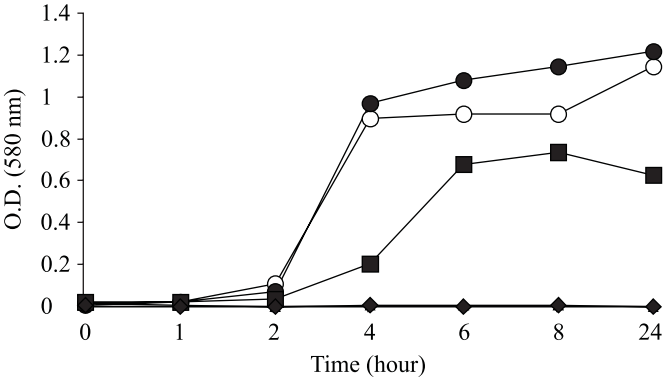

Figure 4. Growth of *Salmonella* sp. in BHI broth reconstituted in distilled water (control) (○) and in $NaHCO_3$-NaOH buffer at different pHs: 7.0 (●), 9.0 (■), 10.0 (◆), and 11.0(▲).

Table 2. Values of pH of the crab meat samples treated with nisin and $NaHCO_3$-NaOH buffer at pH 10.0.

Crab meat sample	pH	
	Storage at 4ºC (days)	
	0	5
Frozen	8.25	-
H_2O distilled-treated	8.25	8.25
Nisin-treated	8.00	8.00
Nisin + EDTA-treated	7.00	7.50
pH 10- treated	9.00	9.00

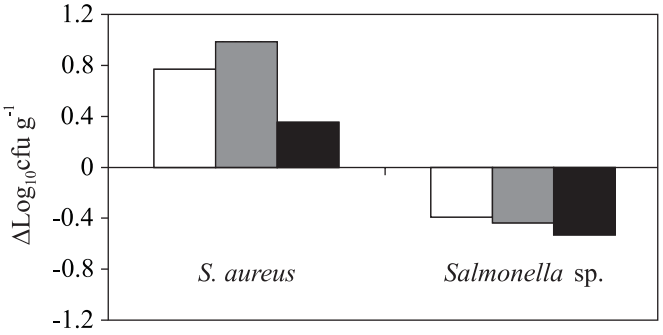

Figure 5. Effect of preservation agents on survival of *Staphylococcus aureus* and *Salmonella* sp. in the samples of crab meat stored for 5 days at 4ºC: (□) washed with distilled water (control), (■) washed with 600 µg/ml of nisin (*S. aureus*) or 100 µg/ml of nisin and 20 mM EDTA (*Salmonella* sp.), (■) washed with $NaHCO_3$-NaOH buffer at pH 10.0. Δlog_{10}cfu g^{-1}: difference between the bacterial counts before washing and after 5 days storage of washed crab meat.

Table 3. Results of two-way ANOVA on the *S. aureus* and *Salmonella* sp. growth in crab meat treatment experiment.

Variable	*S. aureus*				*Salmonella* sp.			
	df	MS	F	P	df	MS	F	P
Treatment	2	64.623	4.165	0.029*	2	53.215	97.27	0.000*
Time	6	97.753	6.300	0.001*	6	36.723	67.13	0.000*
Treatment x Time	12	71.563	4.612	0.001*	12	4.968	9.08	0.000*

Mean Squares (MS) and F values are indicated with degrees of freedom (df);
* Significant at 1 % level.

DISCUSSION

Crab meat is sold commonly as a pre-cooked meat, which can provide a suitable environment for proliferation of meat spoilage microorganisms and common food-borne pathogens, including *S. aureus* and *Salmonella* species (2,9,13). Degnan *et al.* (5) analyzed fresh meat of the blue crab (*Callinectes sapidus*) highly contaminated with total bacteria (mean value 5.1 log_{10} CFU/g) and with *Listeria monocytogenes.* The meat of *U. cordatus* obtained in free markets in Northeast Brazil showed a high contamination level of total bacteria (10^8-10^9 CFU/g)

and of *S. aureus* (10^4-10^8 CFU/g). *S. aureus* was detected in 63% of the meat samples, whereas contamination with *Salmonella* sp. was found in 8% of the samples (10). Chen *et al.* (4) also detected high numbers of total bacteria (5.2 $\log_{10}$ CFU/g) and of *S. aureus* (5.0 $\log_{10}$ CFU/g) in crab meat samples produced industrially. These data show that there is a need for improving sanitary conditions of meat handling and processing, by using some preservatives that could be easily applied in both large and small-scale productions of crab meat.

Information on application of antibacterial agents for preservation of crab meat is scarce. Degnan *et al.* (5) showed that numbers of *Listeria monocytogenes* inoculated into the crab meat of *C. sapidus* decreased after 4 hours of incubation at 4ºC when washed with nisin (10,000 and 20,000 units/mL), maintaining the inhibition effect during 6 days of the experiment.

Scanell *et al.* (14) reported that the growth of *S. kentucky* in culture was inhibited by nisin in the concentration of 500 IU/g over 10 hours incubation at 37ºC and in fresh sausage stored for 10 days at 4ºC. The inhibition of *S. aureus* under the treatment with 500 IU/g of nisin solely or in combination with 1.5 - 2.0% sodium lactate and 1% sodium citrate was also observed (14). Stevens *et al*. (16) reported that cell number of twenty *Salmonella* species was reduced by applying the 50 mg/mL of nisin and 20 mM of EDTA for 1 hour at 37ºC.

In the present study, strong inhibitory effect of nisin, as well as high pH on *S. aureus* and *Salmonella* sp. in broth culture was observed. However, the chemical agents applied to the contaminated crab meat were not very effective in reducing cell numbers of these pathogens.

The effect of nisin can be influenced by pH, the culture medium and the incubation temperature. Huot *et al.* (7) reported that nisin showed the highest antibacterial activity at pH 5.8, and that its activity decreased by 1.4% and 49.0% at pH 6.4 and 7.2, respectively. Thomas and Wimpenny (19) found that the nisin´s effectiveness appeared to increase with the decrease of pH in the range pH 7.9 to 5.0.

In our experiment, where the initial pH of the control and treatment media was 7.5 and decreased to 6.0 after 24 hours of incubation, the nisin effectively prevented the growth of bacterial pathogens in pure cultures. However, the low efficiency of nisin in reducing the number of pathogens in crab meat could be explained partially by the influence of high pH level of nisin treated samples (pH 7 to 8), as well as a high buffer capacity of the crab meat.

High pH treatment has already been shown to be effective in the destruction of *S. enteritidis* on shell eggs (3). Application of this technology to agricultural commodities, such as raw meats, poultry, and plant products, may offer simple and highly effective means for destroying gram-negative food-borne pathogens and thus improving the safety of these valuable foods.

Mendonça *et al.* (12) observed total reduction, in culture medium, of *Salmonella enteritidis* cells after five minutes treatment with buffer at pH 11; after fifteen minutes treatment with buffer at pH 10 the number of viable cells decreased by 1 x 10^4 CFU/mL, and at pH 9 it had no effect. In other study, the $NaHCO_3$-NaOH buffer at pH 7 and 10 decreased partially the growth of *S. enteritidis* and completely at pH 11 (18).

In our study, pH 10 and 11 of $NaHCO_3$-NaOH buffer inhibited completely the growth of *S. aureus* and *Salmonella* sp. over 24 hours of incubation. The buffer at pH 10 showed also significant inhibitory activity on *Salmonella* sp., when applied to contaminated crab meat. It should be pointed out that the pH of the crab meat treated with the pH 10 buffer stabilized at the level of pH 9, due to the buffering capacity of the meat.

Our results suggest that the use of high pH, before freezing, would be an alternative to decrease the contamination level of crab meat. There is a need, however, for further research on high pH effect on crab meat contaminating bacteria, as well as, on sensorial properties of the treated meat, an aspect not deemed in the present work.

RESUMO

Ação da nisina e do pH elevado sobre a multiplicação de *Staphylococcus aureus e Salmonella* sp. em cultura pura e em carne de caranguejo-uçá (*Ucides cordatus*)

O objetivo deste estudo foi avaliar o potencial de inibição de nisina e o pH elevado em relação à multiplicação de *Staphylococcus aureus e Salmonella* sp. em culturas puras e inoculadas na carne de caranguejo-uçá. Em culturas puras, a multiplicação de *S. aureus* foi fortemente inibida por nisina e a de *Salmonella* sp. por nisina-EDTA (20 mM). A multiplicação de *S. aureus* foi inibida até 8h de incubação, enquanto que a de *Salmonella* sp. foi inibida durante todo o experimento (24h). O pH elevado (tampão $NaHCO_3$-NaOH, pH 10 e 11) mostrou-se efetivo na inibição da multiplicação de *S. aureus* e *Salmonella* sp. Nisina e o pH elevado aplicados na carne contaminada não apresentaram o mesmo efeito. A nisina não mostrou eficiência na inibição dos patógenos quando inoculados na carne de caranguejo, enquanto que o tampão em pH 10 demonstrou inibição significante sobre a multiplicação de *Salmonella* sp. Estes resultados sugerem que o pH elevado apresenta um potencial como agente antibacteriano, podendo ser útil na preservação química da carne de caranguejo.

Palavras-chave: carne de caranguejo, nisina, pH elevado, *Staphylococcus aureus* e *Salmonella* sp.

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