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Biopreservation of Brined Shrimp (*Pandalus borealis*) by Bacteriocins from Lactic Acid Bacteria

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In brined shrimp (ca. 3% NaCl), the effects of three different lactic acid bacteria bacteriocins (crude [6.54×10^{10} U of bacteriocin activity {BU}/g] and purified [8.13×10^{23} BU/g] nisin Z, carnocin UI49 [2.32×10^{4} BU/g], and crude bavaricin A [2.78 BU/g]) on bacterial growth and shelf life were compared with those of a benzoate-sorbate solution (0.1% each [wt/wt]) and a control with no preservatives. The shelf life of shrimp subjected to the control treatment was found to be 10 days. Carnocin UI49 did not extend the shelf life, while crude bavaricin A (a cell-free supernatant of *Lactobacillus bavaricus* MI 401) resulted in a shelf life of 16 days, as opposed to 31 days with nisin Z for both its crude and purified forms. The benzoate-sorbate solution preserved the brined shrimp for the whole storage period (59 days). In the control, carnocin UI49, and crude bavaricin A treatments, a gram-positive flora dominated towards the end of the storage period while in the nisin Z treatment a gram-negative flora was more pronounced.

Lactic acid bacteria (LAB) have been used, empirically, for centuries by man to preserve food. It is now generally accepted that the preservative action of LAB is explained by the presence of several factors, including lactic acid, hydrogen peroxide, and bacteriocins. Bacteriocins are protein-containing molecules produced by diverse bacterial species exerting a bactericidal mode of action on susceptible bacteria (16). The bacteriocins of LAB have been extensively studied, and the biochemical and genetic characteristics of these bacteriocins have recently been reviewed (7). One LAB bacteriocin, nisin, has been used as a preservative for decades especially in cheese and other milk products. Other LAB bacteriocins have been proposed and tested as preservatives in food products (17).

For various reasons, there is interest in extending the use of bacteriocins. Consumers have raised concerns about traditional preservatives, e.g., benzoic and sorbic acids, and replacements are sought. Also, benzoic and sorbic acids are of greatest value in low-pH food and in situations where yeasts and molds are the main cause of spoilage, but many food products do not fall into this category. Furthermore, the specific and bactericidal actions of some bacteriocins towards bacteria of special concern, e.g., *Listeria* spp. and *Clostridium* spp., have increased the interest in these compounds.

This report describes a study undertaken to evaluate the effects of three different LAB bacteriocins (crude and purified nisin Z, carnocin UI49, and crude bavaricin A) on bacterial growth in and shelf life of brined shrimp and to compare the effects with those of a benzoate-sorbate solution and a control with no preservatives. Brined shrimp is an example of a food product in which many preservative actions are used to sustain the shelf life and for which bacteriocins could be used. Typically, the product contains 3 to 6% NaCl and an acidulant, as well as sorbic and benzoic acids in concentrations from 0.05 to 0.1%, with pH ranging from 5 to 6, and is stored at temperatures from 0 to 6°C. The bacteriocins used in this study have been tested in laboratory media by coworkers participating in a collaborative biopreservation project.

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MATERIALS AND METHODS

Media and ingredients. The following media and ingredients were used: lactobacilli MRS broth, brain heart infusion (BHI) broth, BiTek agar, plate count agar (PCA), nutrient broth, APT broth, triple sugar iron agar, yeast extract, and Bacto Peptone, all from Difco (Detroit, Mich.); iron agar from Oxoid (Basingstoke, Hampshire, England); and sodium chloride (GR), Tris buffer (0.2 M, pH 7.8), and Triton X-100 from Merck (Darmstadt, Germany). Phosphate buffer (Butterfield) (18) was used as a diluent. The RF-2I medium, developed at the Icelandic Fisheries Laboratories and used to produce the bacteriocin nisin Z, was composed of 15.0 g of Bacto Peptone, 2.0 g of potassium phosphate (dibasic, anhydrous ACS reagent; Sigma, St. Louis, Mo.), 5.0 g of sodium acetate (anhydrous GR; Merck), 0.1 g of magnesium sulfate (heptahydrate GR; Merck) and 10.0 g of α-D(+)-glucose (anhydrous; Sigma) dissolved in 1,000 ml of distilled water and sterilized at 121°C for 15 min.

Bacterial cultures. *Lactococcus lactis* SIK-83 was kindly provided by Rolf Andersson (SIK, Göteborg, Sweden), *Lactobacillus bavaricus* MI 401 by Jytte Josephsen and Anette G. Larsen (The Royal Veterinary and Agriculture University, Frederiksberg, Denmark) and *Pediococcus acidilactici* PAC 1.0 by Ingolf F. Nes (Laboratory of Microbial Gene Technology, Agricultural University of Norway, Aas, Norway). *L. lactis* NCDO 497 was obtained from The National Collection of Dairy Organisms (National Institute for Research in Dairying, University of Reading, Shinfield, Reading, England). *Lactobacillus sake* NCFB2714 was kindly provided by Birthe Jelle from Christian Hansen's Laboratory A.S. (Hørsholm, Denmark). Between experiments, these strains were maintained frozen (−18°C) with 20% (vol/vol) sterile glycerol in 1-ml aliquots in sterile vials (Sarstedt, Nümbrecht, Germany). Working cultures were made by transferring the contents of thawed vials to 5 ml of MRS broth and incubating at 30°C for 24 h.

Bacteriocins. (i) Cell-free supernatants. Two milliliters of working culture of *L. lactis* SIK-83 was transferred to 500 ml of RF-2I medium. Once the pH had reached about 4.5, after approximately 1 day of incubation at 30°C, the resulting fermentation broth was centrifuged at 12,000 rpm for 30 min in a Sorvall RC-5B centrifuge with a GSA rotor (Du Pont Company, Wilmington, Del.). The supernatant was filter (0.45 μm pore size, type HA; Millipore Corp., Bedford, Mass.) sterilized under vacuum.

Two milliliters of working culture of *L. bavaricus* MI 401 was transferred to 500 ml of MRS broth supplemented with 1% (wt/vol) NaCl and incubated at 30°C for 24 h. The fermentation broth was centrifuged and filter sterilized as previously mentioned.

(ii) Carnocin UI49. Carnocin UI49, produced by *Carnobacterium piscicola* UI49 at the Science Institute of the University of Iceland, was kindly provided by Geesje Stoffels and Ágústa Guðmundsdóttir. It was produced as described by Stoffels et al. (15). It was obtained freeze-dried (0.4 g) and was dissolved in sterile distilled water prior to use to obtain a carnocin UI49 solution (0.23%).

(iii) Nisin Z. Four milliliters of working culture of *L. lactis* SIK-83 was transferred to 2,000 ml of RF-2I medium. Once the pH had reached about 4.5, after approximately 1 day of incubation at 30°C, a chromatographic procedure was used to isolate and purify the bacteriocin as described by Stoffels et al. (15), with some modifications.

First, the bacteriocin-containing medium was shaken in a shaker (IKA-Kreisschüttler, model KS 50; Staufen, Germany) for 1 h with amberlite XAD-16 (Sigma), a hydrophobic resin, added in two batches (total, 4% [wt/vol] broth).

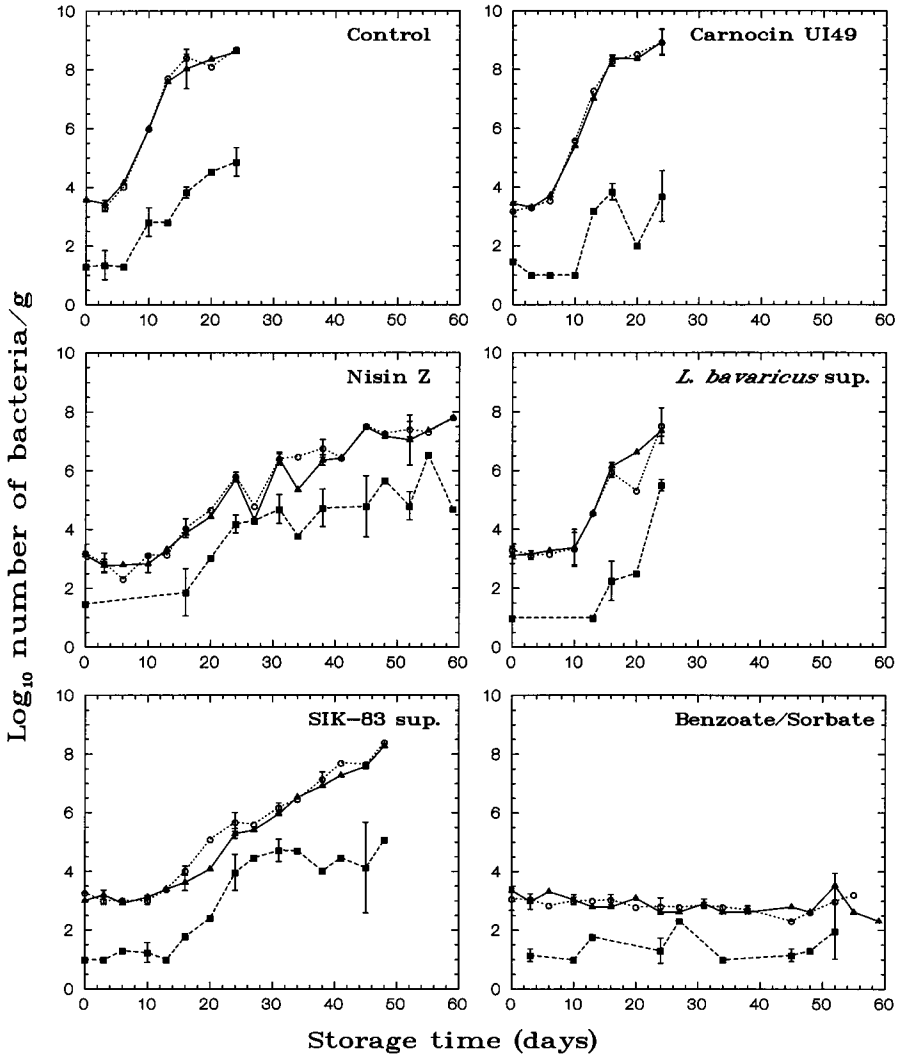

FIG. 1. Microbial growth in brined shrimp containing different preservatives and stored at 4.5 ± 0.4°C. Symbols: ▲, mesophiles; ○, psychrotrophes; ■, MRS count. The values shown are means ± standard deviations of the means (error bars).

The supernatant was poured off, and the resin was applied to a chromatographic column (Econo-Column chromatography column, [2.5 by 50 cm; Bio-Rad Laboratories, Richmond, Calif.]) and was washed with distilled water. The column was subsequently washed with 40% (vol/vol) ethanol (spiritus fortis; Áfengis- og Tóbaksverzlun Ríkisins, Reykjavík, Iceland) until the eluate was colorless; it was then washed with distilled water. The elution of the bacteriocin was carried out with 70% (vol/vol) 2-propanol (GR, Merck) complemented with 10 mM acetic acid (glacial 100%, GR; Merck) and the eluant was adjusted to pH 2.0 with concentrated HCl. The eluate was collected in 25-ml fractions and was checked for bacteriocin activity by the agar spot method with *P. acidilactici* PAC 1.0 as the indicator strain.

The fractions producing inhibition zones were pooled and the 2-propanol was evaporated in a Büchi apparatus (Laboratoriums-Technik AG, Flawil, Switzerland) at room temperature. The resulting sample was applied to an S-Sepharose Fast Flow column, a strong cation exchanger (bed dimensions of 2.5 by 7 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden; and column dimensions of 2.5 by 20 cm; Bio-Rad Laboratories), prerinsed with 20 mM sodium phosphate buffer (monobasic, anhydrous, reagent grade; Sigma), pH 6.0. The column was further washed with the same buffer, and the bacteriocin was eluted with the phosphate buffer supplemented with 1.0 M NaCl, pH 6.0.

The collected 5-ml fractions were checked for bacteriocin activity by the agar spot method. The active fractions were combined and filter sterilized, yielding the nisin Z solution.

Evaluation. (i) Agar spot method. The working culture (150 μl) of the proper indicator strain was transferred to 5 ml of molten BHI soft agar to be poured onto the surface of a BHI agar plate. After drying, 5 μl of each fraction was spotted onto the prepared BHI agar plates which were then incubated at 30°C for 24 h and checked for clear inhibition zones around the spots.

(ii) Assay of bacteriocin activity. A microtiter assay was used to measure the bacteriocin activity. Twofold dilutions of the bacteriocin (100 μl) in MRS broth (100 μl) were prepared in microtiter plates (Flow Laboratories, Irvine, Ayrshire, Scotland). The indicator strains used to evaluate bacteriocin activities were *P. acidilactici* PAC 1.0 for nisin Z and SIK-83 cell-free supernatant, *L. lactis* NCDO 497 for carnocin UI49, and *L. sake* NCFB2714 for *L. bavaricus* MI 401 cell-free supernatant. A diluted working culture of the indicator strain (100 μl of 10^4 to 10^5 CFU/ml) was added to the wells. The blank was made of 50 μl of MRS broth and 150 μl of phosphate buffer (Butterfield); the control was composed of 50 μl of MRS broth, 50 μl of phosphate buffer (Butterfield), and 100 μl of the diluted indicator strain. After the plates had been incubated at 30°C for 24 ± 0.5 h, inhibition was measured with a Titertek Multiskan Plus spectrophotometer, model MK II (Flow Laboratories) and the A_{450} was read. One unit of bacteriocin activity (BU) was defined as the reciprocal of the amount required to inhibit growth of the indicator organism by 50% (50% of the turbidity of the control culture without bacteriocin) after 24 ± 0.5 h of incubation at 30°C.

The MIC is the minimum inhibitory concentration (i.e., the smallest amount of bacteriocin) at which no growth occurs.

The above-mentioned agar spot method was also used to verify whether the antimicrobial activity of the bacterial by-product was due to a proteinaceous compound. This was checked by adding a protease solution to the bacteriocins produced at our laboratory and testing for any further bactericidal activity. The protease solution was made of 4 mg of bromelain (Enzyme Development Corporation, New York, N.Y.) dissolved in 4 ml of sterile 0.1 M sodium phosphate

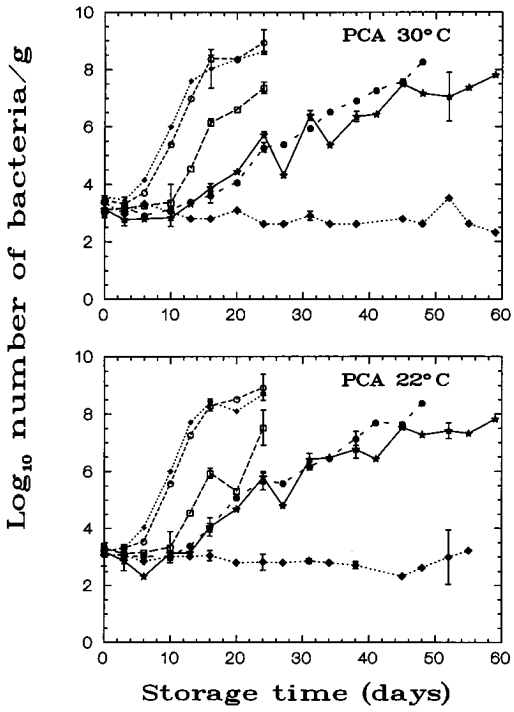

FIG. 2. Mesophilic and psychrotrophic plate counts for each different set of treatment samples stored at 4.5 ± 0.4°C. Symbols: ♦, control; ○, carnocin UI49; □, *L. bavaricus* supernatant; ●, SIK-83 supernatant; ★, nisin Z; ◆, benzoate-sorbate. The values shown are means ± standard deviations of the means (error bars).

buffer (monobasic, anhydrous, reagent grade), pH 6.0; 1.0 ml of this protease solution was added to 0.5 ml of bacteriocin, and the mixture was kept at 37°C for 2 h. Spotting (5 μl) of the protease solution (control), bacteriocin solution (control), and a mixture of the two solutions was done on a prepared BHI plate overlaid with the proper indicator strain (*P. acidilactici* PAC 1.0 for nisin Z and *L. sake* NCFB2714 for the *L. bavaricus* MI 401 cell-free supernatant). The absence of an inhibition zone for the enzyme-bacteriocin mixture was taken as a proof of the proteinaceous nature of the antimicrobial compound.

(iii) Protein concentration. The protein concentrations of the nisin Z and the carnocin UI49 solutions were quantitatively determined by the Micro assay procedure (Pierce, Rockford, Ill.) using the Coomassie protein assay reagent, a dye binding to the protein. This assay reagent is based on the Bradford method (3), which utilizes an absorbance shift in an acidic Coomassie brilliant blue G-250 solution.

A standard curve was prepared by using a protein standard (albumin standard: bovine serum albumin [BSA], fraction V [Pierce]) at a concentration of 0.2 mg/ml, and the data were analyzed by linear regression. The A_{595} of the protein-dye mixture was measured with a UV-visible spectrophotometer (model DMS 80; Varian Techtron Pty. Ltd., Mulgrave, Australia). All assays were performed in duplicate. Volumes of bacteriocins ranging from 5 to 200 μl were transferred to test tubes, and a sample diluent (phosphate buffer, pH 6.0) was used to fill the volume up to 1.0 ml. Then, 1.0 ml of Coomassie protein assay reagent was added and the mixture was properly vortexed. Absorbance was read against that of the phosphate buffer.

(iv) Bacteriocin potency. The potency of the bacteriocin solutions (BU per microgram of protein) can be expressed by dividing the calculated value for BU per gram of shrimp-brine mixture by the calculated value for protein per gram of shrimp-brine mixture.

Storage trial. (i) Shrimp. Shrimp (*Pandalus borealis*) that had been cooked, peeled, and quick-frozen (with 10% glazing) were obtained from Iceland Seafood International Ltd., Reykjavik, Iceland. They were of medium size (300 to 500 pieces per lb [1 lb = 0.45359237 kg]).

(ii) Ingredients used in the preparation of brined shrimp. The brine ingredients were added so that final concentrations of about 3% NaCl, 0.5% citric acid (anhydrous GR; Merck), and 0.75% glucose could be obtained in the buckets. The following preservative solutions were added in amounts corresponding to 10% (wt/wt) of the shrimp-brine mixture, resulting in six different treatments: (i) tap water as the control, (ii) purified nisin Z solution, (iii) SIK-83 cell-free supernatant, (iv) carnocin UI49 solution, (v) *L. bavaricus* MI 401 cell-free supernatant, and (vi) sodium benzoate-potassium sorbate (1% each) solution.

TABLE 1. Preservative actions and bacteriocin activities with various treatments

Treatment	Bacteriocin activity (BU/ml)	Time to reach 10^6 CFU/g (days)
Nisin Z	8.13×10^{24}	31
SIK-83 supernatant	6.54×10^{11}	31
L. bavaricus MI 401 sup.[a]	27.84	16
Carnocin UI49	2.32×10^5	13

[a] sup., supernatant.

Shrimp were added to the brine to obtain a ratio of 1:1. The final concentrations of the sodium benzoate and potassium sorbate in the buckets were therefore 0.1% (wt/wt) for each. Those of the other preservatives are mentioned in Results.

The shrimp-brine mixture stood overnight at 0°C to allow the equilibration of pH and salt to take place. The pH was adjusted with sterile 15% NaOH to about 5.9 to 6.0 to finally reach ca. 5.7 after further equilibration. At this stage, the time was defined as day 0. Each treatment sample was further divided into small containers (transparent polyvinylchloride; capacity, 380 ml; Sigurplast hf, Reykjavík, Iceland), i.e., 25 g of shrimp and 25 g of brine, to render weekly sampling easier. All the treatment samples were stored in a chilled incubator. The actual storage temperature was found to be 4.5 ± 0.4°C.

Estimation of bacterial numbers. Shrimp (12.5 g) and brine (12.5 g) were weighed, 225 ml of phosphate buffer (Butterfield) was added, and the ingredients were mixed by stomacher (Stomacher Lab-Blender 400, A.J. Seward Laboratories, London, England) for 1 min. The total aerobic plate count, determined on PCA with 0.5% NaCl, was evaluated twice a week for analysis of a total of three samples weekly by using a Spiral Plater (model D; Spiral Systems Inc., Cincinnati, Ohio) and a Laser Bacteria Colony Counter (model 500A; Spiral System Instruments), with incubation at both 22 and 30°C (for 3 and 2 days, respectively). Presumptive lactic acid bacteria were counted on MRS medium (MRS broth with 15 g of BiTek agar per liter) by the pour plate method and incubation at 30°C for 3 days. The detection limits were 200 CFU/g for the total aerobic plate count and 10 CFU/g for the MRS count.

Similarly, the microbiological quality of the frozen shrimp was assessed.

pH determination. The pH of three samples, of both the shrimp and the brine, was measured weekly with a PHM 80 pH meter (Radiometer, Copenhagen, Denmark). The pH meter was calibrated at pH 6.00 with buffer-Tritisol (Merck; Art. 9886). The shrimp were mashed, and a few drops of distilled water were added. The mixture was allowed to stand for about 1 h prior to measurement.

Salt concentration. The salt concentration of both the shrimp and the brine of each treatment sample, as well as that of the frozen shrimp, was assessed on day 1 according to AOAC International volumetric method 937.09 (6).

Characterization of bacterial flora. The microbial flora was evaluated on day 0 and when the total aerobic plate counts (22 and 30°C) had reached 10^6 CFU/g, which was defined as the end of shelf life. Up to 24 colonies were randomly selected from the PCA plates (22 and 30°C) used for counting and isolated on PCA (with added NaCl). The Gram reaction was determined by Gram staining (Hucker) (18) and was confirmed by the KOH test with 3% KOH (5).

Further identification of gram-positive colonies was accomplished by a catalase test with 3% H_2O_2 (bacilli found catalase negative when grown at 30°C were also grown at 22°C and were retested for catalase) and a double-tube oxidation-fermentation test for differentiation between *Staphylococcus* and *Micrococcus* spp. (2); by verification of CO_2 production for catalase-negative colonies by using APT broth with a Durham tube and of sugar fermentation and H_2S production by using triple sugar iron agar and iron agar; by microscopic examination of fully grown isolates for the presence of spores; and by using STAA medium (12) to select for *Brochothrix thermosphacta* (22°C for 2 days).

For gram-negative colonies, the oxidase test using Dryslide Oxidase (Difco) and the double-tube oxidation-fermentation test using MOF medium (10) with 3% NaCl instead of the salt solution described were done. Cultures in MOF medium were incubated at 30°C for up to 2 weeks.

Colony appearance, cell morphology, and motility tests done by hanging drop preparations (in nutrient broth at 22°C for 24 to 48 h) were examined for all isolates. Identification of gram-negative bacteria was based on the determinative scheme of Shewan et al. (11) updated according to procedures in Bergey's manual, vol. 1 (8). Gram-positive strains were classified according to the system in Bergey's manual, vol. 2 (13).

Sensory evaluation. Weekly evaluation was done by six to nine Icelandic Fisheries Laboratories-trained panelists for a period of 4 weeks. The samples were smelled, and the results were expressed as percent spoilage, defined on a continuous line scale from 0 to 100% where 0% meant no spoilage and 100% meant excessive spoilage, as well as by hedonic scaling (1 [dislike extremely] to 9 [like extremely]).

On two occasions, a reduction in sample numbers was required. Preevaluation was done by the panel leaders, and samples found (by smelling) to have no

TABLE 2. Bacterial flora of frozen shrimp and brined shrimp on day 0 and at count of about 10^6 CFU/g[a]

Treatment group	Time	Temp (°C)	Bacterial count (CFU/g)			No. of colonies	
			Gram +	Gram −	Total	Total	Chosen
Control	Day 0	22	4	10	2,520	14	14
	Day 3	30	5	12	3,570	17	17
	PC $> 10^6$	22	16	8	5.11×10^7	NA	24
		30	15	9	3.90×10^7	NA	24
Nisin Z	Day 0	22	1	5	1,670	6	6
		30	0	3	1,460	4	3
	PC $> 10^6$	22	0	24	1.83×10^6	NA	24
		30	12	11	1.92×10^6	NA	23
SIK-83 supernatant	Day 0	22	0	7	1,890	7	7
		30	3	2	1,010	5	5
	PC $> 10^6$	22	0	24	1.95×10^6	157[b]	24
		30	0	23	9.33×10^6	96[b]	23
Carnocin UI49	Day 0	22	0	7	1,670	7	7
		30	7	4	2,930	11	11
	PC $> 10^6$	22	23	1	1.89×10^7	NA	24
		30	19	5	1.01×10^7	NA	24
L. bavaricus supernatant	Day 0	22	5	4	2,510	9	9
		30	7	3	2,090	10	10
	PC $> 10^6$	22	17	0	1.14×10^6	67	17
		30	24	0	1.73×10^6	86	24
Benzoate-sorbate	Day 0	22	6	6	2,300	12	12
		30	4	6	2,090	10	10
Frozen shrimp	Day 0	22	6	14	5,480	20	20
		30	11	10	9,190	21	21

[a] Bacterial flora counts were isolated from total aerobic plate counts (PC) of cultures incubated at 22 and 30°C. NA, not available.
[b] Approximative value.

spoilage characteristics were withdrawn from the sensory evaluation (see Fig. 3 and 4: treatment 2, day 10, and treatment 6, day 24).

Statistical analysis. The data were analyzed and plotted with SYSTAT version 5.0 software (19).

RESULTS AND DISCUSSION

One of the main obstacles that had to be overcome prior to conducting this experiment was the difficulty of producing enough bacteriocin from *L. lactis* SIK-83 by means of a simple and cheap substrate. After some elaboration of different combinations of ingredients, a substrate named RF-2I was chosen for this purpose. Other bacteriocins were produced by the donor or as recommended by the donor of the bacteriocin-producing strain. The bacteriocins (nisin Z and its crude version, carnocin UI49, and *L. bavaricus* MI 401 cell-free supernatant) were used in a storage trial of brined shrimp that lasted 59 days at 4.5 ± 0.4°C. The actual ratio of shrimp to brine was 1:1.

Bacteriocin assay. The total bacteriocin activities of the bacteriocin solutions prior to their addition to the brine were determined to be 6.54×10^{11} BU/ml for SIK-83 cell-free supernatant, 8.13×10^{24} BU/ml for the nisin Z solution, 2.32×10^5 BU/ml for the carnocin UI49 solution, and 27.84 BU/ml for the *L. bavaricus* MI 401 cell-free supernatant. The MICs were found to be 3.44×10^{11} BU/ml for SIK-83 cell-free supernatant, 6.04×10^{24} BU/ml for the nisin Z solution, 4.10×10^4 BU/ml for the carnocin UI49 solution, and 20.0 BU/ml for the *L. bavaricus* MI 401 cell-free supernatant.

Once the bacteriocin was incorporated into the shrimp-brine mixture, the bacteriocin activities were calculated to be around 6.54×10^{10} BU/g for the SIK-83 cell-free supernatant, 8.13×10^{23} BU/g for the purified nisin Z, 2.32×10^4 BU/g for the carnocin UI49, and 2.78 BU/g for the *L. bavaricus* MI 401 cell-free supernatant. By the use of the agar spot assay, both the nisin Z solution and *L. bavaricus* MI 401 cell-free supernatant were tested for their sensitivity to a protease (bromelain). They became inactivated, as indicated by the fact that no further antimicrobial action could be detected.

Protein assay of bacteriocins. The protein concentrations were found to be 125.85 μg/ml for the purified nisin Z solution and 104.39 μg/ml for the carnocin UI49 solution. Therefore, the calculated amounts of these bacteriocins in the shrimp-brine mixture were 12.6 and 10.4 μg/g, respectively. Notice that the maximum allowance of nisin in cheese is 12.5 μg/g (4).

Bacteriocin potency. The bacteriocin potencies were calculated to be 6.53×10^{22} BU/μg of protein for the purified nisin Z and 2.23×10^3 BU/μg of protein for the carnocin UI49.

Estimation of bacterial quality, pH, and salt content. The initial bacterial quality of the frozen shrimp was as follows: 6,200 (22°C) and 9,000 (30°C) CFU/g for total aerobic plate counts and 100 CFU/g for the presumptive lactic acid bacteria count.

In Fig. 1, the results of the bacteriological analyses for each different treatment are presented, while in Fig. 2, the comparison of the preservative actions of the six treatments is clearly illustrated. By comparing the control treatment (brine without preservatives) with the other treatments, it is possible to evaluate the preservative action in each treatment. The control treatment shows that within 10 days the microbial flora (at 22 and 30°C) had reached a count of 10^6 CFU/g and the MRS

count was about 10^3 CFU/g. The bacterial growth with the nisin Z treatment was slower than that of the control. A microbial load of about 10^6 CFU/g occurred at day 31. A total count of 10^8 CFU/g was not reached even at the end of the storage period (59 days). The bacteriocin had delayed the proliferation of the microbial flora growing on MRS agar for at least 2 weeks. This bacteriocin has been shown to have an extended activity spectrum on gram-positive bacteria (1). These findings correlate with ours. The results of the SIK-83 cell-free supernatant treatment were very similar to those of the nisin Z treatment, except that a total count of 10^8 CFU/g was reached after 48 days.

The addition of carnocin UI49 did not inhibit microbial growth. This result may be explained by the temperature dependency of the bacteriocin's mode of action. Stoffels et al. (14) found that there was no reduction in the number of viable cells of the indicator strain *L. lactis* NCDO 497 after a 24-h incubation at 4°C at a carnocin UI49 concentration that otherwise had an inhibitory effect at higher temperatures, such as 34°C. It is noteworthy that when compared with the other bacteriocin-containing treatments (Fig. 1), the carnocin UI49 treatment could hold the MRS count below 10^4 CFU/g although its total count became very high (about 10^9 CFU/g).

The *L. bavaricus* cell-free supernatant had a greater preservative effect than carnocin UI49 solution but a lesser effect than nisin Z solution or SIK-83 cell-free supernatant. On the 16th day, the *L. bavaricus* cell-free supernatant treatment samples had reached a count of 10^6 CFU/g, almost a week later than the control but 2 weeks sooner than the nisin Z and SIK-83 cell-free supernatant treatment samples. The bacteria growing on MRS agar were inhibited for about 2 weeks, similarly to inhibition with nisin Z and SIK-83 cell-free supernatant treatments. Even though this bacteriocin has been shown to have a broad range of antimicrobial activity against gram-positive organisms, especially lactic acid bacteria (9), it had poor storage stability when stored at 4 to 6°C in filter-sterilized MRS medium as its biological activity was lost (8a). Also, as it was not purified and hence was less concentrated than the other bacteriocins, its preservative effect could be expected to be inferior to theirs.

Comparison of the results of the bacteriocin activity assay and the preservative actions obtained by each bacteriocin (Table 1) reveals that the preservative action was proportional to the bacteriocin activity but reached a point where it was no longer influenced by the level of the bacteriocin activity. The phenomenon is illustrated well by comparing the purified bacteriocin, nisin Z, and its crude version, SIK-83 cell-free supernatant. A 10^{13}-fold increase in bacteriocin activity did not significantly prolong the preservative effect.

The sodium benzoate and potassium sorbate solution inhibited microbial growth during the whole storage period. Despite the increased preservative effect of the solution, it was observed to cause a serious appearance defect, as decolorization and yellowing of the shrimp occurred around the 5th week (data not shown). On the other hand, the nisin Z and SIK-83 cell-free supernatant treatment samples still had a rather fresh look, as judged by the color, despite their considerable microbial load (over 10^6 CFU/g) at that time. The color of the shrimp receiving these treatments started to fade only around the 7th week, after which yellowing of the shrimp was observed.

The pHs of the brine and the shrimp were similar throughout the experiment (data not shown), with a starting value of approximately 5.7 that was maintained until the end of the shelf life.

The average salt content of the shrimp from the different

TABLE 3. Estimation of composition of the shrimp flora on day 0 and at total count of about 10^6 CFU/g[a]

Group	Temp (°C)	Composition (% of total) by bacterial genus																			
		Moraxella		*Cytophaga, Flavobacteria*		*Pseudomonas, Alcaligenes*		*Acinetobacter*		Unidentified, gram negative		*Micrococcus*		Coryneforms		*Brochothrix*		*Erysipelothrix*	
		Day 0	PC > 10^6	Day 0	PC > 10^6	Day 0	PC > 10^6	Day 0	PC > 10^6	Day 0	PC > 10^6	Day 0	PC > 10^6	Day 0	PC > 10^6	Day 0	PC > 10^6	Day 0	PC > 10^6
Control	22	50.0	33.3			21.4						7.2		21.4	66.7				
	30	47.0	37.5	11.8		11.8						23.5		5.9	62.5				
Nisin Z	22	66.7	100.0			16.6						16.6							
	30	66.7	47.8			33.3									52.2				
SIK-83 supern.[b]	22	71.4	100.0	14.3				14.3											
	30	20.0	100.0	20.0								20.0		40.0					
Carnocin UI49	22	42.9	4.2			28.6				28.6					95.8				
	30	27.2	20.8			9.1						18.2		45.5	79.2				
L. bavaricus supern.	22	22.2						22.2				33.4		22.2			100.0		
	30	10.0												60.0			100.0	10.0	
Benzoate-sorbate	22	33.3	NA[c]	8.3	NA		NA		NA	8.3	NA	25.0	NA	25.0	NA		NA		NA
	30	50.0				10.0						10.0	100.0	30.0					

[a] Plate count (PC) of 10^6 CFU/g.
[b] supern., supernatant.
[c] NA, not available.

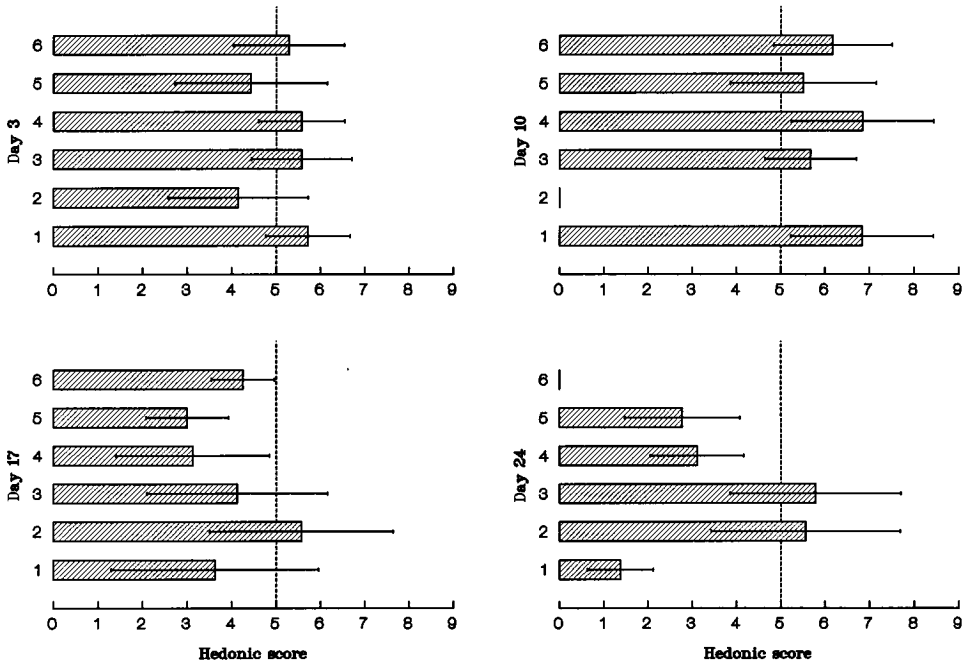

FIG. 3. Hedonic scores on four different days for each treatment (treatment 2, day 10, and treatment 6, day 24, samples were not given to the panel because they were judged good). The numbers on the vertical axis indicate the treatments: 1, control; 2, nisin Z; 3, SIK-83 supernatant; 4, carnocin UI49; 5, *L. bavaricus* supernatant; and 6, benzoate-sorbate. Hedonic scores represent a range from 1 (dislike extremely) to 9 (like extremely) of the mean scores ± the standard deviations of the means (error bars).

treatment samples was 2.84% ± 0.10%, whereas the brine contained 3.28% ± 0.06%.

Characterization of bacterial flora. The initial flora of the shrimp consisted predominantly of gram-negative organisms (Table 2). Once the total count reached 10^6 CFU/g, a different flora had been established. When no preservatives were used (control treatment), a gram-positive flora became dominant. With the nisin Z treatment, the psychrotrophic flora only contained gram-negative organisms whereas the mesophilic flora was almost equally divided between the gram-positive and

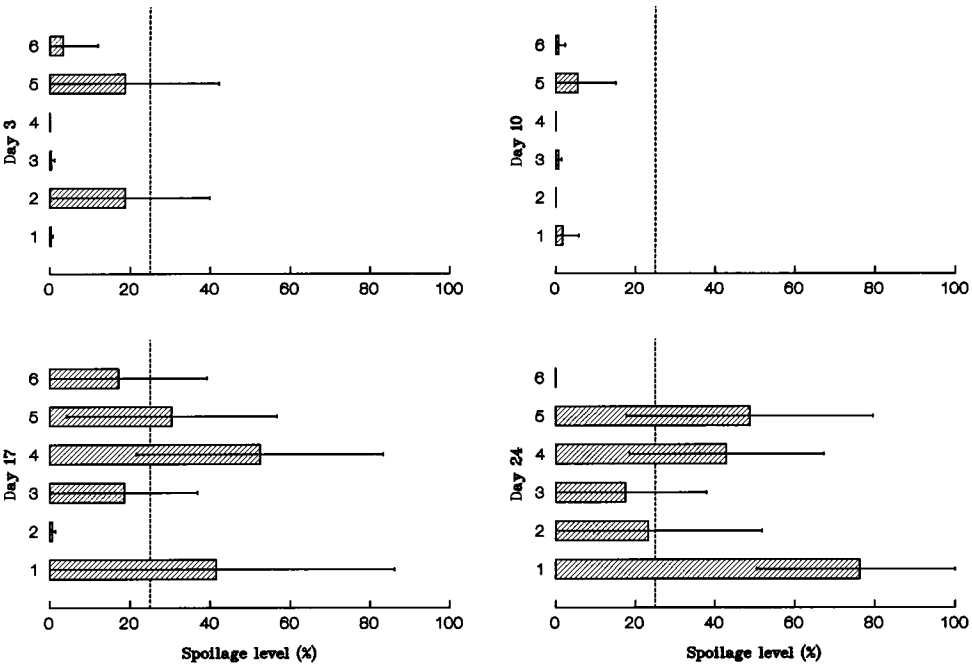

FIG. 4. Evaluation of the extent of spoilage for each treatment on four different days (treatment 2, day 10, and treatment 6, day 24, samples were not given to the panel because they were judged good). Treatment numbers are indicated as for Fig. 3. Spoilage levels represent a range from 0% (no spoilage) to 100% (excessive spoilage), with means ± standard deviations of the means (error bars) indicated.

-negative organisms. An increase in the number of gram-positive organisms occurred with this treatment at 30°C. The SIK-83 cell-free supernatant treatment contained only gram-negative organisms. The carnocin UI49 and *L. bavaricus* cell-free supernatant treatments showed a significant increase in the number of gram-positive organisms.

Further identification has led to estimations of the composition of the shrimp flora (Table 3) that was present at the beginning of the experiment and that established when the total aerobic plate count had reached 10^6 CFU/g. In the control and the carnocin UI49 treatment samples, the dominating flora changed from *Moraxella* spp. to coryneforms during storage. For nisin Z and SIK-83 cell-free supernatant treatments, the initially dominating *Moraxella* flora became composed entirely of *Moraxella* spp. except for the mesophilic flora of the nisin Z treatment sample in which about half of the flora was composed of *Moraxella* spp. whereas the other half was composed of coryneforms. The *L. bavaricus* cell-free supernatant treatment originally contained a diversified flora which changed to 100% *B. thermosphacta*. Because of the low total aerobic count of the benzoate-sorbate treatment, only one colony (on PCA at 30°C) could be isolated on day 59; it was identified as a member of the *Micrococcus* genus.

Sensory evaluation. The sensory evaluation was done four times, once a week during the first 4 weeks of the trial storage. Figures 3 and 4 present the results obtained for the evaluation of the extent of spoilage and acceptability of the brined shrimp. For the extent of spoilage, a 25% limit was defined to express the point at which the shrimp could be considered to have undergone important deterioration. For the hedonic scale, the limit of acceptance was set at 5, defined as "neither like nor dislike."

The hedonic score presented in Fig. 3 shows how much the panelists liked or disliked the samples of the six different treatments. On day 3, most of the treatments were defined as being on the like side of the limit. Notice that the nisin Z and *L. bavaricus* cell-free supernatant treatment samples were disliked during the 1st week but were not disliked in the 2nd week.

The panelists liked all the treatment samples during the 2nd week. In that week, the control and carnocin UI49 were the preferred treatments. The nisin Z treatment samples were not given to the panel, since they were judged to be the best by the panel leaders and the number of samples could thus be reduced.

On day 17, the nisin Z treatment was the only one still on the like side. On the 24th day, the nisin Z and SIK-83 cell-free supernatant treatment samples were liked by the panel, whereas the benzoate-sorbate treatment samples had been judged good by the panel leaders (the benzoate-sorbate treatment samples were not given to the panel to reduce the number of samples).

Figure 4 presents the evaluation of the extents of spoilage for the same days. On days 17 and 24, only the nisin Z, SIK-83 cell-free supernatant, and benzoate-sorbate treatment samples were still considered to be of satisfying quality.

The Icelandic Fisheries Laboratories panelists were not necessarily the same every week, and that may have caused some of the divergences among the results. Some of the results appeared to disagree during the first 2 weeks. For instance, from day 3 to day 10, acceptance for all the treatments increased and percent spoilage scores for most treatments decreased. On one hand, since these samples were judged by smelling, odors unfamiliar to the panelists and originating from the preservatives used could have been responsible for these aberrant results. As time went on, the odors could have become attenuated and could have affected the panel less than before. On the other hand, smelling is unfortunately not as accurate a standard as tasting since compounds formed during spoilage are very volatile and easily lost and therefore it becomes difficult to standardize a sniffing method. In spite of the disagreement of some of the results, they correlate rather well with the estimations of bacterial quality (Fig. 2).

Conclusions. The results of this experiment indicated that, among the LAB bacteriocins evaluated in the storage trial of brined shrimp, nisin Z could be used as a natural preservative to replace the benzoate-sorbate mixture for similar refrigerated, brined products requiring a 3- to 4-week shelf life. The results also showed that purification of nisin Z did not improve its preservative action in this food system. From these results, one can conclude that a combination of bacteriocins and other preserving actions would improve the preservative effect. Also, as bacteriocins have specific and bactericidal action against bacteria like *Listeria* spp., they could be of value where *Listeria* contamination is a potential problem. This and other possible applications of bacteriocins in food preservation are now being investigated in our laboratory.

Nevertheless, the use of nisin Z in brining operations should first be approved by the appropriate government regulatory agencies.

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