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Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins

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Abstract

The effect of food components and ecological factors on the activities of nisin, sakacin P and curvacin A was evaluated. *Lactobacillus curvatus*, *Listeria innocua*, *Salmonella* and *Escherichia coli* including *E. coli* O157:H7 were used as target organisms. Lecithin, casein, and divalent cations were antagonists of the bacteriocins at 0.1%, 0.1% and 10 mmol l^{-1}, respectively. A decrease in pH as well as the presence of EDTA, propyl-parabene or NaCl at concentrations of 0–1 mmol y^{-1}, 0–0.16 g l^{-1}, and 0–6% (w/w), respectively, increased the activity of all bacteriocins. These compounds as well as a pH < 5.5 rendered the Gram-negative target organisms sensitive against bacteriocins. Of practical importance is the respective effect of NaCl at concentrations $> 5\%$ which are achieved in fermentation and ripening processes, e.g. in production of fermented sausages. A characteristic response was observed for each of the bacteriocins. It is suggested that bacteriocins of lactic acid bacteria are effective against a wide range of microorganisms including *E. coli* O157:H7 if applied in combination with other preservative principles prevailing in foods.

Keywords: *E. coli* O157:H7; Nisin; Bacteriocin; *Listeria*; Sakacin P

1. Introduction

Bacteriocins of lactic acid bacteria (LAB) have the potential to prevent microbial food spoilage and to inhibit growth of pathogens (Abee et al., 1995; Montville et al., 1995; Stiles, 1996). Nisin is the only bacteriocin legally approved for use as food additive. The bacteriocinogenic cultures may be used in food production, provided that the amount of bacteriocin formed by the starter or protective culture ensures the desired effect. It has to be taken into account that the applicability of bacteriocin-mediated food preservation may be hampered by the limited inhibitory spectra of bacteriocins of LAB, and the frequent occurrence of resistant mutants in sensitive populations (Mazzotta et al., 1997).

In food matrices the bacteriocin activity may be affected by (i) changes in solubility and charge of the bacteriocins, (ii) binding of the bacteriocins to food components, (iii) inactivation by proteases, and (iv) changes in the cell envelope of the target

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organisms as a response to environmental factors. Recent reports emphasized the importance of interactions of bacteriocins with other antagonistic compounds or food components. For example, a combination of nisin with 2% sodium lactate inhibited growth of *Salmonella kentucky* and *Staphylococcus aureus* on fresh pork sausages more efficiently than the application of nisin alone (Scannell et al., 1997). The temperature, phospholipid content, and pH affected growth inhibition of *Clostridium botulinum* by nisin in a model food system (Roger and Montville, 1994). The dependence of nisin activity on various food components was shown by Henning et al. (1986b). Hugas et al. (1996) investigated the effect of bacteriocin-producing (bac^+) and bac^- starter cultures on the survival of *Listeria innocua* during sausage fermentations. The authors observed that in addition to the competitiveness of the starter culture and its bacteriocin production, the elimination of *L. innocua* depended on the sausage formula and the fermentation technology.

Information on factors determining the sensitivity of bacteria has been gained from the characterization of resistant strains derived from sensitive populations. The cytoplasmic membrane of sensitive cells is the target of bacteriocins (Montville et al., 1995; Abee et al., 1995; Moll et al., 1996). The bacteriocins bind to the charged headgroups of phospholipids of the membrane or a proteinaceous receptor, followed by insertion into the membrane and pore formation leading to a depletion of the proton motive force and efflux of small solutes. A lower cellular phospholipid content as well as an altered membrane fatty acid composition have been found to be associated with nisin resistance (Ming and Daeschel, 1995; Mazzotta and Montville, 1997; Crandall and Montville, 1998). Additionally, changes in cell wall composition may confer resistance towards bacteriocin, as shown by comparison of nisin activity against protoplasts and whole cells of sensitive and resistant strains of *L. monocytogenes* (Davies et al., 1996). An anionic polysaccharide bound to the cell wall of *Lactobacillus casei* was shown to be associated with increased nisin resistance (Breuer and Radler, 1996).

Gram negative bacteria are resistant to bacteriocins of lactic acid bacteria due to the effective barrier function of the outer membrane that is absent in Gram positive bacteria. Accordingly, spheroplasts of *Escherichia coli* and *Salmonella typhimurium* are sensitive to nisin (Schved et al., 1994). Exposure of *E. coli* and *Salmonella* to sublethal stress that disrupts the outer membrane and allows bacteriocins access to the cytoplasmic membrane and leads to an increased sensitivity. The application of hydrostatic pressure, heat, freezing and thawing as well as addition of EDTA or ethyl maltol resulted in a sensitization of *E. coli* and *Salmonella* to nisin (Stevens et al., 1991; Kalchayanand et al., 1992, 1994; Hauben et al., 1996; Schved et al., 1996).

The knowledge of the interactions of bacteriocins with food are limited and restricted mainly to nisin activity towards *L. monocytogenes*. Previous studies have shown that sakacin P efficacy against *L. ivanovii* is enhanced by increasing NaCl and H^+ concentrations (Gänzle et al., 1996). The effects of these factors and other food preservatives on the inhibitory spectrum of bacteriocins have so far received little attention. Therefore, it was the aim of our studies to assess the effect of ecological factors and medium composition on the activity of nisin, sakacin P and curvacin A against several target organisms, including lactic acid bacteria and Gram negatives.

2. Materials and methods

2.1. Microorganisms and media

Lactobacillus curvatus DSM20019, *Listeria innocua* DSM20649, *Escherichia coli* LTH1600, *E. coli* serotype O157:H7 LTH4346, and *Salmonella heidelberg* LTH3658 were used as target organisms. *Lactobacillus sakei* LTH673 and *Lb. curvatus* LTH1174 were used to produce sakacin P and curvacin A, respectively (Tichaczek et al., 1992). All organisms were grown in modified MRS medium (de Man et al., 1960, mMRS) containing the following per litre: 10 g tryptone, 5 g lab lemco powder, 5 g yeast extract, 2 g K_2HPO_4, 2 g diammonium-citrate, 1 g Tween 80, 0.1 g $MgSO_4 \times 7H_2O$, 0.05 g $MnSO_4 \times H_2O$, 20 g glucose (all chemicals from Merck, Darmstadt, Germany). The pH after sterilization was 6.4–6.5. Lactobacilli were grown anaerobically at 30°C, all other strains were incubated agitated (200 rev./min) at 37°C unless otherwise stated.

2.2. Bacteriocin preparations

Nisin was obtained from Aplin and Barret Ltd., UK, and dissolved in H_2O_{demin} to an activity of 4×10^4 IU/ml. Crude preparations of sakacin P and curvacin A were obtained from 2 l culture supernatant of the respective producer organisms which were incubated for 17 h at 20°C. The bacteriocins were adsorbed to 80 g of an Amberlite XAD-7 resin (Sigma, Deisenhofen, Germany) and eluted with a stepwise gradient of isopropanol:0.1% trifluoroacetic acid (TFA) against 0.1% TFA. The active fractions were pooled, evaporated to dryness, and redissolved in 20 mmolar phosphate buffer (pH 8) containing 20% (v/v) ethanol. The stock solutions were stored at −20°C and diluted with mMRS to an activity of 2000–3000 AU ml l^{-1} towards *Lb. curvatus* DSM20019 prior to use, corresponding to an activity at standard conditions of about 5000, 60, 120, and 60 AU ml^{-1} (nisin), 3000, 100, 140, and 80 AU ml^{-1} (sakacin P), and 2700, 250, 350, and 120 AU ml^{-1} (curvacin A) against *L. innocua* DSM20649, *E. coli* LTH1600, *E. coli* serotype O157:H7 LTH4346, and *Salmonella heidelberg* LTH3658, respectively.

2.3. Determination of bacteriocin activity

Bacteriocin activity was determined by measuring the minimum inhibitory concentration (MIC) of the bacteriocin solutions in a critical dilution assay as described previously (Gänzle et al., 1996). Twofold dilutions of the bacteriocin stock solutions were prepared with mMRS on 96-well microtiter plates, inoculated with target organism, and incubated for 16–20 h or until OD 0.3 was reached at 30 or 37°C. The optical density was measured using a microtiter plate reader (model 450, BioRad, Munich, Germany) at 595 nm. The target organisms were prepared as follows: stationary cultures of *Lb. curvatus* DSM20019 in mMRS were diluted 1:30; all other organisms were grown to the early logarithmic growth phase (OD of 0.1), and diluted 1:300 prior to inoculation. The bacteriocin activity was calculated using the following equation (Parente et al., 1995):

$$\frac{OD_{16h}}{OD_{0h}} = a + \frac{(1-a)}{1 + e^{b+c(\ln d)}}$$

where d is the bacteriocin concentration (ml bacteriocin stock solution/ml total volume), and a, b, and c are regression coefficients fitted to each set of OD versus dose (d) data using SigmaPlot 1.02 software (Jandel Scientific, Erkrath, Germany). The amount of bacteriocin solution resulting in a 50% growth inhibition was defined as d_{50}. The bacteriocin activity was calculated as d_{50}^{-1} and expressed as arbitrary units ml^{-1} (AU ml^{-1}). The detection limit of the assay was 60 AU ml^{-1}.

2.4. Determination of the effect of medium composition and pH on bacteriocin activity

Lecithin from egg yolk (Serva, Heidelberg, Germany), glycerin-monooleate, casein, NaCl, para-hydroxybenzoic acid (pHB), propyl-parabene, Na_2EDTA (all obtained from Sigma, Deisenhofen, Germany), $MnSO_4 \times H_2O$, $MgSO_4 \times 7H_2O$, and $CaCl_2 \times 2H_2O$ (Merck, Darmstadt) were dissolved in mMRS and sterilized by filtration. The concentration of these compounds on the microtiter plates was adjusted by mixing with mMRS without any additions. The pH was adjusted with 4 M HCl or 4 M NaOH, and the medium was sterilized by filtration. To compare the effect of these components on bacteriocin activity, the activities measured at the various factor levels were related to that of the same combination of bacteriocin and target organism at standard conditions (mMRS, pH 6.4), i.e. the relative bacteriocin activity of each bacteriocin against each target organism at standard conditions equals one. The variation coefficients for the relative bacteriocin activities were generally <30%. The critical dilution assay applied to measure bacteriocin activity requires growth of the target organism, thus, factor levels that are per se inhibitory to one or several target organisms (no increase of optical density within 48 h) were not evaluated. The inhibitory effects of the compounds alone on growth of the target organisms, however, were taken into account as the assay relates the growth inhibition by bacteriocin addition to the growth of the organisms under the same conditions but in the absence of bacteriocins.

2.5. Inactivation of resting cells of E. coli LTH1600

Cells of an overnight culture on *E. coli* LTH1600 were harvested and washed twice in saline (8.5 g l^{-1}

Table 1
Effect of lecithin on bacteriocin activity

	Relative bacteriocin activity against				
	Lb. curvatus DSM20019		*Listeria innocua* DSM20649		
[lecithin] (%)	0.1	1	0.1	0.5	1
Bacteriocin					
Nisin	0.27[a]	0	0.32	0.01	0
Sakacin P	0.59[a]	0	0.66	0.006	0
Curvacin A	0.66[a]	0	0.91	0.035	0

[a]Relative bacteriocin activity (the activity under standard conditions (no additions, pH 6.4) equals 1).

NaCl, 1 g l^{-1} tryptone). The cells were resuspended in citrate phosphate buffer (70 mmol l^{-1} citric acid and 140 mmol l^{-1} Na_2HPO_4) at pH 6.5, 5.3 or 5.0, or in citrate phosphate buffer pH 6.5 containing 7% (w/v) NaCl. Sakacin P stock solution was added to a final activity of 2500 AU ml l^{-1} and 20 mmol phosphate buffer (pH 8) was added to the controls. Cell counts were determined by plating appropriate dilutions after 0, 4, 8, and 16 h on mMRS agar.

3. Results

3.1. Effect of selected food components on bacteriocin activity

Food components may interact with the bacteriocins themselves, or with their target, i.e. the bacterial cytoplasmic membrane. The activity of bacteriocins is the result of hydrophobic and electrostatic interactions of these amphiphilic, positively charged peptides with the membrane. Therefore, we evaluated the effect of the emulsifier lecithin, casein, and divalent cations on bacteriocin activity towards *Lb. curvatus* and *L. innocua*. As shown in Table 1, lecithin addition at a level of 1% completely abolished bacteriocin activity towards either target organism. At a level of 0.1%, activity was reduced by 40–70%. Nisin activity was more affected than the activity of curvacin A and sakacin P, independent of the choice of target organism. These concentrations are well within the range of foods produced with ingredients rich in lecithin. For example, lecithin is present in egg yolk in concentrations of ca. 6%; milk contains ca. 0.05% phospholipids. Glycerin monooleate at a level of 1% increased the activity of nisin and sakacin P 2.4- and 1.2-fold, respectively (data not shown). Casein at a level of 1 g l^{-1} resulted in an appreciable reduction of bacteriocin activity (Table 2) with nisin activity being less affected than the activity of sakacin P and curvacin A. Compared to *L. innocua* the antagonistic effect of casein was stronger with *Lb. curvatus* as target organism. Addition of divalent cations led to a reduction of bacteriocin activity and magnesium was found to be least effective (Table 3). Generally, the reduction of nisin activity was more pronounced than that of the other two bacteriocins. The reduction of activity was not strain specific. The effective concentrations of Ca^{2+}, Mg^{2+} and Mn^{2+} exceeded those

Table 2
Effect of casein on relative bacteriocin activity

	Relative bacteriocin activity against					
	Lb. curvatus DSM20019			*Listeria innocua* DSM20649		
[casein] (g l^{-1})	0.1	1	10	1	5	10
Bacteriocin						
Nisin	0.96[a]	0.60	0.49	1	0.73	0.70
Sakacin P	1[a]	0.63	0.28	0.83	0.47	0.42
Curvacin A	0.98[a]	0.48	0.21	0.88	0.58	0.40

[a]Relative bacteriocin activity (the activity under standard conditions (no additions, pH 6.4) equals 1).

Table 3
Effect of divalent cations on relative bacteriocin activity

	Relative bacteriocin activity against								
	Lb. curvatus DSM20019						*Listeria innocua* DSM20649		
$[Me^{2+}]$ (mmol l^{-1})	Magnesium		Manganese		Calcium		Calcium		
Bacteriocin	10	100	10	100	10	100	10	50	100
Nisin	0.90[a]	0.69	0.46	0.077	0.59	0	0.53	0.03	0.016
Sakacin P	0.67[a]	0.58	1	0.34	1	0.47	1	0.55	0.27
Curvacin A	0.56[a]	0.33	0.57	0.138	0.69	0.53	0.91	0.40	0.40

[a]Relative bacteriocin activity (the activity under standard conditions (no additions, pH 6.4) equals 1).

commonly encountered in food products, e.g. 30 mmol l^{-1} Ca^{2+} and 2 mmol l^{-1} Mg^{2+} in hard cheese, 0.25 mmol l^{-1} Ca^{2+} and 1 mmol l^{-1} Mg^{2+} in beef or 1.6 mmol l^{-1} Ca^{2+} and 5 mmol l^{-1} Mg^{2+} in rye. The concentrations of Mg^{2+} and Mn^{2+} in mMRS, 1 and 0.3 mmol l^{-1}, respectively, did not affect bacteriocin activity (data not shown).

3.2. Effect of EDTA

EDTA has the potential to counteract the antagonistic effect of divalent cations on bacteriocin activity by complex formation. Furthermore, EDTA is known to disturb the highly ordered structure of the outer membrane and thus allows access of hydrophobic molecules to the cytoplasmic membrane (Vaara, 1992). We evaluated the effect of EDTA on nisin, sakacin P and curvacin A activity against *Lb. curvatus*, *Listeria*, *E. coli*, and *Salmonella*. As shown in Fig. 1, EDTA exerted a stimulatory effect on the activity of all bacteriocins tested. The sensitivities of *Lb. curvatus* DSM20019 and *Salmonella heidelberg* were less affected by EDTA addition than those of *E.coli* LTH1600 and *L. innocua* DSM20649 and the effect of EDTA on nisin efficacy was more pronounced than that on sakacin P or curvacin A.

3.3. Effect of para-hydroxybenzoic acid and propyl-parabene

The methyl, ethyl, and propyl esters of *para*-hydroxybenzoic acid (pHB) are used as food preservatives. Their antimicrobial effect increases with increasing length of the alcohol moiety but the solubility in water decreases. We investigated the effects of pHB and propyl-parabene on bacteriocin activity against *Lb. curvatus* DSM20019, *L. innocua* DSM20649, and *E. coli* LTH1600. As shown in Fig.

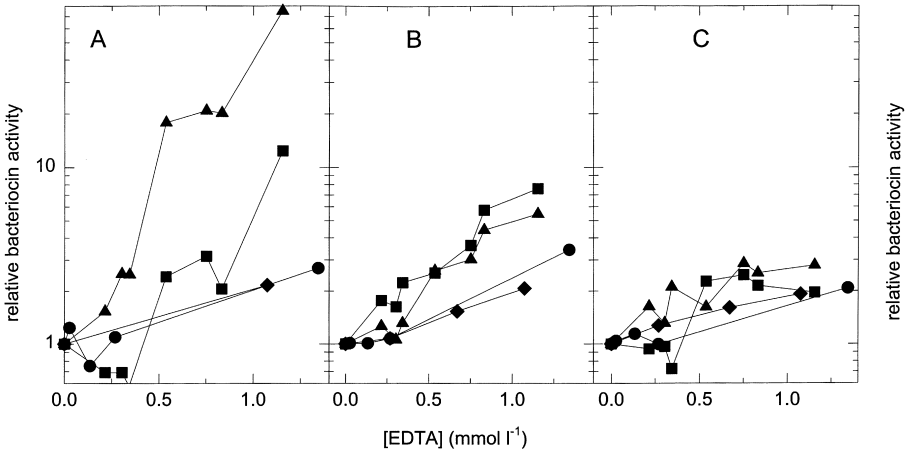

Fig. 1. Effect of EDTA on the activity of nisin (A), sakacin P (B), and curvacin A (C) against *L. innocua* DSM20649 (■), *Lb. curvatus* DSM20019 (●), *E. coli* LTH1600 (▲), and *Salmonella heidelberg* LTH3658 (♦).

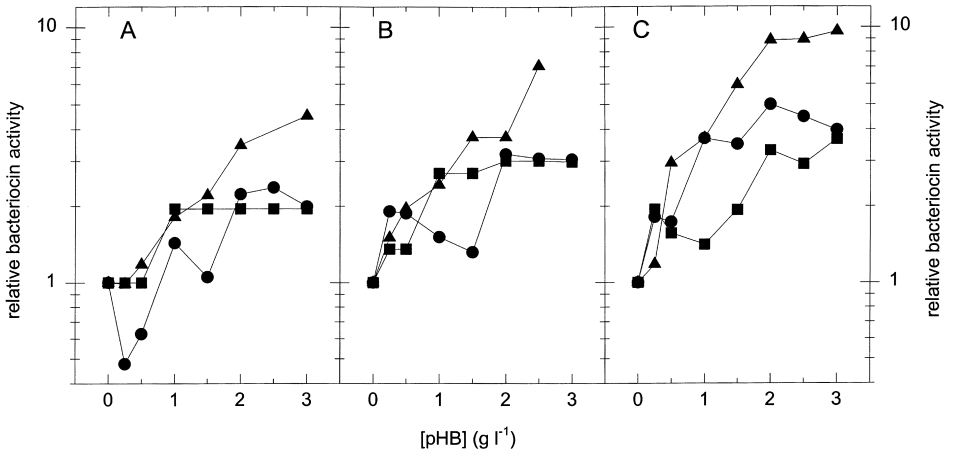

Fig. 2. Effect of para-hydroxybenzoic acid on the activities of nisin (A), sakacin P (B), and curvacin A (C) towards *L. innocua* DSM20649 (■), *Lb. curvatus* DSM20019 (●), and *E. coli* LTH1600 (▲).

2, pHB enhanced the activity of all bacteriocins tested. Whereas the response of *L. innocua* and *Lb. curvatus* to the combined effects of bacteriocin and pHB was rather similar (2–4-fold increase of bacteriocin activity), the bacteriocin activity against *E. coli* LTH1600 was increased up to 10-fold. Nisin activity was less affected by pHB than curvacin A and sakacin P activity.

The effect of propyl-parabene on nisin, curvacin A and sakacin P activity against *Lb. curvatus* DSM10019 is shown in Fig. 3A. As observed with pHB, the activity of all three bacteriocins was enhanced by propyl-parabene, and the effect of the propyl-ester on nisin was less pronounced than that on sakacin P and curvacin A. Propyl-parabene was effective at much lower concentrations than pHB. The effects of propyl-parabene on sakacin P and curvacin A activity against *L. innocua*, *E. coli* or

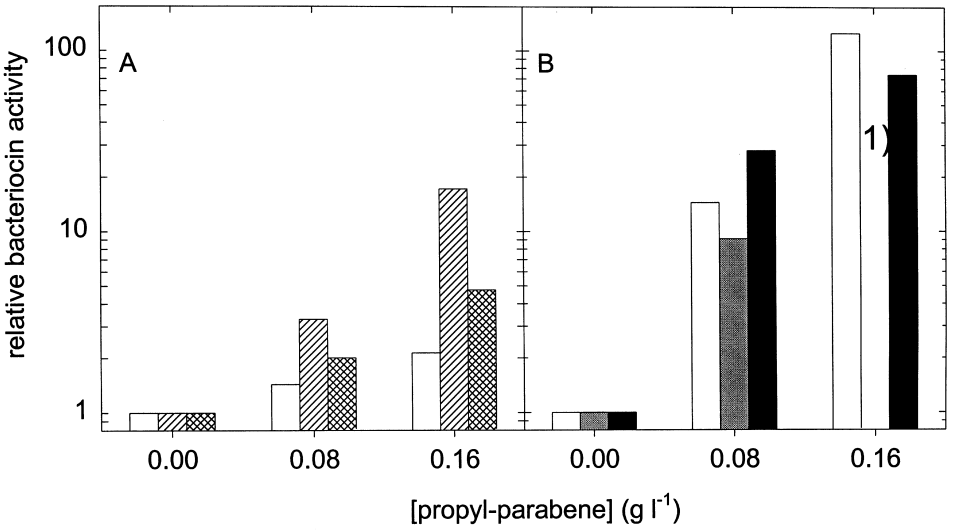

Fig. 3.(A) Effect of propyl-parabene on the activities of nisin (▭), curvacin A (▨), and sakacin P (▩) towards *Lb. curvatus* DSM20019. (B) Effect of propyl-parabene on curvacin A activity towards *E. coli* LTH1600 (▭), and *Salmonella heidelberg* LTH3658 (▭), and sakacin P activity towards *L. innocua* DSM20649 (■). [1]*Salmonella* LTH3658 did not grow at 0.16 g l^{-1} propyl-parabene.

Salmonella heidelberg are shown in Fig. 3B. The concentrations of sakacin P effective against *L. innocua* DSM20649 and *E. coli* LTH1600, respectively, were decreased 100-fold in the presence of 0.16 g l^{-1} propyl-parabene (Fig. 3B). The sensitivity of *Salmonella heidelberg* compared well to that of *E. coli* LTH1600.

3.4. Effect of NaCl and pH on bacteriocin activity

In fermented food, preservation is often achieved by the combined effects of acidification and NaCl addition. In addition, bacteriocins produced by bacteriocinogenic starter or protective cultures can contribute to the protective effect of acid and salt. We investigated the activities of nisin, sakacin P and curvacin A at NaCl concentrations ranging from 0–7% (w/w). In addition to *L. innocua*, *Lb. curvatus* and *E. coli* LTH1600, *E. coli* LTH4346 (O157:H7) were used as target organism. EHEC strains with the serotype O157:H7 are recognized as pathogens of increasing importance. Ground beef as well as dry cured salami have been associated with *E. coli* O157:H7 outbreaks (Buchanan and Doyle, 1997). As shown in Fig. 4, the synergistic effect of NaCl addition on the activity of nisin and curvacin A against *E. coli* O157:H7 was more pronounced than that on any other target organism. Furthermore, *E. coli* LTH4346 (O157:H7) was about 10 times more sensitive than *E. coli* LTH1600 at all pH-values and NaCl concentrations tested (data not shown). *E. coli* LTH1600 grew at 7% NaCl, while 5% NaCl was the highest concentration permitting growth of *E. coli* O157:H7. The activities of nisin, sakacin P and curvacin A activity against *E. coli* LTH1600 were enhanced by NaCl addition approximately to the same extent (7–30-fold). The Gram-positive target-organisms *Lb. curvatus* and *L. innocua* exhibited a similar response to the bacteriocins at NaCl concentrations ranging from 0 to 7% (w/w). Curvacin A and especially sakacin P activity were increased by NaCl addition, but nisin activity against these organisms remained unaffected. The effect of the pH in the range of 6.5–4.9 on bacteriocin activity against *L. innocua*, *E. coli* LTH1600, *E. coli* LTH4346 (O157:H7), and *Salmonella* type Heidelberg was determined (Fig. 5). All target organisms exhibited an increased sensitivity towards nisin, sakacin P and curvacin A at low pH values. As observed for NaCl effects on bacteriocin activity, the effect of pH on nisin and curvacin A activity against *E. coli* O157:H7 was more pronounced than that against *E. coli* LTH1600. The pH effects on sakacin P activity were greater than those on the other two bacteriocins.

3.5. Inactivation of resting cells of E. coli LTH1600 by sakacin P

Because *E. coli* is used as an indicator for fecal contamination and has been implicated with food poisoning, the survival of this organism during food

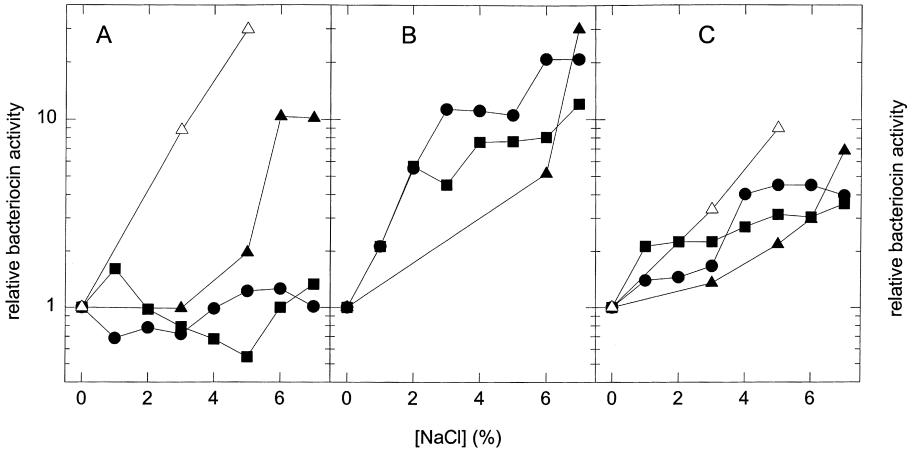

Fig. 4. Effect of NaCl on the activities of nisin (A), sakacin P (B), and curvacin A (C) towards *L. innocua* DSM20649 (■), *Lb. curvatus* DSM20019 (●), *E. coli* LTH1600 (▲) and *E. coli* LTH4346 O157:H7 (△).

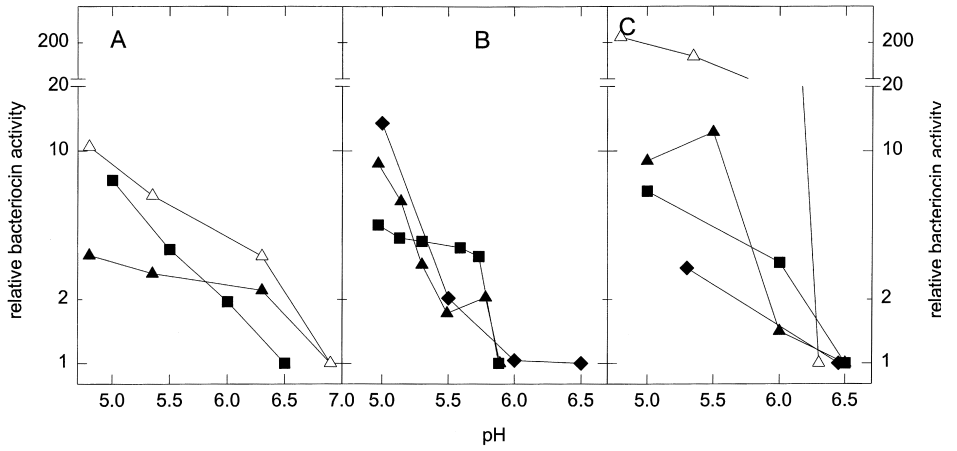

Fig. 5. Effect of pH on the activities of nisin (A), sakacin P (B), and curvacin A (C) towards *L. innocua* DSM20649 (■), *E. coli* LTH1600 (▲), *Salmonella heidelberg* LTH3658 (♦) and *E. coli* LTH4346 O157:H7 (△).

processing and storage is well known (Buchanan and Doyle, 1997). *E. coli* present as contaminant on minimally processed foods such as undercooked ground beef, dry cured salami, lettuce or vegetables remains viable during cold storage. For example, *E. coli* O157:H7 tolerates those pH values and NaCl concentrations encountered in dry cured salami for several weeks without major loss of viability (Glass et al., 1992). Bacteriocins of lactic acid bacteria exhibit a bactericidal effect on sensitive target organisms (Kalchayanand et al., 1992). Accordingly, bacteriocinogenic LAB employed as starter or protective cultures have been shown to contribute to the inactivation of *Listeria* in food (Hugas et al., 1996). In order to determine whether the application of bacteriocins may contribute to the inactivation of *E. coli*, we investigated the effect of pH and NaCl on sakacin P activity towards resting cells of *E. coli* LTH1600. The survival of this strain under the various conditions is shown in Fig. 6. At a pH of 6.5, the cell counts remained unchanged over 24 h in the presence or absence of sakacin P. A reduction of cell counts by 80% was observed at pH 6.5 and 7% (w/w) NaCl. Under those conditions, the inactivation of *E. coli* was not enhanced by bacteriocin addition. Acidification of the buffer with HCl to a pH of 5.3 and 5.0 did not affect the viability of the organism. However, in the presence of sakacin P a reduction of cell counts by one and four orders of magnitude was observed at pH 5.3 and 5.0, respectively. Thus,

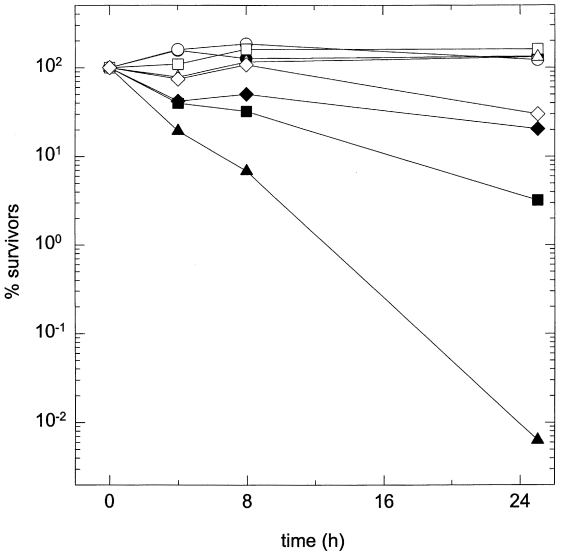

Fig. 6. Inactivation of *E. coli* LTH1600 by sakacin P in citrate-phosphate buffer at pH 6.5 (●), pH 5.3 (■), and pH 5.0 (▲), and pH 6.5, 7% NaCl (♦). Open symbols: control experiment without bacteriocin added.

acidification resulted in an increased sakacin P activity towards growing cells of *E. coli* LTH1600 as well as an increased activity towards resting cells, whereas the addition of NaCl increased sakacin P activity towards growing cells, but did not render resting cells sensitive to the bacteriocin.

4. Discussion

Growth inhibition and elimination of pathogenic microorganisms by bacteriocins during food production and storage is the result of specific interactions between the bacteriocin and the food matrix as well as the target organism. Our studies were designed to provide guidance for the selection of bacteriocins or bacteriocinogenic cultures effective in a given food system against the relevant target organisms. Synergistic as well as antagonistic effects of components of the food matrix on the antimicrobial efficacy of bacteriocins were revealed. Corresponding to the common target for the bactericidal action of nisin and pediocin-like bacteriocins, the cytoplasmic membrane, the same trend was observed with respect to bacteriocin activity when these were applied in combination with other compounds. However, the quantity of the response was specific for the combinations of bacteriocins and target organisms. The results presented in this work therefore extend previous studies dealing mainly with nisin activity against Gram-positive target organisms.

Neutral emulsifiers such as monolaurine, monooleate and Tween 80 were shown to stimulate nisin activity (Blackburn et al., 1989; Jung et al., 1992), whereas the zwitterionic lecithin at concentrations as low as 0.1% was antagonistic (Henning, 1984). The effect of lecithin was attributed to the formation of stable complexes between nisin and the zwitterionic phospholipid (Henning et al., 1986a). The effects of lecithin and glycerol-monooleate on nisin activity presented in our study are well in accordance with these data. The effects of emulsifiers on sakacin P and curvacin A activity were comparable to that on nisin, however the synergistic and antagonistic effects were less pronounced. A protective effect of casein has previously not been described. It is explained by interactions between bacteriocins and casein, which is an amphiphilic molecule consisting of negatively charged and hydrophobic domains.

The antagonistic effect of calcium on nisin activity has been reported by Henning (1984) and Blackburn et al. (1989) using lactic acid bacteria as target organisms. Our data on the effect of Ca^{2+} on nisin activity towards *Listeria* are inconsistent with the results of Abee et al. (1994). These authors found that the nisin Z induced K^{+} efflux from cells of *L. monocytogenes* was reduced by about 31 and 60% in the presence of 5 mmol l^{-1} Mg^{2+} and Ca^{2+}, respectively. Remarkably, the activity of sakacin P and curvacin A is much less affected by addition of divalent cations. The protective effect of divalent cations was explained by the binding of Mg^{2+} to anionic phospholipids resulting in an enhanced rigidity of the cytoplasmic membrane and a reduced affinity of nisin to the cytoplasmic membrane (Abee et al., 1994; Demel et al., 1996; Crandall and Montville, 1998).

Treatment of Gram-negative organisms with EDTA disturbs the outer membrane function (Vaara, 1992). The synergistic effect of EDTA on bacteriocin activity is completely abolished by addition of calcium and magnesium (Cutter and Siragusa, 1995a). At concentrations of 1–20 mmol l^{-1}, EDTA allows nisin-mediated inactivation of strains of *E. coli*, including *E. coli* O157:H7, and *Salmonella* (Blackburn et al., 1989; Stevens et al., 1992; Cutter and Siragusa, 1995a). Our data on nisin activity against *E. coli* and *Salmonella* are consistent with these observations. In agreement with the observation that sakacin P and curvacin A are less affected by Ca^{2+} addition, the stimulatory effect of EDTA on those bacteriocins was less pronounced than that on nisin. The synergism between EDTA and nisin may be of practical importance as it was shown that their combined application reduced the cell counts of *Salmonella* and *E. coli* on broiler carcasses (Shefet et al., 1995). On the other hand, nisin and EDTA exerted only a slight effect on *E. coli* attached to beef (Cutter and Siragusa, 1995b).

The parabenes are used at concentrations of up to 0.1% as preservatives in food. These compounds enhanced growth inhibition of Gram-negative bacteria by bacteriocins. In addition to the synergistic effect of these preservatives and bacteriocins on the prevention of bacterial growth, it was shown that similar combinations (nisin and sorbate, low pH, or NaCl) also prevented or reduced the occurrence of resistant strains upon exposure to bacteriocins (Buncic et al., 1995; de Martinis et al., 1997; Mazzotta et al., 1997).

The data available on combined effects of bacteriocins, pH and NaCl on bacteria are restricted to Gram-positive target organisms. Gänzle et al. (1996) reported synergistic effects of pH, NaCl, and sakacin P against *L. ivanovii*, and observed that the inhibitory effects of pH, NaCl, and nitrite on growth of *Listeria*

were rather independent of the effects of these factors on sakacin P activity against this organism. The activities of nisin, sakacin P, and curvacin A against *Lb. sakei*, *Staphylococcus aureus*, and *L. monocytogenes* were enhanced at low pH values and high NaCl concentrations (Thomas and Wimpenny, 1996; Blom et al., 1997; Datta and Benjamin, 1997). Our data on sakacin P and curvacin A activity are in good agreement with these results, but do not suggest an effect of NaCl on nisin activity against *Lb. sakei* and *Listeria*. This difference can be attributed to the choice of the assay. By calculating the MIC from OD data, changes in the diffusion rate of nisin in the agar do not occur. We could demonstrate that *E. coli*, including *E. coli* O157:H7, and *Salmonella* are inhibited by nisin, sakacin P and curvacin A at low pH and high salt concentrations. Thus, bacteriocins produced by lactic acid bacteria may contribute to the inactivation of these Gram-negative bacteria in food. The pH and the NaCl concentrations found to be effective in the microtiter-plate assay match those encountered in a wide variety of foods, including cheeses, fermented meats and delicatessen type salads. The effect of the pH on bacteriocin activity against both growing and resting cells of *E. coli* could be demonstrated. In contrast, NaCl addition enhanced bacteriocin activity on growing, but not on resting cells. This indicates that the outer membrane of cells growing in the presence of salt allowed penetration of the bacteriocin to the cytoplasmic membrane, whereas the outer membrane of cells grown at low salt concentrations remained functional even if the cells are transferred to a high salt buffer. Transfer to cells grown at a pH of 6.5 to an acidic environment resulted in the loss of the integrity of the outer membrane and sensitivity towards sakacin P.

The medium composition and the pH have been found to affect not only the activity of nisin, sakacin P and curvacin A, but also to enhance their inhibitory spectra. These included *E. coli* and *Salmonella* under certain conditions. Several effects were found to be specific for a given bacteriocin, indicating that pediocin-like bacteriocins may be effective where nisin is not, or vice versa. Furthermore, several effects, especially those of pH and NaCl concentration, appear to be species or even strain specific, with characteristic differences between *Listeria*, lactic acid bacteria and Gram negatives. The test system employed in our studies allowed a rapid assessment of the effects of single factors on bacteriocin efficacy.

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