Biotechnology Letters **23:** 605–608, 2001.
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Sensitivity variations of *Listeria* strains to the bacteriocins, lactocin 705, enterocin CRL35 and nisin

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Received 19 December 2000; Revisions requested 4 January 2001; Revisions received 6 February 2001; Accepted 7 February 2001

Key words: bacteriocin, *Listeria*, sensitivity

Abstract

Five strains of *Listeria monocytogenes*, four strains of *Listeria innocua* and a strain of *Listeria seeligeri* showed different sensitivities to lactocin 705 (17 000 AU ml^{-1}), enterocin CRL35 (8500 AU ml^{-1}) and nisin (2500 IU ml^{-1}) at different pHs (5, 6 and 7). The susceptibility of *Listeria* strains to bacteriocins at each pH was strain dependent, and it was enhanced at the low pH. *L. monocytogenes* had enhanced nisin tolerance while the non-nisin bacteriocins were more inhibitory with viability losses of 3–3.4 in contrast with 1.5–1.8 log cycles, respectively. Lower viability loss values were obtained with *L. innocua* strains with all three bacteriocins while *L. seeligeri* was more sensitive to nisin than to lactocin 705 or enterocin CRL35.

Introduction

Bacteriocins from lactic acid bacteria inhibit the growth of microorganisms involved in both pathogenicity and food spoilage (Jack *et al*. 1995, Stiles 1996, Moll *et al.* 1999). This effect makes these bacteria potentially useful in food manufacturing as sources of biopreservatives. The best known bacteriocin is nisin which is commercially available and, at present, the only one with GRAS (generally regarded as safe) status (Delves-Broughton 1990). The anti-listerial activity of nisin has been reported and there are a number of studies on the sensitivity of *Listeria monocytogenes* and other *Listeria* spp. to nisin (Harris *et al*. 1991, Ukuku & Shelef 1997). Many other bacteriocins also demonstrating anti-listeria properties have been reported (Muriana 1996), and others have been investigated for the inhibition of this pathogen in foods, either through bacteriocin producing cultures or by the addition of pure or semi-pure bacteriocins (Winkowski *et al*. 1993, Vignolo *et al.* 1996, Farías *et al*. 1999).

The narrow spectrum of activity of lactic acid bacteria bacteriocins, their failure to act against Gram-negative bacteria and yeasts, and the emergence of naturally-resistant isolates as well as adaptional tolerance constitute the main limitations in their use. The need for an increased database on the activity of bacteriocins, particularly those other than nisin constituted the aim of this study. The sensitivity of ten *Listeria* strains to nisin, lactocin 705 and enterocin CRL35 in liquid media and the effect of broth pH (5, 6 and 7) as well as the effect of initial cell numbers on the anti-listerial activity have been examined

Materials and methods

Bacterial strains and culture conditions

Listeria monocytogenes FBUNT (Facultad de Bioquímica, Química y Farmacia, UNT, Argentina), ScottA, WR129, SR215 and 4ab, *L. seeligeri* WS2253 (Institute of Hygiene and Toxicology, IHT, Karlsruhe, Germany) and *L. innocua* 7, 11, 12 and L1PE (Unité de Recherches Laitiéres et Génétique Appliqueé, INRA, France) were used. Before use they were grown twice in TSBYE (Tryptic Soy Broth,

Table 1. Growth inhibition of *Listeria monocytogenes* strains by nisin, lactocin 705 and enterocin CRL35 at 30 °C and different pH.

Strains	Inoculum	Viability loss (log N_0/N)[a]								
		Nisin			Lactocin 705			Enterocin CRL35		
		pH 5	pH 6	pH 7	pH 5	pH 6	pH 7	pH 5	pH 6	pH 7
FBUNT[b]	Low	3.0	2.5	2.5	3.4	3.2	3.2	3.0	2.5	2.5
	High	1.5	1.0	1.0	1.0	0.6	0.6	0.7	0.5	0.5
Scott A[c]	Low	1.5	0.8	0.5	2.0	1.8	1.8	1.5	1.2	1.2
	High	0.6	0.3	0.3	0.7	0.7	0.6	0.6	0.6	0.6
WR129[c]	Low	1.8	1.5	1.5	2.5	2.3	2.3	2.2	2.0	2.0
	High	1.0	1.0	0.8	1.0	1.0	0.8	0.9	0.7	0.7
SR215[c]	Low	2.5	1.5	1.5	2.8	2.5	2.4	2.5	2.4	2.4
	High	1.2	1.0	1.0	1.4	1.1	1.1	1.2	1.0	0.8
4ab[c]	Low	2.0	1.8	1.8	3.0	2.8	2.7	2.4	2.2	2.2
	High	1.0	0.8	0.8	1.5	1.5	1.2	1.0	0.8	0.8

[a]Viability loss: log N_0/N. Low inoculum: 10^3–10^4 c.f.u. ml^{-1}; high inoculum: 10^6–10^7 c.f.u. ml^{-1}.
[b]Strain from Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán.
[c]Strains from IHT, Karlsruhe, Germany. Nisin: 2500 IU ml^{-1}; lactocin 705: 17 000 AU ml^{-1}; enterocin CRL35: 8500 AU ml^{-1}.

supplemented with 0.6% yeast extract, pH 6.7) at 30 °C.

Bacteriocins preparations

Lactocin 705 was prepared according to the procedure described by Palacios *et al.* (1999). Briefly, an overnight culture in MRS broth (De Man *et al.* 1960) of *Lactobacillus casei* CRL705, was heated to inactivate proteases and kill cells and the adsortion-desortion, pH-dependent method developed by Yang *et al.* (1992) was applied. The active extract was further subjected to reverse phase-HPLC and SDS-PAGE. Enterocin CRL35, produced by *Enterococcus faecium* CRL35, was obtained from an overnight culture supernatant precipitated with $(NH_4)_2SO_4$ at 60% (w/v) at 4 °C. After separation by a Biogel-P6 column, the bacteriocin preparation was then applied to a CM-Sephadex cation exchange column. The pooled concentrated fractions were then loaded on a C18 reverse-phase column (Farías *et al.* 1996). Nisin (Nisaplin, 1×10^6 IU g^{-1}) was kindly provided by Aplin & Barret Ltd. (Trowbridge, UK). Stock solutions (10^5 IU ml^{-1}) were prepared by solubilizing appropriate amounts of powder in 0.02 M HCl. The pH was adjusted to 2 with 1 M NaOH, the solution was then filter-sterilized (pore size 0.22 μm) and stored at 20 °C. The activity of the purified stock solutions of lactocin 705 and enterocin CRL35, expressed as arbitrary units per ml (AU ml^{-1}), was determined using serial twofold dilutions of the extracts and the well diffusion assay. The indicator lawn was prepared by adding 70 μl of an overnight culture of *Lactobacillus plantarum* CRL691.

Bacteriocin sensitivity measurement

Listeria strains at 10^3 c.f.u. ml^{-1} and 10^7 c.f.u. ml^{-1} were inoculated in tubes containing 5 ml TSBYE (triptic soy agar + yeast extract 0.6%) previously adjusted to pH 5, 6 and 7 by the addition of 0.02 M HCl. Nisin (2000 IU ml^{-1}), lactocin 705 (17 000 AU ml^{-1}) and enterocin CRL35 (8500 AU ml^{-1}) were added to the tubes before being inoculated. Finally, *Listeria* strains were incubated for 24 h at 20 °C. At intervals samples were taken for viable counts in TSAYE and incubated at 30 °C for 48 h. All results presented in this paper are the mean of two independent replicate assays. The variations were less than 10%.

Results and discussion

Ten strains of *Listeria* were tested for sensitivity against lactocin 705, enterocin CRL35 and nisin. The viability loss of the different strains of *Listeria monocytogenes* to bacteriocins comparing treatment of high (10^6–10^7 c.f.u. ml^{-1}) and low inocula (10^3–10^4 c.f.u. ml^{-1}) at pH 5, 6 and 7 are shown in Table 1. In preliminary experiments (data not shown) the growth of

Table 2. Growth inhibition of *Listeria innocua* by nisin, lactocin 705 and enterocin CRL35 at 30 °C and different pH.

Strains	Inoculum	Viability loss (log N_0/N)[a]								
		Nisin			Lactocin 705			Enterocin CRL35		
		pH 5	pH 6	pH 7	pH 5	pH 6	pH 7	pH 5	pH 6	pH 7
7[b]	Low	2.0	2.0	1.9	2.3	2.1	2.1	2.1	2.0	2.0
	High	1.2	1.0	1.0	1.0	0.8	0.8	1.2	1.0	1.0
11[b]	Low	2.5	2.4	2.4	2.5	2.4	2.4	2.3	2.2	2.2
	High	1.3	1.1	1.1	1.5	1.5	1.5	1.5	1.5	1.5
12[b]	Low	2.4	2.2	2.2	2.5	2.3	2.4	2.2	2.0	2.0
	High	1.1	1.0	1.0	1.4	1.2	1.2	1.5	1.5	1.4
L1PE[b]	Low	2.0	2.0	2.0	2.5	2.2	2.0	2.4	2.2	2.0
	High	1.2	1.0	1.0	1.8	1.5	1.5	1.7	1.5	1.3

[a]See footnote a Table 1.
[b]Strains from Unité de Recherches Laitiéres et Génetique Appliqueé, INRA, France.

Table 3. Growth inhibition of *Listeria seeligeri* by nisin, lactocin 705 and enterocin CRL35 at 30 °C and different pH.

Strains	Inoculum	Viability loss (log N_0/N)[a]								
		Nisin			Lactocin 705			Enterocin CRL35		
		pH 5	pH 6	pH 7	pH 5	pH 6	pH 7	pH 5	pH 6	pH 7
WS2253[b]	Low	2.7	2.5	2.4	2.5	2.0	2.0	2.2	1.8	1.8
	High	1.6	1.5	1.5	1.5	1.5	1.5	1.2	1.2	1.0

[a]See footnote a in Table 1.
[b]Strain from IHT, Karlsruhe, Germany.

Listeria in bacteriocin-free TSYBE was found to be slightly slower at pH 5 than at pH 7 and a re-growth of survivors after 2 h in the presence of bacteriocins was observed even when comparable viable cell counts after 24 h were obtained. When the effect of 2500 IU nisin ml^{-1} on *L. monocytogenes* strains was compared, strain Scott A and WR129 proved to be the most resistant, the viability loss being 1.5 and 1.8 log cycles for low inocula, respectively. The inhibitory action of nisin was dependent on the pH, producing a higher decrease in viable cells at pH 5 than at pH 6 and 7 both for low and high inocula. This enhanced nisin tolerance of *L. monocytogenes* strains, as well as the strongest bactericidal effect when pH decreases, is well documented (Muriana 1996, Schillinger *et al*. 1998). When the non-nisin bacteriocins were added to *L. monocytogenes* culture, higher viability losses were obtained as well as lower pH differences when compared to nisin. Lactocin 705 (17 000 AU ml^{-1}) was more effective in inhibiting *L. monocytogenes* strains than enterocin CRL35 (8500 AU ml^{-1}), strains FBUNT and 4ab being the most sensitive exhibiting a viability loss of 3.4 and 3.0 log cycles, respectively, with a low inocula after 2 h and pH 5. Enterocin CRL35 was more effective in the inhibition of strains FBUNT and SR215 with viability losses of 3 and 2.5 log cycles, respectively. Nevertheless, when high inocula were used, lower viability loss values were observed independently of the bacteriocin and pH values. Observations of these effects on high inocula of *L. monocytogenes* enabled comparison of sensitivity of the individual strains to bacteriocins, these effects being clearly strain dependent and, despite some variations, strain FBUNT appeared to be the most sensitive to the action of the studied bacteriocins, from which lactocin 705 demonstrated to be the most effective. These results agree with those of Ukuku & Shelef (1997) who reported a strain dependence when the sensitivity of *L. monocytogenes* to nisin was studied. Bankerroum & Sandine (1988) also reported strain differences when the well assay was used and inhibition-zone diameters measured noting that strain Scott A was particularly resistant.

The effects of different strains of *Listeria innocua* and *Listeria seeligeri* subjected to the action of the three bacteriocins at pH 5, 6 and 7 are summarized

in Tables 2 and 3. Lower viability loss values as well as less differences between pH values were obtained both at low and high inocula when comparing to *L. monocytogenes* strains. *L. innocua* 11 and 12 showed to be the most sensitive to the effect of all three bacteriocins at low inocula while *L. innocua* L1PE showed higher inhibitory effectiveness with lactocin 705 and enterocin CRL35 reaching viability loss values of 2.5 and 2.4 log cycles (low inocula) and 1.8 and 1.7 log cycles (high inocula) at pH 5, respectively. Moreover no re-growth was produced after the initial decrease in the viable counts when *L. innocua* 11 and 12 at 10^3 cells ml^{-1} was treated with nisin (data not shown). On the other hand, *L. seeligeri* showed a higher viability loss with nisin than with lactocin 705 and enterocin CRL35 (Table 3).

The natural variation in the susceptibilities of Gram-positive bacteria towards bacteriocins is considerable. The inhibitory action of these antimicrobial peptides can vary between different genera, species of genera, identical species, and even identical cultures under different environmental conditions (Bennik *et al*. 1997). Both cell wall constitution and membrane lipid composition have been demonstrated to be involved in bacteriocin action as well as bacteriocin resistance. To explain differences in bacteriocin sensitivities among bacteria, the role of membrane fluidity has also been postulated (Davies *et al*. 1996, Masnier-Patin & Richard 1996, Ming & Daeschel 1993). Finally, Bennik *et al*. (1997) also reported evidence that the association of bacteriocins with the cell membrane and their subsequent insertion take place in a similar way for the cells that have a high or a low natural tolerance towards bacteriocins. The factors that account for the naturally occurring variability in bacteriocin susceptibility have yet to be clarified. The effects of bacteriocins were clearly strain dependent and the lower pH enhanced the bactericidal effect mainly of nisin. In view to bacteriocin applications in food studies of individual strain sensitivity to different bacteriocins must be considered.

Acknowledgements

This study was supported by funds from PICT98 No. 09-04632 (ANPCyT), Argentina. The authors would like to thank A.Y. Borchia for her excellent technical assistance and Aplin & Barret Ltd. for the gift of a nisin sample.

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