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ORIGINAL ARTICLE

Antilisterial activity of carnocin 54, a bacteriocin from *Leuconostoc carnosum*

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Carnocin 54, a bacteriocin produced by Leuconostoc carnosum *LA54A acts very rapidly in a bactericidal manner on* Listeria *cells. The killing kinetics strongly depend on bacteriocin concentration and pH. At a high concentration, more than 99·99% of the cells were killed within 1 min. Lowering carnocin concentration and increasing pH from 4·9 to 6·5 resulted in a slower reduction of* Listeria *viable counts. Carnocin was able to inactivate* Listeria *cells to the same extent at pH 4·5 and 6·5 within 5 h. After 24 h, however, a slight resurgence of* Listeria *was observed at pH 6·5. The bactericidal efficiency was* Listeria *strain dependent, but was not influenced by the growth phase. At a given bacteriocin concentration, the level of inactivation of* Listeria *was not affected by the initial cell number.*

Introduction

In recent years, many lactic acid bacteria isolated from various foods were shown to be producers of bacteriocins (Klaenhammer 1988, 1993). As a large number of these bacteriocins inhibit spoilage bacteria and foodborne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*, several authors suggested a possible application of these antibacterial compounds as biological additives in the preservation of foods (Marugg 1991, Earnshaw 1992, Ray 1992). This is especially attractive in minimally processed foods such as vacuum-packaged refrigerated meats and new types of foods which lack multiple barriers to the growth of pathogenic and spoilage bacteria formerly conferred by traditional preservation techniques.

Most of the known bacteriocins act in a bactericidal manner, some of them resulting in cell lysis. Sensitive cells are killed after exposure to the bacteriocin. Little, however, is known about the factors affecting the killing kinetics and the bactericidal efficiency of such bacteriocins.

The purpose of this study was to investigate factors affecting the bactericidal efficiency of carnocin 54, an antilisterial bacteriocin produced by a strain of *Leuconostoc carnosum* (Keppler et al. 1994).

Materials and Methods

Bacterial strains and media

Leuconostoc carnosum LA54A which produces carnocin 54 was grown in MRS broth (Merck, Darmstadt, Germany) at 25°C.

The strains of *Listeria* used in this study were the same as those used by Uhlman et al. (1992). Additionally, *Listeria monocytogenes* DSM 20600 was included. They were cultivated in Standard I

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broth (Merck, Darmstadt, Germany) at 30°C and were maintained at –80°C as frozen stock cultures with 20% glycerol.

Bacteriocin preparation

Bacteriocin was obtained from cultures of *Lc. carnosum* LA54A grown in MRS broth for 20–24 h. Cells were removed by centrifugation (10 000 *g*, 15 min) and the supernatant fluids were adjusted to different pH levels with NaOH and HCl, respectively. The supernatants were sterilized either by using 0·2 μm pore-size filter discs (Schleicher and Schüll, Dassel, Germany) or by heating at 100°C for 10 min. These crude bacteriocin preparations were kept frozen at –20°C until use.

A stock preparation of carnocin 54 which had an activity of 51 200 AU ml^{-1} against *Listeria innocua* WS 2257 was used for all the following experiments.

Bacteriocin activity assay

The activity of the crude bacteriocin preparation was determined using an agar spot test as described previously (Schillinger et al. 1993). *L. innocua* WS 2257 was used as indicator strain.

Killing kinetics of carnocin 54

Carnocin preparations (51 200 AU ml^{-1}) adjusted with NaOH to pH 4·9 and 6·5, respectively, were diluted with MRS broth (pH 4·9 and pH 6·5) to contain 5120, 512 and 51·2 AU ml^{-1} and were inoculated with *L. innocua* WS 2257 ($1·5 \times 10^6$ ml^{-1}). MRS broth of pH 4·9 and 6·5 was used as a control (0 AU ml^{-1}). 1 ml samples were withdrawn at 0, 1, 3, 5, 10, 30 and 60 min and were immediately put into sterile Eppendorf tubes containing 20 μl proteinase K (10 mg ml^{-1}). After mixing, these samples were plated onto Standard I agar to determine the number of survivors.

Influence of type, growth phase and cell number of the indicator strain on the bactericidal efficiency of carnocin 54

Carnocin preparations (51 200 AU ml^{-1}) were inoculated with the indicator strain ($1·5 \times 10^6$ ml^{-1}) and the optical density and number of survivors were determined at different time intervals by plating on Standard I agar. *L. monocytogenes* WS 2250, *L. innocua* WS 2257 and *L. seeligeri* WS 2253 were used as indicator strains.

To determine the influence of the growth phase of the indicator strain on the bactericidal efficiency of carnocin 54, *L. monocytogenes* WS 2250 and *L. innocua* WS 2257 were inoculated in Standard I broth at 3×10^5 ml^{-1} and their growth at 30°C was followed by plating appropriate dilutions on Standard I agar after different time intervals. Samples taken after 5, 10, 14 and 24 h (representing different phases of growth) were used to inoculate the carnocin preparation at 10^5–10^6 ml^{-1}.

The influence of inoculum density of the indicator strain on the bactericidal efficiency of carnocin 54 was assessed. Thus, carnocin preparations (51 200 AU ml^{-1}) were inoculated with different dilutions of an overnight culture (18 h, 30°C) of *L. innocua* WS 2257 to give initial cell numbers of 3×10^4, 3×10^5, 3×10^6, 3×10^7 and 3×10^8 ml^{-1}.

Results

Antilisterial activity

Sixteen strains of different species of *Listeria* including the pathogen *L. monocytogenes* were screened for sensitivity to carnocin 54. All strains were inhibited by the culture supernatants of *Lc. carnosum* LA54A in the agar spot test.

L. innocua WS 2257 was chosen as indicator strain for the quantitative estimation of bacteriocin activity. The activity of crude carnocin preparations to be used in the following experiments was determined to be 51 200 AU ml^{-1}.

Killing kinetics of carnocin 54

A rapid bactericidal effect of carnocin 54 on *L. innocua* WS 2257 was demonstrated by exposing *Listeria* cells to these bacteriocin preparations for different time periods. Fig. 1 shows the effect of different concentrations of carnocin 54 on viability of *L. innocua* WS 2257 at pH 4·9. The highest rate of decrease in viable counts was observed with undiluted supernatant as well as with a 10^{-1} dilution (51 200 and 5120 AU ml^{-1}, respectively). Viable cell numbers decreased from $1·5 \times 10^6$ ml^{-1} to $4·2 \times 10^1$ ml^{-1} and $2·0 \times 10^2$ ml^{-1}, respectively, within 1 min. With more diluted carnocin

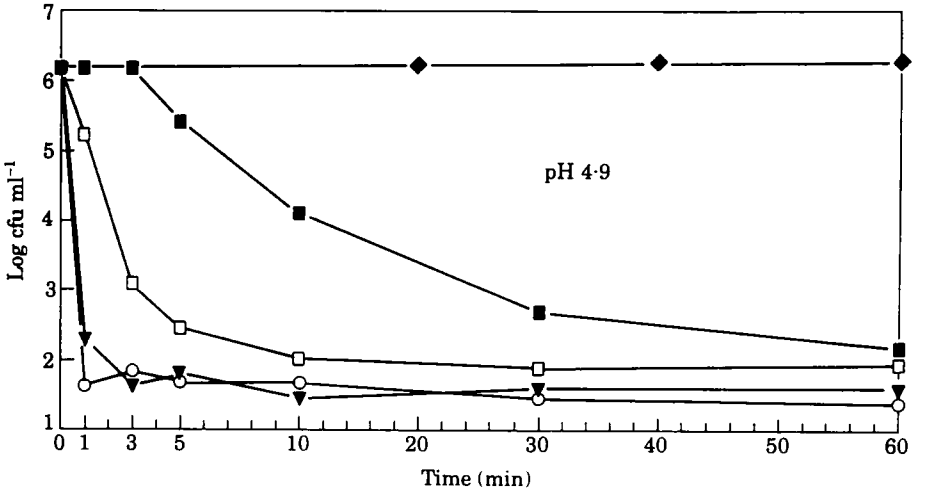

Figure 1. Bactericidal action of carnocin 54 on cells of *Listeria innocua* WS 2257 in MRS broth at pH 4·9. (♦), Control (no bacteriocin); (○), 51200 AU ml^{-1}; (▼), 5120 AU ml^{-1}; (□), 512 AU ml^{-1}; (■), 51·2 AU ml^{-1}.

preparations indicator cells were killed at a slower rate, reaching comparable cell densities not before 60 min. In the control without bacteriocin, viable cell numbers did not decrease over the same incubation period.

When the pH of carnocin preparations was adjusted to 6·5, a slower reduction of viable cell numbers was observed for all carnocin concentrations (Fig. 2). For example, in the presence of 512 AU ml^{-1}, 1·5% of the cell population was still viable after 3 min at pH 6·5, whereas less than 0·1% survived at pH 4·9. Carnocin at a concentration of 51·2 AU ml^{-1} acted even slower and needed 30 min to reduce cell numbers by 98·5% at pH 6·5.

Factors affecting bactericidal efficiency of carnocin 54

Type of indicator strain and pH. Cells of three different *Listeria* species were exposed to carnocin 54 (Fig. 3). The bacteriocin was more effective against *L. seeligeri* WS 2253

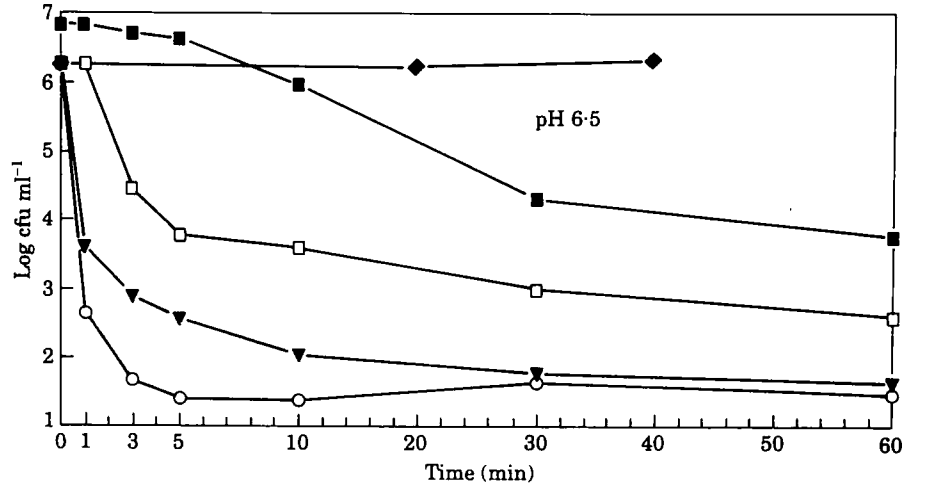

Figure 2. Bactericidal action of carnocin 54 on cells of *Listeria innocua* WS 2257 in MRS broth at pH 6·5. See Fig. 1. for explanation of symbols.

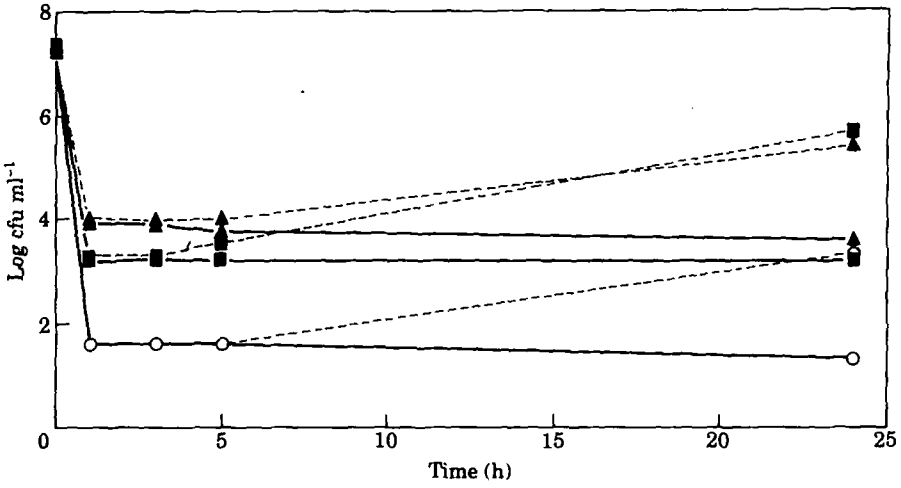

Figure 3. Effect of carnocin 54 (51200 AU ml^{-1}) on viable cells of *Listeria innocua* WS 2257 (■), *Listeria monocytogenes* WS 2250 (▲), and *Listeria seeligeri* WS 2253 (○) at pH 4·5 (—) and pH 6·5 (– –).

than against *L. monocytogenes* WS 2250 and *L. innocua* WS 2257. The cell population of *L. seeligeri* WS 2253 decreased by 5·8 log cycles whereas viable numbers of *L. monocytogenes* WS 2250 and *L. innocua* WS 2257 were reduced by about 3 log cycles only.

No change in the optical density occurred, indicating that carnocin 54 is listericidal without causing cell lysis.

The killing effect observed 5 h after exposure was about the same at pH 6·5 and pH 4·5. After 24 h, however, an increase in viable cell numbers of *Listeria* was detected at pH 6·5.

Growth phase. *Listeria* cells of different phases of growth (early and late log and stationary phases) did not differ in their response to the bactericidal action of carnocin (Fig. 4). Log and stationary phase cultures of

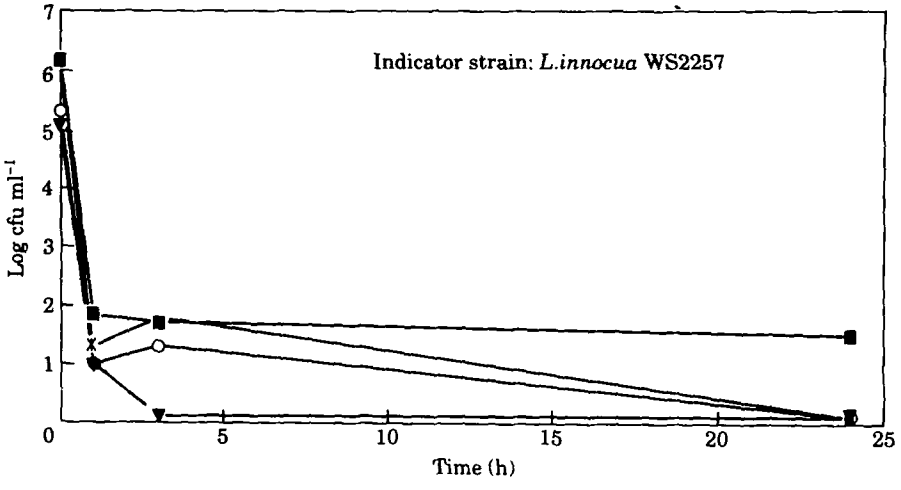

Figure 4. Effect of the growth phase of the indicator strain on the bactericidal efficiency of carnocin 54. Cells of *Listeria innocua* WS 2257 at different growth phases [(▼), early log phase; (○), late log phase; (⋆), early stationary phase; (■), late stationary phase] were used to inoculate carnocin preparation (51200 AU ml^{-1}) and viable cell numbers were determined at selected time intervals.

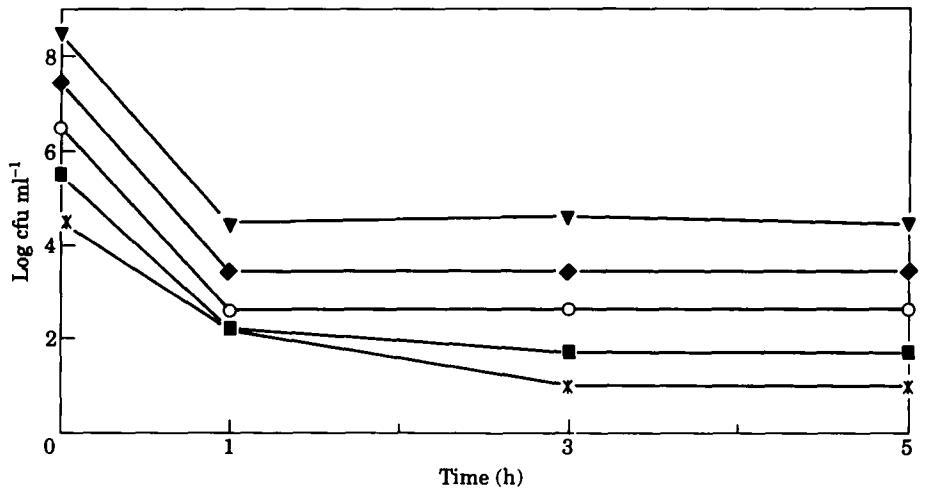

Figure 5. Effect of initial number of the indicator strain on the bactericidal efficiency of carnocin 54. Carnocin preparations were inoculated with *Listeria innocua* WS 2257 at log cfu ml^{-1} 4·5 (⋆); 5·5 (■); 6·5 (○); 7·5 (♦); and 8·5 (▼).

L. innocua WS 2257 and *L. monocytogenes* WS 2250 (data not shown) exhibited similar survivor curves.

Inoculum density of indicator cells. Exposure of different *Listeria* inoculum densities between 3×10^8 ml^{-1} and 3×10^4 ml^{-1} to carnocin 54 (51 200 AU ml^{-1}) resulted in a 4 log reduction of viable counts (Fig. 5). In all cases, 0·01% of the cells of *L. innocua* WS 2257 survived.

Discussion

Several bacteriocins from different species of *Leuconostoc* are known to be active against the foodborne pathogen *L. monocytogenes* (Harding and Shaw 1990, Daba et al. 1991, Hastings and Stiles 1991, Lewus et al. 1992, Van Laack et al. 1992, Mathieu et al. 1993, Keppler et al. 1994). Carnocin 54 belongs to this group of antilisterial bacteriocins. It is produced by *Lc. carnosum* LA54A isolated from a vacuum-packaged meat product. Carnocin 54 is a heat-resistant bacteriocin of a molecular weight of about 4000 Da (Keppler et al. 1994) and differs from most other *Leuconostoc* bacteriocins by its sensitivity to amylase. Production of a similar antilisterial bacteriocin by another strain of *Lc. carnosum* (LA44A) was reported by Van Laack et al. (1992). This bacteriocin, however, shows an inhibitory spectrum different from that of carnocin 54.

A rapid reduction of *Listeria* viable numbers was observed in the presence of carnocin 54. More than 99·9% of the listeriae were killed within 1 min at pH 6·5. A similar high killing rate of a bacteriocin was reported by Ahn and Stiles (1990) who observed a reduction of viable cell numbers by 99·9% within 5 min by the bacteriocin(s) produced by *Carnobacterium piscicola* LV 17. The lactostrepcin Las 5 inactivated more than 99% of the indicator cells within 3 min. (Zajdel and Dobrzanski 1983). Other bacteriocins were shown to reduce a cell population by 96% within 4 h (Vaughan et al. 1992), by 92% within 1 h (Bhunia et al. 1988), by 99% within 2 h (Piard 1994, Nes et al. 1994) and by 99·96% within 3 h (Schillinger and Lücke 1989). In contrast to carnocin 54, enterocin 1146 did not show an instant killing effect on *L. innocua* (Parente and Hill 1992). The authors concluded from protease rescue experiments that significant cell death did not occur before 2 min after exposure to the bacteriocin.

The killing kinetics of carnocin 54 were affected by the pH. When cells of *L. innocua* WS 2257 were treated with carnocin 54, a higher inactivation rate was observed at pH

4·9 than at pH 6·5. The pH effect was more pronounced at low bacteriocin concentrations (Figs 1 and 2). Similarly, Harris et al. (1991) observed an increase in the effectiveness of nisin when the pH of the medium was decreased from 6·5 to 5·5.

The higher killing rate at acidic pH may either be the result of the higher activity of carnocin 54 or may be due to higher susceptibility of the indicator strain at lower pH. A synergistic effect between bacteriocin action and reduced pH may explain the enhanced destruction rate of the indicator strain (Becker et al. 1994). Keppler et al. (1994) found carnocin to be most active at pH 3–5 and observed a 50% reduction of activity at pH 6–7.

When *Listeria* cells were exposed to carnocin 54 for 24 h, a resurgence of the indicator strain was observed at pH 6·5 while cell counts remained at the low level at pH 4·5 (Fig. 3). Cells that survived the effect of carnocin were able to multiply again at neutral but not at acidic pH indicating an irreversible effect in the lower pH range. This pH effect on the bactericidal action of carnocin was much more pronounced when *Leuconostoc* was used as indicator organism. Carnocin 54 caused a reduction in *Lc. mesenteroides* cell counts by 5·8 log cycles within 5 h at pH 4·5 whereas cell numbers decreased by 3·9 log cycles only at pH 6·5 (Becker et al. 1994).

Carnocin 54 inhibited all *Listeria* strains used in this study in the agar spot assay; however, its bactericidal efficiency varied between different strains of *Listeria*. Some strains were less sensitive than others. Other antilisterial bacteriocins from *Leuconostoc* spp. like mesenterocin 5 (Daba et al. 1991) and mesentericin Y105 (Hechard et al. 1992) neither inhibited all *Listeria* strains to the same extent.

The bactericidal efficiency of carnocin 54 was not dependent upon the growth phase of the indicator strain. Log and stationary phase cells showed the same susceptibility to the lethal action of carnocin 54. By contrast, Vaughan et al. (1992) and Davey (1994) reported that stationary phase indicator cells were more resistant to the effect of helveticin V-1829 and diplococcin, when compared to log phase cells, respectively. Other bacteriocins like pediocin SJ-1 (Schved et al. 1993) or enterocin 1146 (Parente and Hill 1992) were more effective against stationary phase cells than against log phase cells.

The relative bactericidal efficiency of a given concentration of carnocin 54 was not dependent upon the number of indicator cells. Reducing inoculation density did not result in a higher bactericidal efficiency.

A certain percentage of cells within the *Listeria* population appears to be resistant, or less susceptible to the bacteriocin. This was also confirmed by checking the bacteriocin sensitivity of the survivors. Most of them showed a reduced susceptibility to carnocin; they were, however, still sensitive to nisin (unpublished observations). An improvement of the bactericidal efficiency may be achieved by using a combination of carnocin 54 and nisin. In a recent study, Hanlin et al. (1993) showed that pediocin AcH and nisin were more effective in combination than when these bacteriocins were used alone. These findings, however, still need to be confirmed in food systems.

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