Bactericidal effect of acidophilin 801, a bacteriocin produced by *Lactobacillus acidophilus* IBB 801

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Abstract

The aim of this study was to investigate the mode of inhibition of acidophilin 801, a small and thermorezistant bacteriocin produced by Lactobacillus acidophilus IBB 801. The effect of acidophilin 801 on the growth, glycolytic activity and ultrastructure of the sensitive cells was investigated by measuring the cell counts, the change in pH value and by electron microscopy studies, respectively. Acidophilin 801 displayed a concentration-dependent bactericidal mode of inhibition towards sensitive strains, but it did not induce cell lysis. Moreover, treatment with acidophilin 801 resulted in a significant decrease of the glycolytic rate and changes in the ultrastructure of the sensitive cells. The most important step in its mode of action seems to be the pore formation that causes the release of intracellular molecules. Consequently, the glycolytic rate is reduced and hence many vital processes might be blocked.

Keywords: lactic acid bacteria, bacteriocins, bactericidal effect, acidophilin

Abbreviation: LAB = lactic acid bacteria

Introduction

Among the various antimicrobial compounds produced by lactic acid bacteria (LAB), bacteriocins have been studied for many years now. They are antibacterial, single polypeptides or polypeptide complexes that are active against strains or species closely related to the producer strain [1,2]. However, many of the LAB bacteriocins are also active towards food spoilage and foodborne pathogenic bacteria, being interesting for use in the food industry. Bacteriocins form a heterogeneous group with respect to molecular mass, biochemical properties, range of sensitive hosts, and mode of action. Several attempts have been made to classify LAB bacteriocins [1, 3-5]. Currently, bacteriocins are classified into three groups [6]: class I, the lantibiotics; class II, the small, non-modified peptide bacteriocins, further subdivided into three subclasses (IIa - the pediocin-like, antilisterial peptides; IIb – bacteriocins requiring two different peptides for full activity, and IIc – other peptides); and class III, the large, heat-labile protein bacteriocins.

Although active towards related taxa only, the inhibitory spectrum of the LAB bacteriocins can vary significantly, from relatively narrow, as in the case of the lactococcins, which have been found to kill only other *Lactococcus* species [7], to extraordinarily broad, as in the case of the lantibiotic nisin A [8]. Moreover, particular bacteriocins (mutacin B-Ny266, nisin A) are active *in vitro* against a number of medically important Gram-negative bacteria, including *Campylobacter, Haemophilus, Helicobacter*, and *Neisseria* [8].

It is generally accepted that most bacteriocins produced by LAB act by altering the permeability barrier of the target cell membrane through pore formation, resulting in the loss of the cell's viability [9-16]. One of the common mechanisms of inhibition is the dissipation of the membrane potential [11,12, 16, 18-20]. Moreover, it has been shown that nisin interferes with cell wall biosynthesis, which was later found to be based on the interaction with the membrane-bound cell wall precursor lipid II [undecaprenylpyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc], used as docking molecule [21-23].

Apart from damaging cell membranes, some bacteriocins have also been reported to cause loss of cell viability by inducing cell lysis of the target strains, as indicated by the decrease in optical density of the culture [24,25]. Apparently, the bacteriocins themselves are not capable of lysing sensitive cells. In fact, it was shown that the

dissipation of the proton motive force caused by the bacteriocins has a direct effect on autolysis, when active autolysins are present in the sensitive cells [26].

Lactobacillus acidophilus IBB 801 produces a heat stable, strongly hydrophobic, low-molecular-mass bacteriocin, referred to as acidophilin 801, characterised by a narrow inhibitory spectrum [27]. The aim of this paper was to study the mode of inhibition of acidophilin 801 and to assess the ultrastructural changes induced in sensitive cells after treatment with this bacteriocin.

Materials and Methods

Bacterial strains and culture media. The bacterial strains used in this study were *L. acidophilus* IBB 801, as the bacteriocin-producing strain, and *L. helveticus* 102 and *L. helveticus* rosyjski as the acidophilin 801-sensitive strains. The latter strains were kindly provided by the Institute for Food Research (Bucharest, Romania) and Rhodia Food Biolacta (Olsztyn, Poland), respectively. All the strains were maintained at -75 °C in MRS medium, in the presence of 25% (v/v) glycerol. To obtain fresh cultures, the strains were propagated twice in MRS medium [28], at 37 °C for 12 h. MRS agar (1.5%, w/v) was used as solid medium. For the bioassays, soft agar was used by adding 0.7% (w/v) agar to MRS broth.

Detection and quantitative determination of bacteriocin activity. Bacteriocin activity was assayed by an adaptation of the critical dilution method as described by De Vuyst *et al.* [29]. Briefly, serial twofold dilutions in ultrapure water of samples were spotted (10 µl) onto fresh indicator lawns of the indicator strains. The bacteriocin activity was defined as the reciprocal of the highest dilution which demonstrated complete inhibition of the indicator lawn and was expressed in activity units (AU) per millilitre of sample.

Production of an acidophilin 801 preparation. For bacteriocin isolation, the producing strain was grown in MRS medium at 37 °C for 12 h. Acidophilin 801 was concentrated by ammonium sulphate precipitation followed by chloroform/methanol extraction, as described elsewhere [27]. Briefly, cells were centrifuged (25000 *g*, 15 min) and the cell-free culture supernatant (pH adjusted to 6.5) was precipitated with ammonium sulphate (40% saturation) at 4 °C, overnight, with gentle stirring. The floating pellicle was collected after centrifugation, dissolved in potassium phosphate buffer (pH 6.5), and extracted with 15 volumes of a mixture of chloroform/methanol (2/1, v/v). After l h at 4 °C, the white precipitate formed was centrifuged at 25000 *g* for 1 h and resuspended in ultrapure water. Alternatively, the method described by Yang *et al.* [30] was used to concentrate the acidophilin from the culture medium: the culture broth was adjusted to pH 6.5 and heated at 70 °C for 30 min to kill the cells. Cells were harvested by centrifugation at 25000 *g* for 15 min, washed with 50 mM potassium phosphate (pH 6.5) and resuspended in 100 mM NaCl, pH 2.0 (adjusted with phosphoric acid). After 1 h at 4 °C, the cell suspension was centrifuged at 25000 *g* for 20 min and the pH of the supernatant was adjusted to pH 6.5. The acidophilin 801 preparation (activity ranging between 1600 and 6400 AU ml^{-1}) thus obtained was used for further study.

Inhibitory effect of acidophilin 801 on sensitive strains. In a first experiment, the acidophilin 801 preparation was diluted with sterile potassium phosphate buffer (pH 6.5) to obtain activities of 62.5, 125, 250 and 500 AU ml^{-1}. Buffer solely was used as a control. A 10-ml sample of each defined preparation was added to a separate sterile test tube and pre-warmed to 37 °C. Cells from a log-phase culture of the sensitive strain *L. helveticus* 102 were washed in potassium phosphate buffer and added to each test tube. At intervals of 0, 1, 3 and 5 h, viable cell counts (CFU ml^{-1}) were determined.

In a second experiment, the sensitive strains, *L. helveticus* 102 and *L. helveticus* rosyjski, were inoculated (1%, v/v) into fresh MRS medium and incubated at 37 °C for 3 h. An amount of acidophilin 801 preparation was added to this culture to obtain a final activity of about 250 AU ml^{-1} and 500 AU ml^{-1}, respectively, and the cell counts were determined at regular time intervals on MRS agar containing trypsin (1 mg ml^{-1}) to stop the inhibitory activity of the bacteriocin. Cultures without bacteriocin were used as controls. All experiments were done in duplicate.

Effect of acidophilin 801 on the glycolytic activity of sensitive cells. *L. helveticus* rosyjski cells (OD_{660}=1) were suspended in 0.5 mM potassium phosphate, pH 6.5, containing 70 mM KCl, and 1 mM $MgSO_4$, to which

0.5% (w/v) of glucose was added [31]. The change in pH value was monitored with a pH-meter, both at 30 °C and 37 °C, and using two different activities of acidophilin 801, namely 250 and 500 AU ml^{-1}. The experiment was done in duplicate.

Electron microscopy. Samples were taken from an exponentially growing sensitive culture (both *L. helveticus* 102 and *L. helveticus* rosyjski) before adding the bacteriocin, and after 15 and 30 min after addition. An adaptation of the method described by Hayat [32] was used to prepare the samples. Cells were harvested by centrifugation (11000 *g*, 15 min), washed, and fixed in sodium cacodylate buffer, containing 3% (v/v) of glutaraldehyde and 0.01% (w/v) $CaCl_2$ x $2H_2O$, for 1 h, all at 4 °C. Samples were washed once with cacodylate buffer. Cells were then included in agar and washed four times with the same buffer, for about 1 h. The preparations were postfixed in 2% (w/v) OsO_4 at room temperature for 2 h, washed with distilled water for 1 h, dehydrated with ethanol, and embedded in Epon 812 (Fluka). Thin sections were stained applying the Reynolds method [33], using lead citrate and uranyl acetate, and they were observed with a transmission electron microscope (TESLA BS500).

Results

The inhibitory effect of acidophilin 801 on the sensitive strains *L. helveticus* 102 and *L. helveticus* rosyjski. In the first experiment, the addition of acidophilin 801 to a culture of *L. helveticus* 102 did not permit growth of this strain, indicating a bactericidal effect (Fig. 1). Moreover, after 1 h of incubation, the cell count was approximately 1-2 log cycles lower than in the control, indicating a fast effect. After 5 h of incubation, cell counts decreased with 1, 3 and 4 log, respectively, after the addition of 62.5, 250 and 500 AU ml^{-1} of acidophilin 801, respectively, indicating a concentration-dependent effect.

In the presence of 500 AU ml^{-1} of acidophilin 801 a culture of *L. helveticus* 102 resulted in a reduction of the cell counts with 3 log within 4 h, as compared with the control (Fig. 2A). In the case of *L. helveticus* rosyjski, the bacteriocidal effect of acidophilin 801 was faster, as after only 30 min cell counts decreased with 1 and 2 log after the addition of 250 and 500 AU ml^{-1}, respectively (Fig. 2B). For both experiments, the OD_{660} of the samples after bacteriocin addition remained approximately constant, indicating that no cell lysis occurred.

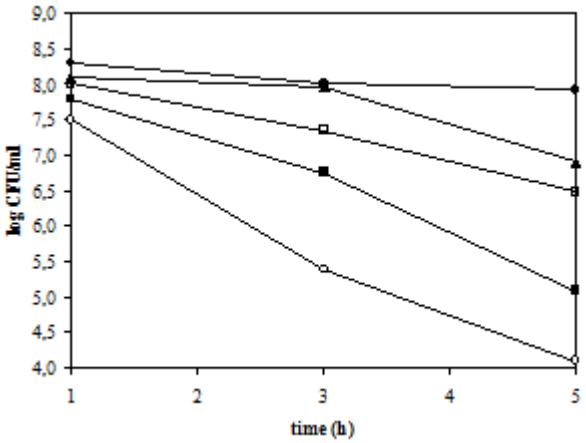


Figure 1. Effect of the addition of increasing amounts of acidophilin 801 towards a culture of *L. helveticus* 102. Graphs are representative for all experiments.

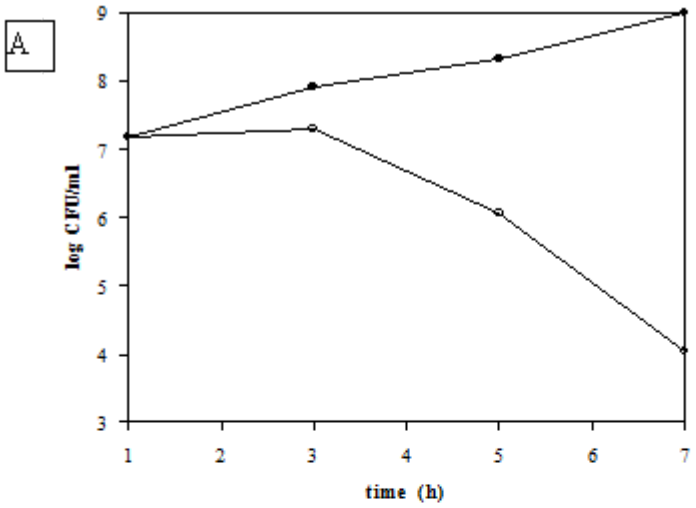
● control; ▲, 62.5 AU ml^{-1};□, 125 AU ml^{-1};■ , 250 AU ml^{-1};○ , 500 AU ml^{-1}.

Effect of acidophilin 801 on the glycolytic activity of sensitive cells. Acidophilin 801 caused a significant reduction of the glycolytic activity of sensitive cells, indicating a loss of cell viability (Fig. 3). The effect was higher when the activity of the bacteriocin was higher, but there was no significant difference between the effect at 30 °C and at 37 °C.

Ultrastructural changes induced in sensitive cells by the addition of acidophilin 801. Exponentially growing cells of *L. helveticus* 102 and *L. helveticus* rosyjski possess a thick, uniform cell wall to which the cytoplasmic membrane tightly adheres, and a granulated cytoplasm. Mesosomes or other intracytoplasmic membrane formations were not observed in these cells during our study (Fig. 4).

Bacteriocin addition induced a change in the structure of the sensitive cells. After 30 min of incubation with 250 AU ml^{-1} of acidophilin 801, mesosome-like membranous formations were observed protruding into the cytoplasm of the *L. helveticus* 102 cells, indicating that the structure of the cytoplasmic membrane was affected by the bacteriocin (Fig. 5A, 5B). The presence of cytoplasmic vesicles could also be detected (Fig. 5A). In some cells, changes were observed even at the level of the cell wall, as shown by the formation of an electron-transparent layer between the plasma membrane and the outer wall layer (Fig. 5C).

In the case of *L. helveticus* rosyjski cells, the changes induced in their ultrastructure by the acidophilin 801 treatment (250 AU ml^{-1} and 500 AU ml^{-1}, respectively) were more complex. The vesiculization of the protoplasm (Fig. 6G), pore formation (Fig 6A, 6B), and disintegration of the cell wall with loss of the protoplasmic material through a cell wall pore (Fig 6C, 6D) were detected, while some cells were completely disintegrated (Fig. 6E, 6F).



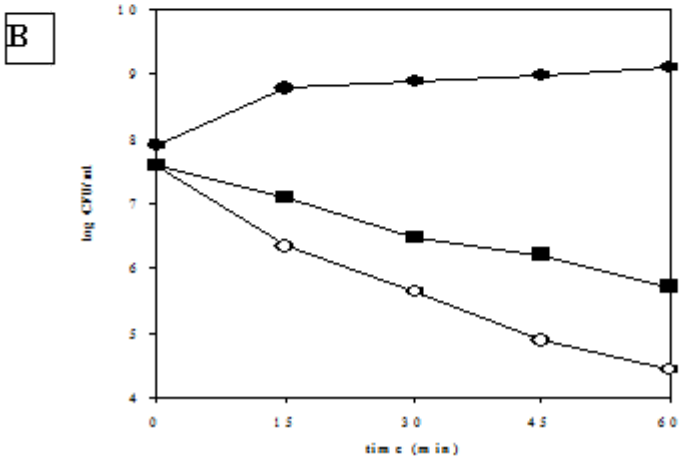


Figure 2. Effect of the addition of acidophilin 801 towards an exponentially growing culture of *L. helveticus* 102 (A) and *L. helveticus* rosyjski (B). Graphs are representative for all experiments.

●, control; ■, 250 AU ml^{-1}; ○, 500 AU ml^{-1}.

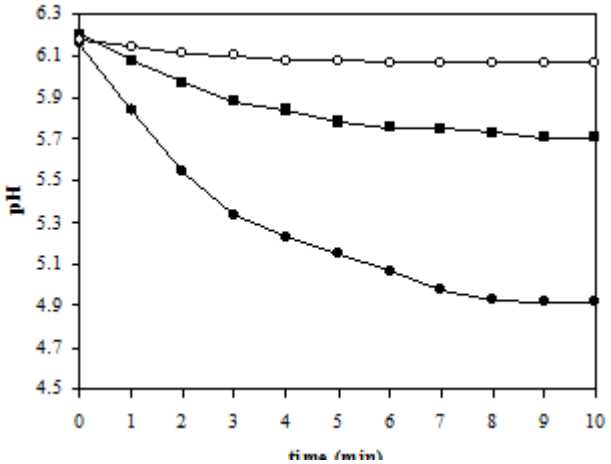


Figure 3. Effect of acidophilin 801 on the glycolytic activity of sensitive cells of *L. helveticus* rosyjski. Graphs are representative for all experiments.

●control; ■, 250 AU ml^{-1}; ○, 500 AU ml^{-1}.

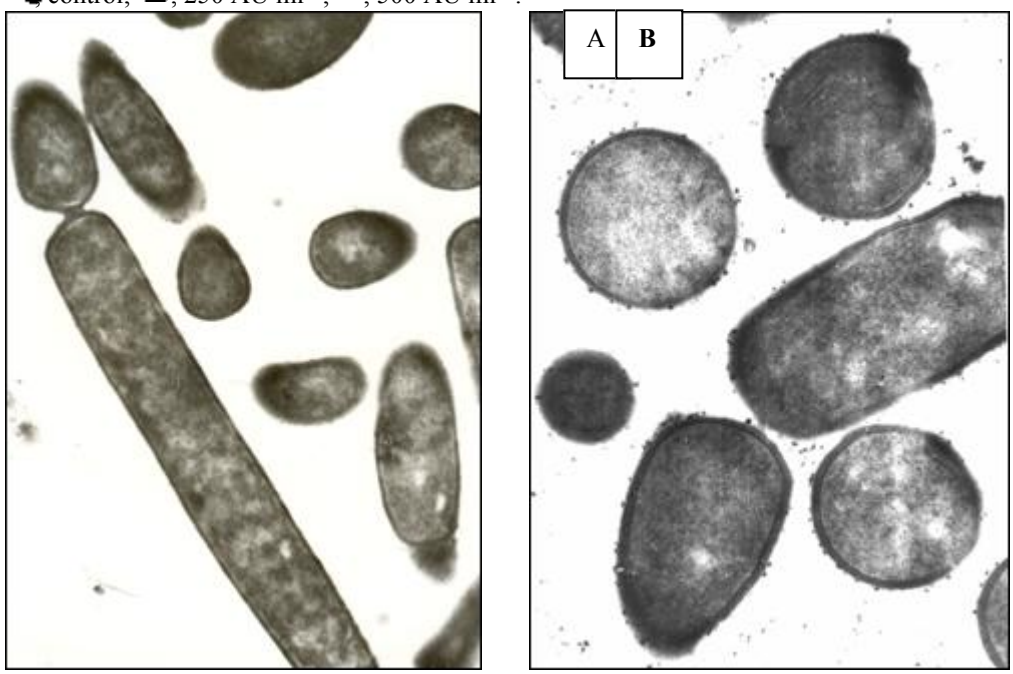


Figure 4. Electronomicroscopical aspect of normal cells of L. helveticus 102 (A) and L. helveticus rosyjski (B).

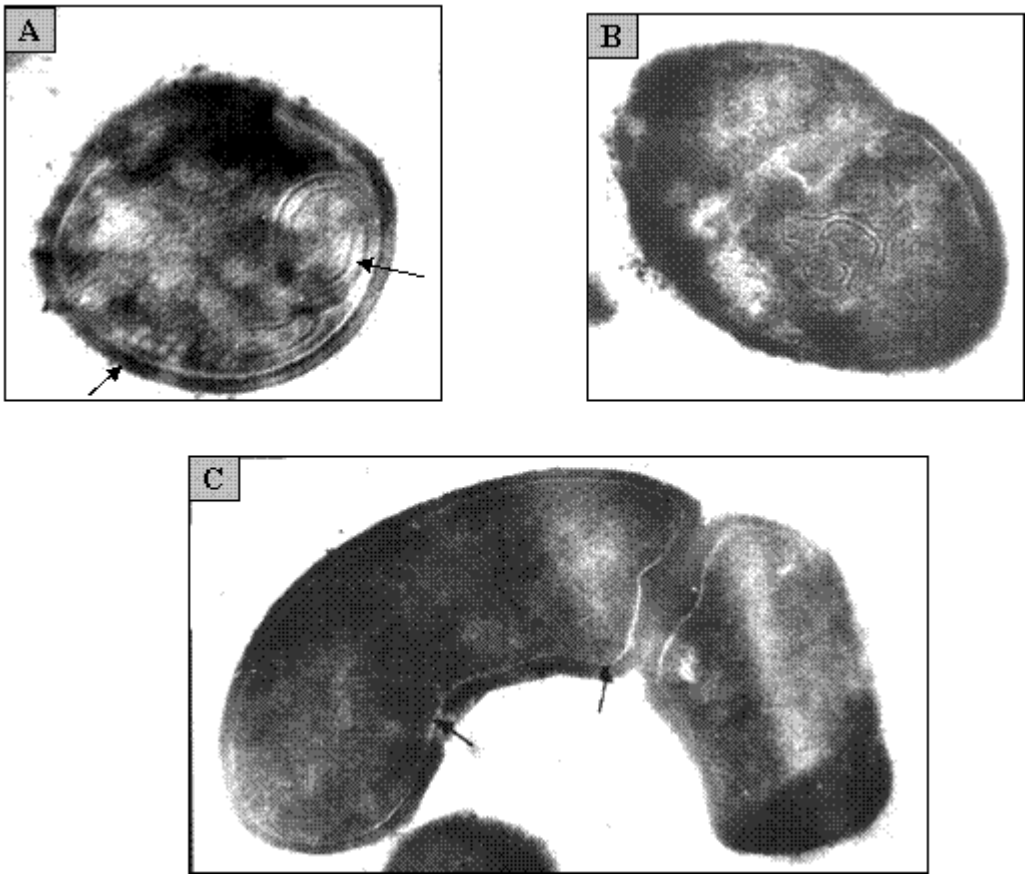


Figure 5. Ultrastructural changes in the sensitive cells of L. helveticus 102 induced by the treatment with acidophilin 801: vesiculization of the protoplasm (A); mesosomes formation (B); formation of an electron-transparent layer between the plasma

membrane and the outer wall layer (C).

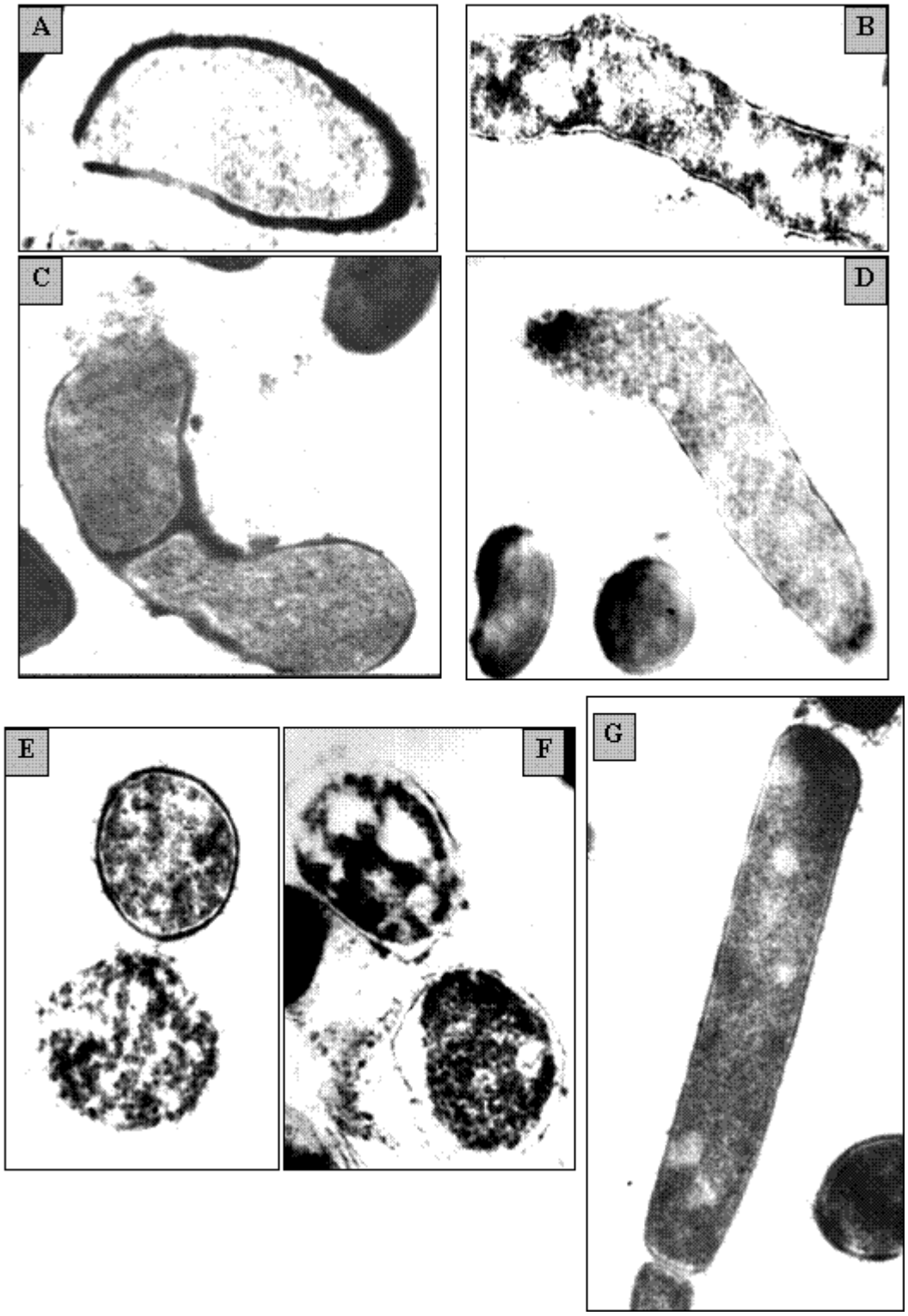


Figure 6. Ultrastructural changes in the sensitive cells of L. helveticus rosyjski induced by the treatment with acidophilin 801: pore formation (A, B); exclusion of the protoplasmic material (C, D); cell disintegration (E, F); vesiculization of the protoplasm (G).

Discussion

The low-molecular-mass bacteriocins of the Gram-positive lactic acid bacteria demonstrate bactericidal activity principally directed against other Gram-positive bacteria. As most bacteriocins produced by *L. acidophilus* strains, acidophilin 801 produced by *L. acidophilus* IBB 801 displays a narrow inhibitory spectrum [27]. It seemed to have a concentration-dependent bactericidal effect towards the sensitive strains and the degree of sensitivity to this bacteriocin varied even within a given species. For example, *L. helveticus* 102 strain was less sensitive to acidophilin 801 compared with *L. helveticus* rosyjski. Such variations in the levels of sensitivity to a certain bacteriocin were previously reported by other researchers. They suggested that the ability of a bacteriocin to interact with the cytoplasmic membrane can be influenced by factors such as the composition of the cell envelope, including the peptidoglycan layer, and the lipid composition of the membrane [19, 34, 35].

The addition of acidophilin 801 caused in a very short time significant changes in the ultrastructure of the sensitive cells. The frequency of damaged cells in a treated culture seemed to be dependent on the concentration of acidophilin added and the duration of the treatment (data not shown). The variation in the sensitivity to acidophilin 801 of the two strains tested was reflected in the ultrastructural changes induced by their treatment. In the case of *L. helveticus* 102, mesosomes and intracytoplasmic vesicles could be observed in treated cells. Mesosomes, which are intracytoplasmic membrane inclusions, have been previously regarded as structural artefacts induced by the chemical fixatives used on the cells prior to embedding and thin sectioning [36]. Yet mesosomes must be regarded as being an indication of cytoplasmic membrane alteration, in this case induced by bacteriocins, since untreated cells did not contain them. Mesosome formation was also reported in Gram-positive bacteria treated with plantaricin C [14] or with cationic peptides with antibacterial action [37]. As the cytoplasmic membrane is important in cell wall biosynthesis and turnover, these authors consider that a perturbation of this membrane may also affect the cell wall integrity.

No mesosomes were detected in *L. helveticus* rosyjski cells, but the formation of transmembrane pores, with the loss of intracellular material, and the formation of intracytoplasmic vesicles could be observed in a large number of cells after treatment with acidophilin 801.

Acidophilin 801 did not permit the growth of the two sensitive strains used in our study. Moreover, cell death occurred in a short time, but the OD of the cultures remained approximately constant, proving that acidophilin did not induce cell lysis. This was confirmed by the electron microscopy studies. A very small number of lysed ghost cells could be observed however, but this might be explained by the activation of the autolytic systems in dead cells, as a secondary effect.

Acidophilin 801 caused a decrease in the glycolytic rate of *L. helveticus* rosyjski cells, as shown by measuring the rate of acidification of the medium after addition of glucose. The same effect was observed by Gonzalez *et al.* [14] for plantaricin C and it was explained as a result of the sugar uptake inhibition.

On the basis of the data presented in this paper, it can be concluded that acidophilin has a bactericidal, concentration-dependent effect towards sensitive strains, without causing concomitant cell lysis of the indicator cells. The most important step in its mode of action seems to be the pore formation that causes the release of intracellular molecules. Consequently, the glycolytic rate is reduced and hence many vital processes might be blocked. All these effects cause growth inhibition and result in the ultimate death of sensitive cells.

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References

1. Klaenhammer, T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev* **12**, 39-86.
2. Jack, R.W., Tagg, J.R. and Ray, B. (1995) Bacteriocins of Gram-positive bacteria. *Microbiol Rev* **59**, 171-200.
3. Nes, I.F., Diep, D.B., Håverstein, I.S., Brurberg, M.B., Eijsink, V. and Holo, H. (1996) Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van Leeuwenhoek* **70**, 113-128.

4. Nes, I.F., Holo, H. (2000) Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers* **55**, 50-61.
5. McAuliffe, O., Ross, R.P., Hill, C. (2001) Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol Rev* **25**, 285-308.
6. Eijsink, V.G.H., Axelsson, L., Diep, D.B., Havarstein, L.S., Holo, H., Nes, I.F. (2002) Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek* **81**, 639-654.
7. Ross, R.P., Galvin, M., McAuliffe, O., Morgan, S.M., Ryan, M.P. (1999) Developing applications for lactococcal bacteriocins. *Antonie Van Leeuwenhoek* **76**, 337-346.
8. Mota-Meira, M., Lapointe, G., Lacroix, C., Lavoie, M.C. (2000) MICs of mutacin B-Ny266, nisin A, vancomycin, and oxacillin against bacterial pathogens. *Antimicrob Agents Chemother* **44**, 24-29.
9. van Belkum, M.J., Kok, J., Venema, G., Holo, H., Nes, I.F., Konings, W.N., Abee, T. (1991) The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. *J Bacteriol* **173**, 7934-7941.
10. Venema, K., Abee, T., Haandrikman, A.J., Leenhouts, K.J., Kok, J., Konings, W.N. and Venema, G. (1993) Mode of action of lactococcin B, a thiol-activated bacteriocin from *Lactococcus lactis*. *Appl Environ Microbiol* **59**, 1041-1048.
11. Bruno, M.E.C., Montville, T.J. (1993) Common mechanistic action of bacteriocins from lactic acid bacteria. *Appl Environ Microbiol* **59**, 3003-3010.
12. Abee, T. (1995) Pore-forming bacteriocins of Gram-positive bacteria and self-protection mechanism of producer organism. *FEMS Microbiol Lett* **129**, 1-10.
13. Winkowski, K., Ludescher, R., Montville, T.J. (1996) Physicochemical characterization of the nisin-membrane interaction with liposomes derived from *Listeria monocytogenes*. *Appl Environ Microbiol* **62**, 323-327.
14. Gonzalez, B., Glaasker, E., Kunji, E.R.S., Driessen, A.J.M., Suarez, J.E. and Konings, W.N. (1996) Bactericidal mode of action of plantaricin C. *Appl Environ Microbiol* **62**, 2701-2709.
15. Moll, G., Ubbink-Kok, T., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I.F., Konings, W.N., Driessen, A.J.M. (1996) Lactococcin G is a potassium ion-conducting, two-component bacteriocin. *J Bacteriol* **178**, 600-605.
16. Moll, G., Hildeng-Hauge, H., Nissen-Meyer, J., Nes, I.F., Konings, W.N., Driessen, A.J.M. (1998) Mechanistic properties of the two component bacteriocin lactococcin G. *J Bacteriol* **180**, 96-99.
17. Abee, T., Klaenhammer, T.R., Letellier, L. (1994) Kinetic studies of the action of lactacin F, a bacteriocin produced by *Lactobacillus johnsonii* that forms poration complexes in the cytoplasmic membrane. *Appl Environ Microbiol* **60**, 1006-1013.
18. Montville, T.J., Winkowski, K., Ludescher, R.D. (1995) Models and mechanism for bacteriocin action and applications. *Int Dairy J* **5**, 797-814.
19. McAuliffe, O., Ryan, M.P., Ross, R.P., Hill, C., Breeuwer, P., Abee, T. (1998) Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. *Appl Environ Microbiol* **64**, 439-445.
20. Herranz, C., Chen, Y., Chung, H.J., Cintas, L.M., Hernandez, P.E., Montville, T.J., Chikindas, M.L. (2001) Enterocin P selectively dissipates the membrane potential of *Enterococcus faecium* T136. *Appl Environ Microbiol* **67**, 1689-1692.
21. Reisinger, P., H. Seidel, H. Tschesche, and P. Hammes. (1980) The effect of nisin on murein synthesis. *Arch Microbiol* **127**, 187–193.
22. Brötz, H., M. Josten, I. Wiedemann, U. Schneider, F. Götz, G. Bierbaum, and H.-G. Sahl. (1998) Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Mol Microbiol* **30**, 317–327.
23. Wiedemann, I., Benz, R., Sahl, H.-G. (2004) Lipid II-mediated pore formation by the peptide antibiotic nisin: a black lipid membrane study. *J Bacteriol* **186**, 3259–3261.
24. Morgan, S., Ross, R.P., Hill, C. (1995) Bacteriolytic activity caused by the presence of a novel lactococcal plasmid encoding lactococcins A, B, and M. *Appl Environ Microbiol* **61**, 2995-3001.
25. Martínez-Cuesta, M.C., Pelaez, C., Juarez, M., Requena, T. (1997) Autolysis of *Lactococcus lactis* ssp. *lactis* and *Lactobacillus casei* ssp. *casei*. Cell lysis induced by a crude bacteriocin. *Int J Food Microbiol* **38**, 125-131.

26. Martínez-Cuesta, M.C., Kok, J., Herranz, E., Peláez, C., Requena, T., Buist, G. (2000) Requirement of Autolytic Activity for Bacteriocin-Induced Lysis. *Appl Environ Microbiol* **66**, 3174–3179.
27. Zamfir, M. Callewaert, R., Cornea, C.P., Savu, L., Vatafu, I., De Vuyst, L. (1999) Purification and characterization of a bacteriocin produced by *Lactobacillus acidophilus* IBB 801. *J Appl Microbiol* **87**, 923-931.
28. de Man, J.C., Rogosa, M. and Sharp, M.E. (1960) A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130-135.
29. De Vuyst, L., Callewaert, R. and Pot B. (1996) Characterisation of the antagonistic activity of *Lactobacillus amylovorus* DCE 471 and large scale isolation of its bacteriocin amylovorin L471. *Syst Appl Microbiol* **19**, 9-20.
30. Yang, R., Johnson, M.C., Ray, B. (1992) Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Appl Environ Microbiol* **58**, 3355-3359.
31. Bhunia, A.K., Johnson, M.C., Ray, B. and Kalchayanand, N. (1991) Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. *J Appl Bacteriol* **70**, 25-30.
32. Hayat, M.A. (1972) *Basic Electron Microscopy Techniques* ed. Hayat, M.A. New York: Van Nostrand Reinhold Company.
33. Reynolds, R.S. (1963) The use of lead citrate at high pit as an electron opaque stain electron microscopy. *J Cell Biol* **17**, 208-209.
34. Gao, F.H., Abee, T., Konings, W.N. (1991) Mechanism of action of the peptide antibiotic nisin in liposomes and cytocrome c oxidase-containing proteoliposomes. *Appl Environ Microbiol* **57**, 2164-2170.
35. Garcia-Garcera, M.J., Elferink, M.G.L., Driessen, A.J.M., Konings, W.N. (1993) In vitro pore-forming activity of the lantibiotic nisin: role of protonmotive-force and lipid composition. *Eur J Biochem* **212**, 417-422.
36. Beveridge, T.J. (1989) The structure of bacteria,. In: *Bacteria in Nature: A Treatise on the Interaction of Bacteria and their Habitats* ed. Leadbetter, E.R. and Poindexter, J.S. vol.3., pp. 1-65. New York: Plenum Publishing Co.
37. Friedrich, C.L., Moyles, D., Beveridge, T.J., Hancock, R.E.W. (2000) Antibacterial action of structurally diverse cationic peptides on Gram-positive bacteria. *Antimicrob Agents Chemother* **44**, 2086-2092.