



Original Contribution

ATOMIC FORCE MICROSCOPY ON THE EFFECT OF BACTERIOCINS ON TARGET CELLS: A NEW METHOD FOR VISUALISING ITS MODE OF ACTION

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ABSTRACT

The aim of this study was to visualise the effect of various bacteriocins on target cells using Atomic Force Microscopy (AFM) and to evaluate this method as a standard in the determination of the mode of action of bacteriocins. The interaction between various bacteriocins and target cells was studied using different methods, such as Atomic Force Microscopy (AFM), cell lysis, and extracellular leakage of β -galactosidase. Vesiculation was clearly visible on cells of *Lactobacillus sakei* DSM 20017 after treatment with bacteriocin ST62BZ, bacteriocin ST63BZ, bacteriocin ST611BZ and bacteriocin ST612BZ produced by *E. faecium* and *Lactococcus lactis*, respectively. Changes in morphology, such as collapse and the formation of pores of *Enterococcus faecium* HKLHS, were observed after treatment with bacteriocin ST69BZ produced by *L. plantarum*. An inhibitory effect as observed by reduced growth of sensitive strains in presence of bacteriocins or as result of detection of extracellular levels of β -galactosidase was recorded when *L. sakei* DSM 20017 and *E. faecium* HKLHS were treated with bacteriocins ST62BZ, ST63BZ, ST611BZ, ST612BZ and ST69BZ.

Key Words: lactic acid bacteria; bacteriocins; mode of action; AFM

INTRODUCTION

Lactic acid bacteria (LAB) are known for their production of antimicrobial compounds, including bacteriocins or bacteriocin-like peptides [1]. Bacteriocins of LAB are defined as ribosomally synthesized proteins or protein complexes usually antagonistic to genetically closely related organisms [1,2]. Bacteriocins are generally low molecular weight proteins that gain entry into target cells by binding to cell surface receptors. Their bactericidal mechanism vary and may include pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis [1,3,4].

AFM images were obtained in air with tapping mode. The images clearly showed changes in cell morphology, such as collapse

of the apical ends or the cell centre, signs of cytoplasm leakage and vesiculation. Differences observed among the bacteriocins suggest different modes of action, such as the barrel stave model and the toroidal model, which describe the formation of pores in the cell membrane or the carpet model, which describes the vesiculation of the outer cell membrane.

MATERIALS AND METHODS

Strains and media

Bacteriocin producer strains *L. plantarum* ST69BZ, *E. faecium* ST62BZ, *L. lactis* ST63BZ, *L. lactis* ST611BZ and *L. lactis* ST612BZ were previously characterised as bacteriocin producers [5]. Bacteriocin producers were cultured in a MRS medium (Biolab, Biolab Diagnostics, Midrand, SA) at 30°C for 24h. The test microorganisms *L. sakei* DSM 20017 and *E. faecium* HKLHS were cultured in MRS and BHI broth (Biolab), respectively at 30°C for 18h. Cultures were stored at -80°C in a growth medium, supplemented with glycerol (15%, v/v, final concentration).

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Bacteriocin activity

Bacteriocin producing strains were inoculated (2%, v/v) into 100 ml MRS broth and incubated at 30°C for 24h. The cells were harvested (1000 x g, 15 min, 4°C), the pH of the cell-free supernatant containing bacteriocin adjusted to 6.0 with sterile 1 M NaOH, heated for 10 min at 80°C, and then filter-sterilized (0.20 µm, Minisart®, Sartorius). The bacteriocin activity was tested against target organisms by using the agar-spot test [6].

In a separate experiment, 20 ml cell-free supernatant containing bacteriocin (pH 6.0) was filter-sterilized (0.20 µm, Minisart®, Sartorius) and added to 100 ml 3-h-old cultures (early exponential growing culture) of target microorganisms. Incubation was at 30°C. Optical density readings were recorded at 600 nm, hourly for 10 h.

Effect of bacteriocins on cell morphology

Target cells were harvested by centrifugation (8000 x g, 10 min, 4 °C), washed 5 times with 10 ml sterile distilled water, and re-suspended in 10 ml bacteriocin-containing filter-sterilized supernatant (pH 6.0). The suspension was incubated for 1 h at 4°C, after which the cells were harvested by centrifugation (8000 x g, 10 min, 4 °C) and washed five times with 10 ml sterile distilled water. The pellet was re-suspended in 1 ml sterile water and then visualized by either a Multimode AFM from Veeco (USA) or an EasyScan II from Nanosurf (Switzerland). The cell suspension was applied onto a freshly cleaved mica surface and allowed to dry for 5 min before subjected to AFM. A silicon non-contact cantilever from Nanosensors (Switzerland) with a resonance frequency of 160 kHz and a spring constant of approximately 50 Nm⁻¹ was used. Height and size information were acquired by using the imaging software of the AFM. Untreated target cells served as control.

Effect of bacteriocins on the cell membrane permeability

Extracellular levels of β-galactosidase activity were monitored. Eleven-h-old cultures of target microorganisms (10 ml each) were harvested and the cells washed twice with 0.03 M sodium phosphate buffer (pH 6.5) and re-suspended in 2 ml of the same buffer. The cell suspensions were treated with 2 ml bacteriocin (between 1600 AU/ml and 6400

AU/ml, pH 6.0) for 5 min at 25°C, followed by the addition of 0.2 ml 0.1 M ONPG (O-nitrophenyl-β-D-galactopyranoside) in 0.03 M sodium phosphate buffer (pH 6.8). After 10 min at 30°C, the reaction of β-galactosidase was stopped by the addition of 2.0 ml 0.1 M sodium carbonate. The cells were harvested (8000 x g, 15 min, 25°C) and absorbance readings of the supernatant recorded at 420 nm. Controls cells were prepared the same way, but not treated with bacteriocin [7,8]. All experiments were done in duplicate.

RESULTS AND DISCUSSION

Cell-free supernatants from 24-h-old cultures of *L. plantarum* ST69BZ, *L. lactis* ST63BZ, ST611BZ and ST612BZ and *E. faecium* ST62BZ (pH neutralized) inhibited the growth of several bacterial strains as shown on **Table 1**. Identical results were obtained with the agar-spot and well diffusion methods. Early-log phase cells of test micro-organisms treated with bacteriocins resulted in immediate and complete growth inhibition for at least 10 h (**Figure 1**), suggesting that the mode of action is bactericidal. Similar results were recorded for plantaricin 423 against *Oenococcus oeni* 19Cl [9], *Ent. faecium* HKLHS and *L. sakei* DSM 20017 [10], pediocin N5p against *Pediococcus pentosaceus* E5p [11], and buchnericin LB against *L. monocytogenes* and *Bacillus cereus* [12].

Cells of test microorganisms treated with bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ were clearly deformed or vesiculated as visualised by AFM (**Fig. 2**). Sensitive strains treated with bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ resulted in leakage of DNA, RNA, proteins and β-galactosidase (data not shown).

The results obtained by AFM (**Fig. 2**) and leakage of DNA, RNA, proteins and β-galactosidase confirm that bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ are destabilizing the permeability of the cell membrane. Similar results have been reported for buchnericin LB [12,13], plantaricin 423 [10] and pediocin AcH [14]. Vesiculation was clearly visible on cells of *L. sakei* DSM20017 after treatment with bacteriocin ST62BZ produced by *E. faecium* ST62BZ, bacteriocins ST63BZ, ST611BZ and ST612BZ produced by *L. lactis* ST63BZ, ST611BZ and ST612BZ, respectively.

Table 1. Spectrum of activity recorded for bacteriocins ST69BZ, ST62BZ, ST63BZ, ST611BZ and ST612BZ

Strain	Media	bacteriocins				
		ST69BZ	ST62BZ	ST63BZ	ST611BZ	ST612BZ
<i>Enterococcus faecalis</i> 1071	MRS	+	-	-	-	-
<i>E. faecalis</i> E88	MRS	-	+	-	+	-
<i>E. faecalis</i> E90	MRS	-	+	+	+	-
<i>E. faecalis</i> E92	MRS	-	+	-	+	-
<i>E. faecium</i> HKLHS	MRS	+	+	+	+	+
<i>Escherichia coli</i> P40	BHI	+	+	+	+	+
<i>L. curvatus</i> DF38	MRS	+	-	-	-	-
<i>L. sakei</i> DSM 20017	MRS	-	+	+	+	+
<i>L. salivarius</i> 241	MRS	+	-	-	-	-
<i>L. innocua</i> LMG 13568	BHI	+	+	+	+	+
<i>L. ivanovii</i> subsp. <i>ivanovii</i> ATCC 19119	BHI	+	+	+	+	+
<i>L. monocytogenes</i> NCTC 4885	BHI	+	+	-	-	+
<i>L. monocytogenes</i> ScottA	BHI	+	+	-	-	+
<i>L. monocytogenes</i> NCTC 11944	BHI	+	+	-	-	+
<i>S. caprinus</i> ATCC 700066	BHI	-	+	-	+	-
<i>Streptococcus</i> spp. TL2R	BHI	-	+	-	+	-

(+) = inhibition; (-) = no inhibition

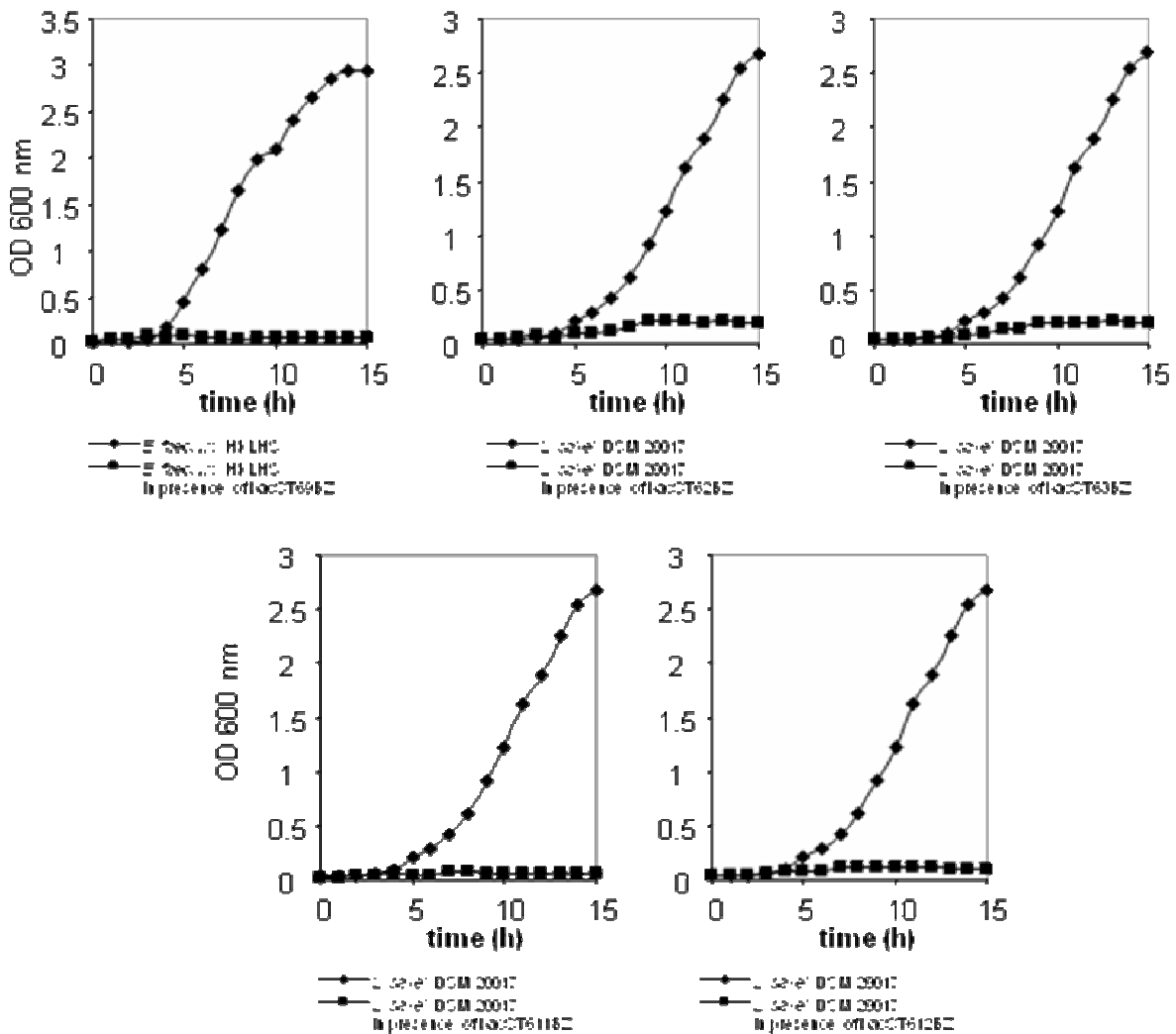


Figure 1 Effect of bacteriocin ST69BZ, bacteriocin ST62BZ, bacteriocin ST63BZ, bacteriocin ST611BZ and bacteriocin ST612BZ on the growth of test microorganisms *E. faecium* HKLHS and *L. sakei* DSM 20017.

Changes in morphology, such as collapse and formation of pores of *E. faecium* HKLHS were observed after treatment with bacteriocin ST69BZ produced by *L. plantarum* ST69BZ. Leakage was observed in exponentially growing cells of *L. sakei* DSM20017 after

treatment with bacteriocin AMA-K and bacteriocin JW6BZ, produced by *L. plantarum* AMA-K and JW6BZ, respectively and *L. innocua* LMG13568 treated with bacteriocin ST8KF produced by *L. plantarum* ST8KF [5,15,16].

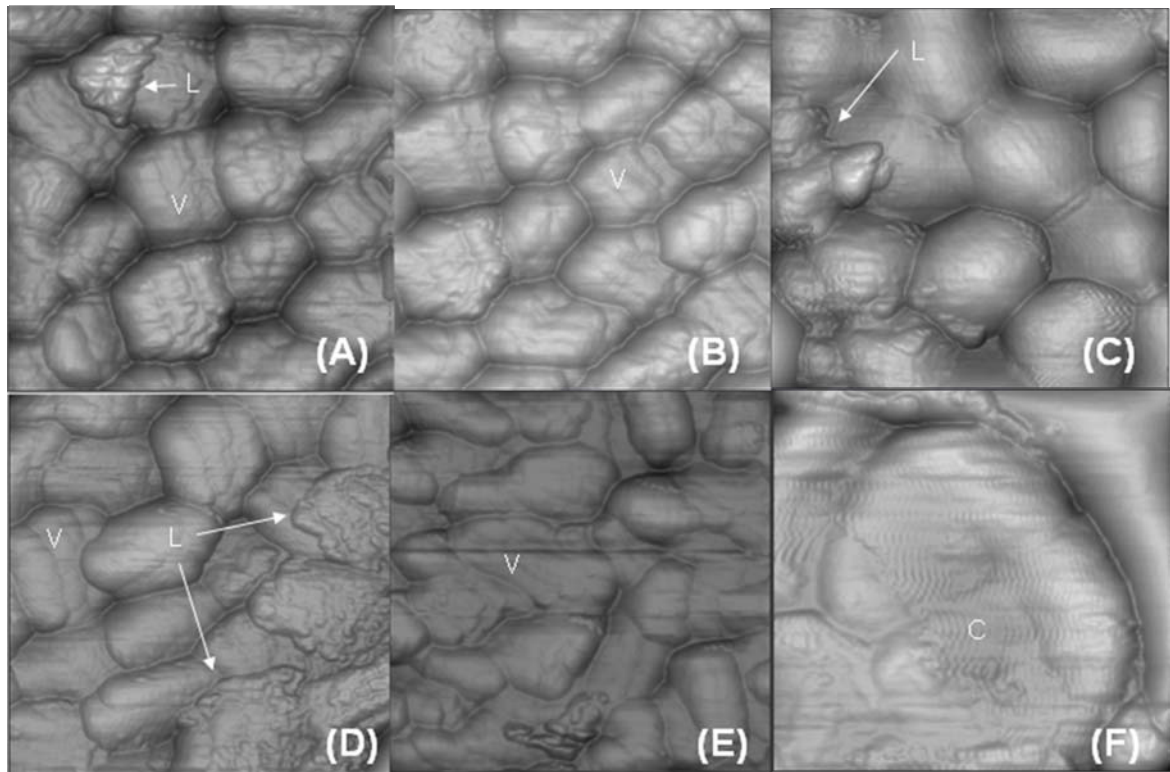


Figure 2: Effect of bacteriocins on target cells visualized by AFM. (A): *L. sakei* DSM20017 treated with bacteriocin ST62BZ; (B): *L. sakei* DSM20017 treated with bacteriocin ST612BZ; (C): *E. faecium* HKLHS treated with bacteriocin ST69BZ; (D): *L. sakei* DSM20017 treated with bacteriocin ST611BZ; (E): *L. sakei* DSM20017 treated with bacteriocin ST63BZ; (F): *E. faecium* HKLHS treated with bacteriocin ST69BZ. L – leakage; V – vesiculation; C – collapse.

The resulting images clearly showed changes in cell morphology, such as collapse of the apical ends or the cell centre, signs of cytoplasm leakage or vesiculation. Differences observed between the bacteriocins suggests different modes of action, such as the barrel stave model and the toroidal model, which describe the formation of pores in the cell membrane or the carpet model, which leads to a vesiculation of the outer cell membrane.

CONCLUSIONS

Methods commonly used to study the mode of action of bacteriocins are mostly based on the indirect observation of cell damage, in the form of leakage of intracellular compounds or by observing the reduction of the bacterial growth. With AFM these processes and the effect of bacteriocins on target cells can be made visible and additional information on mode of action can be obtained.

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REFERENCES

1. De Vuyst, L. and Vandamme, E.J., Bacteriocins of lactic acid bacteria: Microbiology, genetics and applications. London: Blackie Academic and Professional, 1994.
2. Klaenhammer, T., Bacteriocins of lactic acid bacteria. *Biochimie* 70:337-349, 1988.
3. Heu, S., Oh, J., Kang, Y., Ryu, S., Cho, S.K., Cho, Y. and Cho, M., Glycinecin A, a bacteriocin produced

- by *Xantomonas campestris* pv. *Glycines* 8ra. *Appl Environ Microbiol* 67:4105-4110, 2001.
4. James, R., Lazdunski, C. and Pattis, F., Bacteriocins, microcins and lantibiotics. New York: Springer-Verlag pp.519, 1991.
 5. Von Mollendorff, J.W., Todorov, S.D. and Dicks, L.M.T., Comparison of bacteriocins produced by lactic acid bacteria isolated from boza, a cereal-based fermented beverage from the Balkan Peninsula. *Curr Microbiol*, 53:209-216, 2006.
 6. Todorov, S.D. and Dicks, L.M.T., Characterization of bacteriocins produced by lactic acid bacteria isolated from spoiled black olives. *J Basic Microbiol*, 45:312-322, 2005.
 7. Nagy, Z., Kiss, T., Szentirmai, A. and Biro, S., β -Galactosidase of *Penicillium chrysogenum*: Production, purification, and characterization of the enzyme. *Prot Exp Purif*, 21:24-29, 2001.
 8. Hsu, C.A., Yu, R.C. and Chou, C.C., Production of β -galactosidase by bifidobacteria as influenced by various culture conditions. *Int J Food Microbiol*, 104:197-206, 2005.
 9. Van Reenen, C.A., Dicks, L.M.T. and Chikindas, M.L., Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *J Appl Microbiol* 84:1131-1137, 1998.
 10. Todorov, S.D. and Dicks, L.M.T., Parameters affecting the adsorption of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum* 423 isolated from sorghum beer. *Biotechnol J*, 1:405-409, 2006.
 11. Manca de Nadra, M.C., Sandino de Lamelas, D. and Strasser de Saad, A.M., Pediocin N5p from *Pediococcus pentosaceus*: Adsorption on bacterial strains. *Int J Food Microbiol* 39:79-85, 1998.
 12. Yildirim, Z., Avşar, Y.K., Yildirim, M. Factors affecting the adsorption of buchnericin LB, a bacteriocin produced by *Lactobacillus buchneri*. *Microbiol Res*, 157:103-107, 2002.
 13. Yildirim, Z., Johnson, M.G. and Winters, D.K. Purification and mode of action of bifidocin B produced by *Bifidobacterium bifidum* NCFB 1454. *J Appl Microbiol*, 86:45-54, 1999.
 14. Bhunia, A., Johnson, M.C., Ray, B. and Kalchanand, N., Mode of action of pediocin AcH from *Pediococcus acidilactici* H on sensitive bacterial strains. *J Appl Bacteriol*, 70:25-33, 1991.
 15. Todorov, S.D., Nyati, H., Meincken, M., Dicks, L.M.T. Partial characterization of bacteriocin AMA-K, produced by *Lactobacillus plantarum* AMA-K isolated from naturally fermented milk from Zimbabwe. *Food Control*, 18:656-664, 2007.
 16. Powell, J.E., Todorov, S.D., van Reenen, C.A., Dicks, L.M.T. and Witthuhn, R.C., Growth inhibition of *Enterococcus mundtii* in Kefir by *in situ* production of bacteriocin ST8KF. *Le Lait*, 86:401-405, 2006.