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ORIGINAL ARTICLE

Morphological changes in *Salmonella* Typhimurium caused by the lantibiotic bovicin HC5 in association with EDTA

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Abstract Bacteriocins, particularly those produced by Gram-positive bacteria, have in recent years been considered promising antimicrobial agents to inhibit bacterial growth in food, and thus are potential food preservatives. These peptides generally exhibit a spectrum of action limited to Gram-positive bacteria. However, their action can be extended to Gram-negative bacteria through association with chelating agents. In the present study, we evaluated the occurrence of morphological changes on the cell envelope of *Salmonella* Typhimurium cells treated with bovicin HC5—a lantibiotic from *Streptococcus bovis* HC5—in association with EDTA. The morphological changes of the cells were visualized by atomic force microscopy (AFM), and the increase in cell membrane permeability was confirmed by the leakage of potassium ions (K^+). The images displayed changes in the cell envelope, with increased surface roughness and a decreased cell volume. These changes indicate that EDTA plays a role in the destabilization of the outer membrane, allowing bovicin HC5 to act on the cytoplasmic membrane through the formation of pores, which was confirmed by the detection of potassium in the cell supernatant. These results suggest that bovicin HC5 combined with EDTA has potential for use on *Salmonella* cells.

Keywords Bacteriocins · Biocontrol · Lantibiotics · Atomic force microscopy · Outer membrane · Gram-negative bacteria

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Introduction

Salmonella continues to be identified as an important foodborne agent and recent records show that it is the most relevant pathogen in terms of mortality and necessity for hospitalization (CDC 2012). The need to ensure food safety drives the constant search for alternative methods to preserve food. Of special interest are mild treatments that aim to maintain the food in its most natural form, thus minimizing the loss of nutritional and sensorial properties (Chalón et al. 2012). Among the methods considered, the use of bacteriocins stands out as a promising alternative and is the focus of research in the field of food science and technology (Balciunas et al. 2013).

Bacteriocins produced by Gram-positive bacteria are ribosomally synthesized and exert a bacteriostatic or bactericidal effect on other bacteria (Héchard and Sahl 2002). These peptides belong to a heterogeneous group, differing in their spectrum of activity, biochemical properties, molecular mass, mode of action and genetic origin (Abee et al. 1995). Generally, bacteriocins have a non-enzymatic mode of action, causing depolarization of the cytoplasmic membrane, by forming pores and/or inhibition of cell wall synthesis, by sequestering of lipid II during anchoring in the membrane (Dischinger et al. 2014; Nes et al. 2006). The most well-studied bacteriocins are those produced by lactic acid bacteria, and their effect on Gram-positive bacteria has been extensively demonstrated, especially with use of nisin (Ávila et al. 2014; Millette et al. 2007; Pinto et al. 2011; Ruiz et al. 2010; Settanni and Corsetti 2008; Solomakos et al. 2008; Wijnker et al. 2011).

Gram-negative bacteria, such as *Salmonella*, are usually resistant to the action of bacteriocins produced by Gram-positive bacteria. This resistance is due to the outer membrane, which functions as a barrier, rendering the organism less

permeable (Chalón et al. 2012; Gálvez et al. 2014; Gyawali and Ibrahim 2014). However, this resistance can be reduced with the use of compounds or treatments that destabilize the outer membrane, such as chelating agents, plant essential oils, freezing, heating, high pressure processing and pulsed electric fields (Boziaris and Adams 1999; Cobo Molinos et al. 2008; Prudêncio et al. 2014, 2015a).

Bovicin HC5 is a lantibiotic produced by *Streptococcus bovis* HC5—a bacterium isolated from the bovine rumen. The ability of this bacteriocin to inhibit several Gram-positive bacteria, such as *Listeria monocytogenes* (Mantovani and Russell 2003), *Bacillus cereus*, *Bacillus thuringiensis* (de Carvalho et al. 2007a), *Clostridium tyrobutyricum* (de Carvalho et al. 2007b) and *Alicyclobacillus acidoterrestris* (de Carvalho et al. 2008) has already been demonstrated. Activity on Gram-negative bacteria was also recently demonstrated (Prudêncio et al. 2014, 2015a).

The environmental stresses used to destabilize the outer membrane and the bacteriocin action may produce morphological changes in the cell envelope, but this topic is still poorly understand (Thongbai et al. 2006). In this context, atomic force microscope (AFM) allows the acquisition of high-resolution images without the requirement for chemical drying, metal coating, or exposure to an ultrahigh vacuum. Therefore, AFM is an ideal tool to study those morphological changes (Deupree and Schoenfisch 2009). The effect of bovicin HC5 on Gram-negative bacteria was recently demonstrated, but morphological changes resulting from bacteriocin activity have not been detected. The present study was designed to demonstrate the occurrence of morphological alterations on *Salmonella* Typhimurium cells caused by bovicin HC5 associated to EDTA.

Materials and methods

Bacterial strains and growth conditions

Salmonella enterica serovar Typhimurium ATCC 14028 was grown in brain heart infusion broth (BHI, Himedia, Mumbai, India), at 37 ± 1 °C for 18 to 20 h. *Streptococcus bovis* HC5 was grown according to the method of Mantovani and Russell (2003). *Lactococcus lactis* ATCC 19435 was grown in Man, Rogosa, and Sharpe broth (MRS, Himedia, Mumbai, India) at 37 ± 1 °C for 18 to 20 h and was used to determine the antimicrobial activity of the bacteriocin.

Bovicin HC5 preparation and activity

Bovicin HC5 extracts were prepared in accordance with the protocol described by Mantovani and Russell (2003). The

antimicrobial activity was determined through the agar-diffusion method (Tagg et al. 1976) using *L. lactis* as indicator, and quantified by the critical dilution method (Hoover and Harlander 1993). The concentration was expressed in arbitrary units (AU/mL).

Effect of bovicin HC5 in association with EDTA on *Salmonella* growth

The effect of antimicrobial action of bovicin HC5 on *Salmonella* growth, in association with ethylenediamine-tetraacetic acid (EDTA, Reagen, Colombo, Brazil), was evaluated in microplate assays and in viability tests. Prior to treatments, the *Salmonella* Typhimurium cells were centrifuged at $2500\,g$ for 15 min at 4 °C (Sorvall RT 6000D, DuPont, Gilroy, CA), washed and resuspended in 0.85 % saline. The BHI broth was inoculated at an initial concentration of 5 log of number of colony-forming units ($\log_{10}$ CFU/mL) supplemented with EDTA (1.6 mM) and bovicin HC5 (100 AU/mL). These concentrations have been demonstrated previously to have an inhibitory effect on *Salmonella* Typhimurium (Prudêncio et al. 2014).

Microplates were incubated at 37 ± 1 °C and growth was assessed by measuring the absorbance at 600 nm (Thermo Scientific, Marietta, OH) at different time intervals over a period of 24 h. Each treatment was performed three times, with at least four replicates. Viability was assessed at the same times of samples of microscopy, i.e., after 0, 3, 6, 12 and 24 h of incubation at 37 ± 1 °C, using the drop plate method on plate count agar (PCA, Difco, Sparks, USA) (Morton 2001). Plates were counted after 8–12 h of incubation at 37 ± 1 °C. The test was repeated twice and differences between the treatments were evaluated by Tukey's test, with significance level of 0.05, using the Assistat program, version 7.7 beta, 2009 (Silva and Azevedo 2009).

Evaluation of potassium loss by *Salmonella* Typhimurium cells

To determine potassium leakage, 7 $\log_{10}$ CFU/mL of *Salmonella* Typhimurium cells was suspended in sodium phosphate buffer (5 mM, pH 6.5), supplemented with bovicin HC5 (100 AU/mL) and EDTA (1.6 mM). Aliquots of approximately 5 mL were collected at different time intervals, filtered (0.22 μm membranes, Millipore®, Darmstadt, Germany), and stored in tubes that had been washed previously with hydrochloric acid and sterilized. The potassium concentration was determined in a flame photometer (Corning, Cambridge, UK) (Minahk et al. 2000).

Each experiment was executed three times. Statistical analyses were performed using the Assistat program, version 7.7 beta, 2009 (Silva and Azevedo 2009). Tukey's test was used
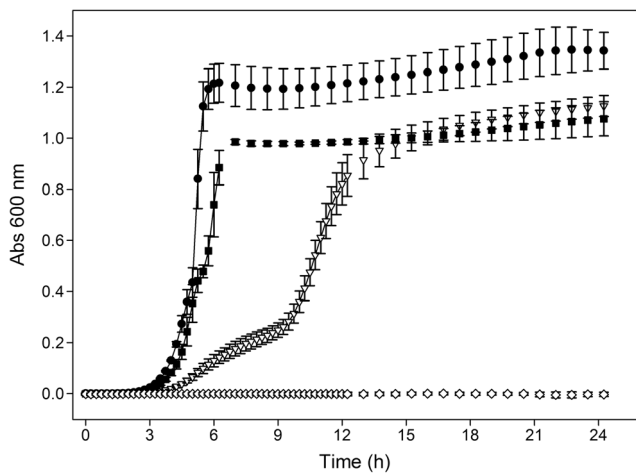
Fig. 1 Growth of *Salmonella* Typhimurium in brain heart infusion (BHI) broth, with or without bovicin HC5 (100 AU/mL) and EDTA (1.6 mM) at 37±1 °C for 24 h. ● BHI broth, ▽ BHI broth + EDTA, ■ BHI broth + bovicin HC5, ◊ BHI broth + bovicin HC5 + EDTA. *Error bars* Standard deviation (*n*=3)

to determine the existence of differences between the treatment and the control groups. A significance level of 0.05 was adopted.

Evaluation of the morphology of *Salmonella* Typhimurium cells treated with bovicin HC5 and EDTA

To prepare the samples, 5 $\log_{10}$ CFU/mL of *Salmonella* Typhimurium cells was inoculated in BHI broth, supplemented with EDTA (1.6 mM) and bovicin HC5 (100 AU/mL), and incubated at 37±1 °C. Controls were performed with cells cultured in BHI broth supplemented with sodium phosphate
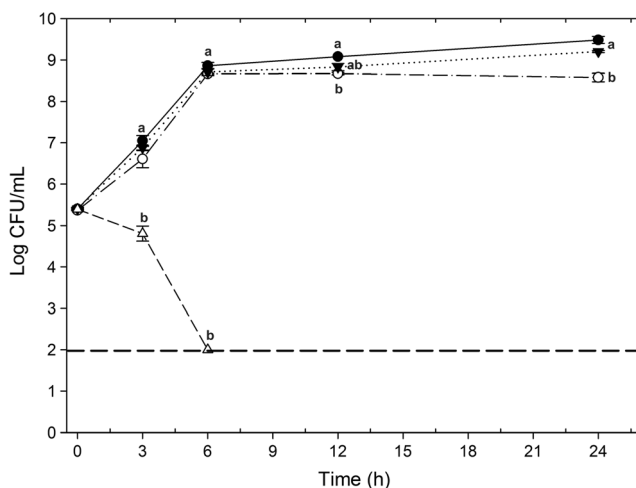

Fig. 2 Viability of *Salmonella* Typhimurium in BHI broth, with or without bovicin HC5 (100 AU/mL) and EDTA (1.6 mM) at 37±1 °C for 24 h. ● BHI broth, ○ BHI broth + EDTA, ▼ BHI broth + bovicin HC5, △ BHI broth + bovicin HC5 + EDTA. *Errors bars* Standard deviation (*n*=2). *a, b* Counts with the same letter in each time interval do not differ from each other by Tukey's test (*P*>0.05). *Dotted horizontal line* Detection limit of the technique

solution (5 mM, pH 2.0), and added of EDTA or bovicin HC5 separately, at the same concentrations. Next, 500 μL aliquots were collected at different time intervals, centrifuged at 5000*g* for 5 min, washed five times with 0.85 % saline, and resuspended in 0.85 % saline. The cells were spread onto previously sterilized glass slides (1 cm×1 cm) .

Changes in the *Salmonella* Typhimurium cell envelope were observed by AFM (NT-MDT, N Ntegra Prima, Moscow, Russia). Topographic measures were performed using the intermittent mode.

Results and discussion

Effect of bovicin HC5 in association with EDTA on *Salmonella* growth

Figures 1 and 2 show the growth of *Salmonella* Typhimurium in the presence of EDTA and bovicin HC5 separately or in association. In the absence of both antimicrobials, the maximum population achieved after 24 h of incubation was 9.5 $\log_{10}$ CFU/mL. The presence of EDTA decreased the growth, with a difference of approximately 0.9 log cycle (*P*<0.05). Bovicin HC5, at a concentration of 100 AU/mL did not alter *Salmonella* Typhimurium growth (*P*>0.05), as expected for a Gram-negative bacterium (Figs. 1, 2). These results corroborate those presented by Prudêncio et al. (2014), in which concentrations up to 200 AU/mL did not inhibit the microorganism under optimal growth conditions. Bacteriostatic activity of bovicin HC5 alone was observed at specific conditions, namely low temperature and low pH (Prudêncio et al. 2015a).

In the presence of minimal concentrations of EDTA, bovicin HC5 inhibited completely the growth and bactericidal activity of bacteriocin was observed, with a reduction of approximately 3.5 log cycles (Figs. 1, 2), corroborating the results of Prudêncio et al. (2014, 2015a), who demonstrated the bactericidal activity of this bacteriocin. These results suggest that EDTA destabilizes the outer membrane, allowing bovicin HC5 to access the cytoplasmic membrane.

Bovicin HC5 exhibits a dual mechanism of action by (1) pore formation that promotes the release of electrolytes and metabolites, and by (2) inhibition of bacterial cell wall biosynthesis through binding of lipid II (Paiva et al. 2011). The finding of potassium in the cell supernatant confirmed pore formation by bovicin HC5 (Fig. 3). In the absence of bovicin HC5, *Salmonella* Typhimurium cells lost significantly smaller amounts of potassium (*P*<0.05) (Fig. 3). Surprisingly, cells treated with bovicin HC5 only demonstrated considerable leakage of potassium, although without growth effects, and in smaller amounts than cells treated with bovicin HC5 and EDTA, especially after 30 min of treatment (Fig. 3). The presence of potassium in the cell supernatant is commonly

Fig. 3 Extracellular potassium leakage of *Salmonella* Typhimurium cells treated with or without bovicin HC5 (100 AU/mL) and EDTA (1.6 mM) in sodium phosphate buffer at 37± 1 °C for 60 min. *Black bars* Sodium phosphate buffer, *light gray bars* sodium phosphate buffer + EDTA, *dark gray bars* sodium phosphate buffer + bovicin HC5, *hatched bars* sodium phosphate buffer containing bovicin HC5 + EDTA. *a–c* Bars with the same letter at each point do not exhibit significant differences according to Tukey's test ($P<0.05$). *Error bars* Standard deviation ($n=3$)
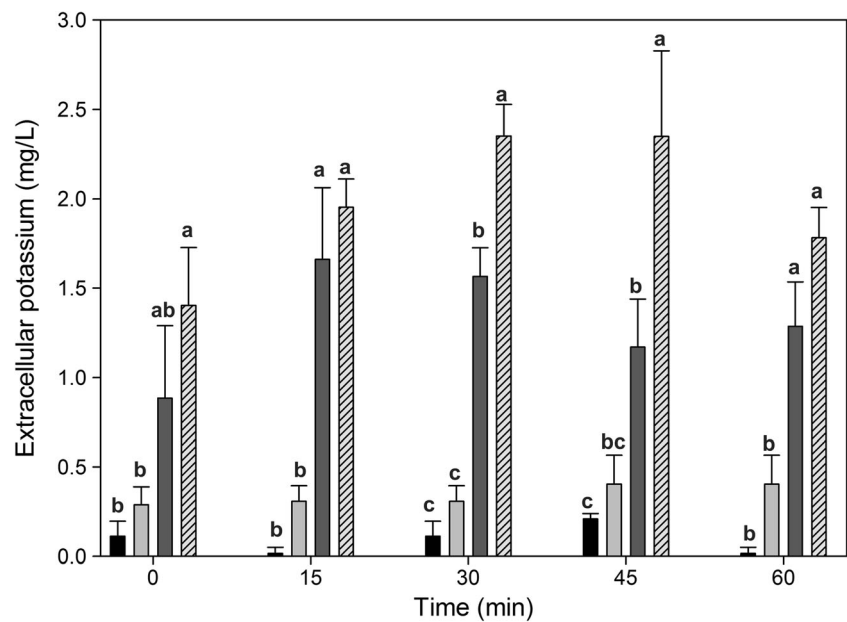
detected after treatment with bacteriocins because the mechanism of action of these peptides generally involves the formation of pores in the cytoplasmic membrane of the target cells (Abee et al. 1995; Mantovani et al. 2002). These results confirm that, when EDTA is present, *Salmonella* Typhimurium cells became sensitized to bovicin HC5.

The effectivity of minimal concentrations of bovicin HC5 and EDTA demonstrate the potential use of this bacteriocin for
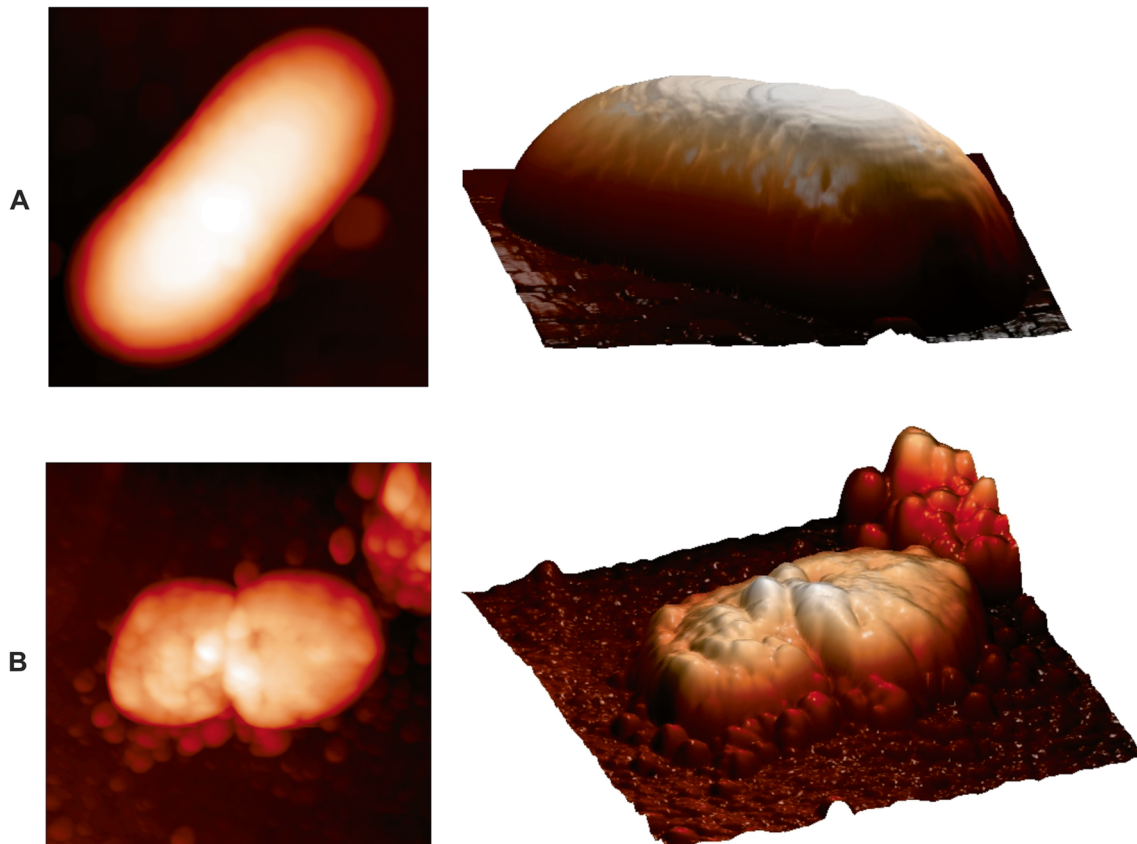

Fig. 4a,b Atomic force microscopy (AFM) images of *Salmonella* Typhimurium cells (3 μm scan). **a** Cultured in BHI broth for 12 h. **b** Cultured in BHI broth added of bovicin HC5 (100 AU/mL) and EDTA (1.6 mM) for 12 h
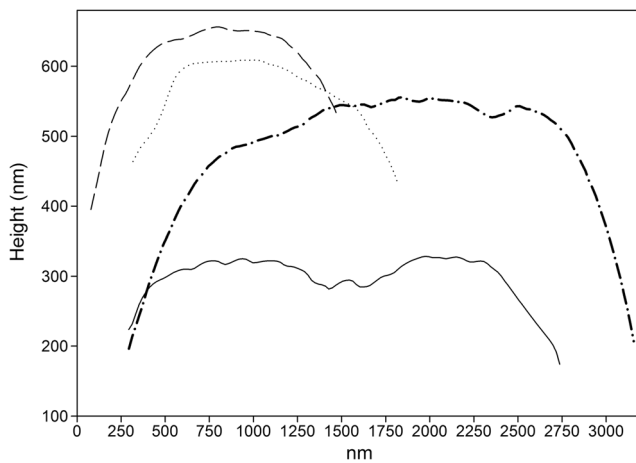
Fig. 5 Topographic profile of *Salmonella* Typhimurium cells in BHI broth with or without bovicin HC5 (100 AU/mL) and EDTA (1.6 mM) at 37±1 °C for 24 h. BHI broth (− − −); BHI broth + EDTA (— • —); BHI broth + bovicin HC5 (……); BHI broth + bovicin HC5 + EDTA (——)

controlling Gram-negative microorganisms, which is of fundamental importance because this is a relevant group of bacteria among pathogenic and food spoilage microorganisms (Boziaris and Adams 1999). However, the antimicrobial activity of bovicin HC5 can be influenced by environmental conditions, such as temperature and pH, thus the efficiency of bacteriocin varies according to treatment conditions. Therefore, such parameters should be considered before

establishing optimal conditions for application (Prudêncio et al. 2015a).

EDTA is used broadly in several countries to minimize oxidation or any other reactions related to metal-catalyzed food spoilage (Branen and Davidson 2004). The activity of EDTA is related, in part, to release of the lipopolysaccharide (LPS) layer, by binding to calcium and magnesium ions that destabilize the structure of LPS, favoring access of bacteriocin molecules to the cytoplasmic membrane (Alakomi et al. 2003). The efficacy of EDTA for sensitizing Gram-negative bacteria to bacteriocins produced by Gram-positive bacteria has been demonstrated extensively on different bacteria, such as *Salmonella*, *Escherichia coli*, *Enterobacter aerogenes*, *Citrobacter freudii*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Arcobacter butzleri* (Boziaris and Adams 1999; Elliason and Tatini 1999; Phillips and Duggan 2001; Belfiore et al. 2007; Lappe et al. 2009; Martin-Visscher et al. 2011; Prudêncio et al. 2014, 2015a). Usually, low concentrations of EDTA (10–20 mM) are sufficient to produce sensitization to the activity of bacteriocins, which are used at different concentrations (100–3200 AU/mL) (Prudêncio et al. 2015b).

The efficacy of association of bacteriocins with EDTA in inhibition of the growth of Gram-negative bacteria is well demonstrated. However, few studies have evaluated the effect of this treatment on cell morphology, especially with bovicin HC5—the main object of this study.
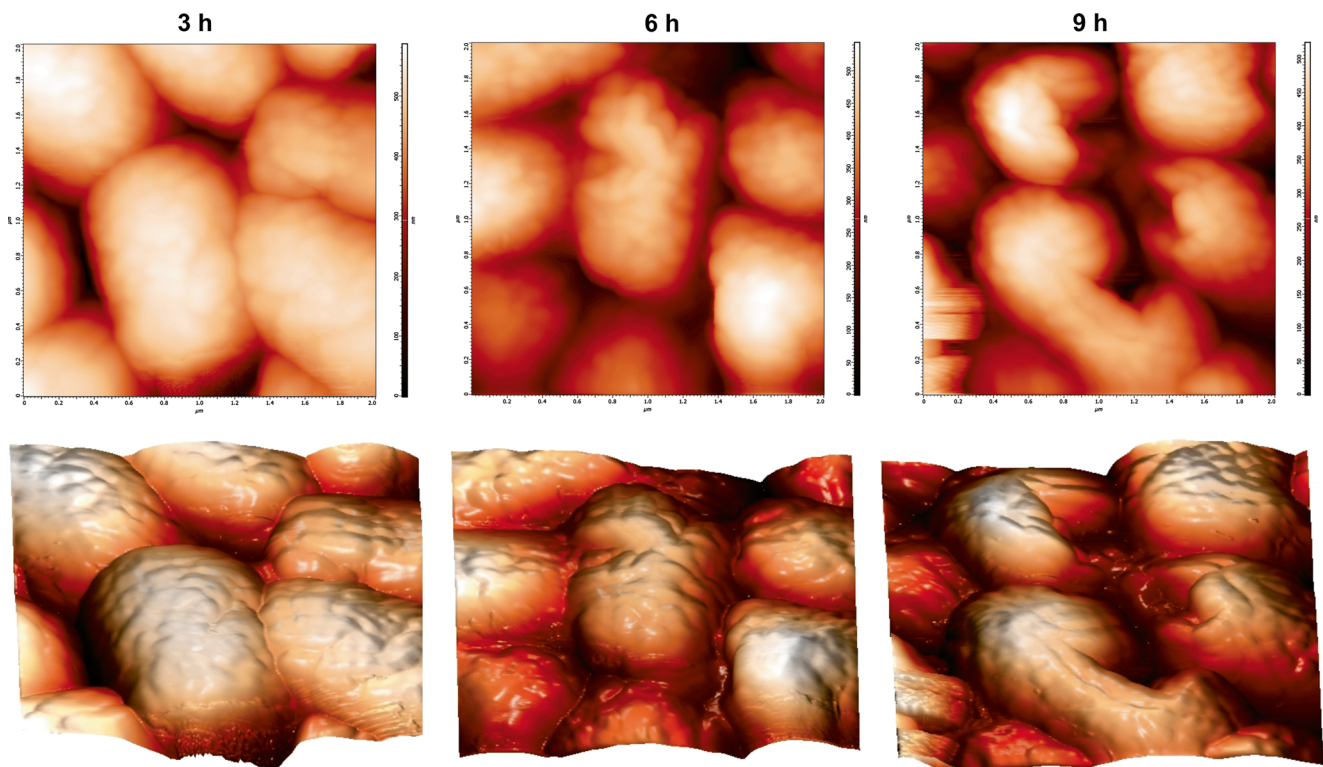
3 h	6 h	9 h

Fig. 6a–c AFM images of *Salmonella* Typhimurium cells cultured in BHI broth added of bovicin HC5 (100 AU/mL) and EDTA (1.6 mM) (2 μm scan). **a** 3 h, **b** 6 h, **c** 9 h

Morphology of the cell envelope of *Salmonella* Typhimurium cells treated with bovicin HC5 and EDTA

Salmonella Typhimurium cells cultured in BHI and imaged by AFM appeared as short rods with a compact, uniform surface and a smooth and slightly irregular texture due to the LPS layer, but with no signs of rupture (Fig. 4a). This morphological aspect is common among Gram-negative bacteria visualized by this technique (Alakomi et al. 2006; Cui et al. 2012).

The topographic profiles demonstrated that the presence of EDTA or bovicin HC5 separately did not affect cell volume even after 24 h of treatment, although the cell surface demonstrated a topographic variation that was slightly more irregular in the presence of EDTA (Fig. 5). Cells treated with bovicin HC5 and EDTA demonstrated a considerable decrease in cell volume, visualized by the significant difference in cell height, and cell surface irregularity, with higher roughness and depressions (Figs. 4b, 5). This phenomenon occurred due to the loss of turgidity, most likely caused by the efflux of intracellular components, such as potassium, as shown by our results (Fig. 3), and it may be related to the formation of pores in the cell membrane due to the action of bovicin HC5. Analysis of the topographic profiles corroborated these observations. Compared within the same scan interval, the amplitude of the topographic variation in cells treated with bovicin HC5 and EDTA cells was higher than in the control cells, with or without nisin and EDTA (Fig. 5). Increased duration of treatment caused an increase in surface roughness, most likely due to an increase in the number of extrusion sites (Fig. 6). These results suggest that the increase in cell surface roughness was caused by the sites of cytoplasmic leakage.

Similar morphological changes have been observed in other Gram-negative bacteria. An antimicrobial peptide with a mechanism of action identical to that of bacteriocins, i.e., the formation of pores in the plasma membrane and disruption of the outer membrane, produced similar morphological changes in *P. aeruginosa* and *E. coli* (Li et al. 2007). The first morphological alterations observed by these latter authors were the appearance of small indentations and outer membrane residues around the cells. Additionally, the rupture of the cell envelope was initiated by an increase in the peptide concentration.

The increase in cell surface indentations, observed as an increase in cell roughness, appears to be a common response of Gram-negative cells to stress conditions. This morphological pattern has been observed by several authors in other Gram-negative bacteria, such as *E. coli* cells treated with antimicrobial peptides (Meincken et al. 2005), toluidine blue and ultra-violet radiation (Sahu et al. 2009), and anionic antimicrobial peptides, alone or in association with lysozyme (Zdybicka-Barabas et al. 2012).

Conclusion

Salmonella Typhimurium cells treated with bovicin HC5 in association with EDTA exhibited morphological changes, with an increase in cell surface roughness and a loss of turgidity, possibly due to leakage of intracellular metabolites such as potassium. This observation suggests that bovicin HC5 might be used to inhibit the growth of Gram-negative bacteria, provided that it is associated with an outer membrane-disrupting agent. However, additional studies are required to investigate the effect of this bacteriocin in other Gram-negative bacteria.

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