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Atomic force microscopy study of magainin 2 *versus* human platelet extract action on *Escherichia coli* and *Bacillus cereus*

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Some similarities and differences between action of two antimicrobial preparations - cationic antimicrobial peptide (CAMP) magainin 2 and human platelets extract (HPE) demonstrating CAMP properties - on morphological and mechanical properties of *E. coli* and *B. cereus* cells were revealed by atomic force microscopy. Both CAMPs induced formation of pore-like lesions on the *E. coli* surface and resulted in the cell collapse at the apical ends. It was established that HPE damaged the greater quantity of the *E. coli* cells as well as formed the larger pores as compared with magainin 2. On the other hand, HPE *versus* magainin 2 was more active against *B. cereus* cells; this was reflected in a greater number of damaged cells with a significant decrease of cell wall rigidity.

Key words: atomic force microscopy, antimicrobial cationic peptides, magainin 2, human platelet extract, morphological properties, mechanical properties.

INTRODUCTION

Cationic AntiMicrobial Peptides (CAMPs) are specific antibiotic agents with strong bactericidal properties and are considered as a one of the alternative ways to treat the resistant forms of bacteria (Rautenbach & Hastings, 1999; Hancock & Patrzykat, 2002; Alves *et al*., 2010). CAMPs have been isolated from single-cell microorganisms, plants, amphibians, birds, fish, and mammals (Hancock & Diamond, 2000; Zasloff, 2002). They have also been found in human platelets (Krijgsveld *et al*., 2000). Many of these peptides have been extensively studied in order to elucidate their antimicrobial mode of action.

Study of the efficacy of new antimicrobial drugs should be carried out using different methods. Microscopy is one of the informative methods for understanding effects of various factors on bacterial cells. Among the various microscopic techniques, the Atomic Force Microscopy (AFM) can be distinguished (Dufrêne, 2002). AFM is a relatively new technique that has provided new opportunities for the surface analysis of biological specimens with nanoscale resolution and minimal effect on the sample structure. AFM can be used not only for visualization but also to probe local surface forces and mechanical properties of biomaterials (Gaboriaud & Dufrêne, 2007). These benefits make it possible to use AFM for cellular and intracellular structures investigations, yielding information on cell wall assembly and dynamics that cannot be obtained with traditional microscopy techniques (Dorobantu & Gray, 2010). AFM can also be used to study the effect of drugs on microbial cell walls (Cerf *et al*., 2009; Perry *et al*., 2009). One of the first works concerning the application of AFM to investigate the antibiotics effect on bacteria was made by Braga & Ricci (1998). In this work, the authors studied qualitatively the morphological and surface alterations of *E. coli* induced by various concentrations of the β-lactam antibiotic cefodizime. Meincken *et al*. (2005) used AFM for the analysis of the membranolytic effects in the mechanisms of action of three antimicrobial peptides by using *E. coli* cells as the live bacterial target. A high-speed AFM technique has allowed investigating kinetics of antimicrobial peptide activity measured on

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E. coli cells and characterizing the initial stages of the action of the drug (Fantner *et al*., 2010).

In this study we compared effects of two different antimicrobial preparations on the morphology and mechanical properties of bacteria by AFM method. The first is a well-studied CAMP - magainin 2 (Mag 2) - and the other one is a Human Platelets Extract (HPE) demonstrating antibacterial activity with poorly known mechanism. As model microorganisms, a gram-negative (*Escherichia coli* K12 TG1) and a gram-positive bacteria (*Bacillus cereus* IP 5832) were chosen.

MATERIALS AND METHODS

Preparation of bacterial samples with antibiotics

Magainin 2 was acquired from Anaspec Inc. (San Jose, CA, USA). Human platelets extract was obtained from the Institute of Cellular and Intracellular Symbiosis, Russian Academy of Sciences (Orenburg, Russia) and contained up to 90 mg ml^{-1} of protein. Detailed HPE preparation procedure is described by Ivanov (2005).

Studying the actions of each antibiotic has been performed in separate experiments. The procedure of sample preparation was identical for each experiment. Cultures of *Escherichia coli* K12 TG1 and *Bacillus cereus* IP 5832 strains were added to 2.5 ml nutrient LB-broth (Sigma-Aldrich, USA) at 37˚C for 24 hrs to produce $\approx 10^9$ CFU. Cells were harvested by centrifugation (5 min @ 1100 x g). A sample of the bacterial suspension (~10^8 CFU) in buffer solution was incubated for 30 min at 37˚C with each antimicrobial peptide separately.

Determined concentration of antibiotics corresponding to LD_{50} for *E. coli* cells was 50 μg ml^{-1} for Mag 2 and 78 μg ml^{-1} for HPE. In case of *B. cereus* cells, LD_{50} was 156.8 μg ml^{-1} for Mag 2 and 245 μg ml^{-1} for HPE. To reveal differences between gram-negative and gram-positive bacteria damages induced by cationic peptides, *B. cereus* cells were treated with an equal for *E. coli* peptide LD_{50} concentration dose. After incubation, the cell cultures were centrifuged down to pellet and washed twice with dH_2O. The drop of the cell suspension was deposited then on pieces of freshly cleaved mica (5 × 5 mm) which were placed in exsiccators. To avoid dehydration effects of bacteria, relative humidity and temperature values inside exsiccators were kept at 93% and 20-22˚C, respectively (Nikiyan *et al*., 2010).

Atomic Force Microscopy imaging

Bacteria were imaged in contact mode, using SMM-2000 AFM (JSC "PROTON-MIET Plant", Russia). Images were obtained using V-shaped silicon nitride cantilevers MSCT-AUNM from Veeco Instruments Inc. with a spring constant of 0.01 N m^{-1}. Calibration of cantilever spring constant included measurements of the thermally induced motion of the unloaded cantilever (Hutter & Bechhoefer, 1993). For each group of cells, the following morphological parameters were measured: length, width and height. Relying on this data, perimeter section, area section and volume of the cells were calculated. At least 30 cells were processed to calculate mean values for the each parameter. The reliability of the difference was estimated according to non-parametric Wilcoxon's signed-rank test. Root mean square roughness R_q (the standard deviation of the *Z* values) for the height images was determined by drawing section plot of the cell surface and was calculated using SMM-2000 software. The images were flattened and plane fitted prior to analysis.

Determination of Young's modulus

The Young's modulus was determined quantitatively using AFM force measurements in combination with the widely used Hertz model of contact theory (Zhu *et al*., 2000). Although there are more sophisticated models that go beyond the Hertz approximation and include the effects of surface adhesion, finite thickness of the sample and cell homogeneity, the Hertz model is still useful for achieving information about cell elasticity (Hertz, 1881). The model relates the applied loading force (F) to the indentation depth or deformation (δ). For a sphere:

$$F = \frac{4}{3} \cdot \frac{E}{1-\nu^2} \cdot \delta^{3/2} \cdot \sqrt{R},$$

where E is the Young's modulus, R is the probe sphere radius and ν is the Poisson ratio. Cells were assumed to be linear elastic, isotropic (Zhu *et al*., 2000) and incompressible at small strains. Therefore, Poisson ratio ν of the cells was chosen to be 0.5 (Domke & Radmacher, 1998). The sample indentation δ is calculated by subtracting the piezo displacement from the cantilever deflection.

Force-indentation curves were obtained at various locations in each cell and were derived from the measured force *versus* displacement relationship using the mica surface to calibrate the deflection of the can-

tilever. Young's modulus calculation procedure from force-indentation relations is described in Salerno & Bykov (2006). Force-distance curves acquired with an approach speed of 5 μm sec^{-1}. The forces were chosen to keep the indentation depth to less than 10% of the cell height such that errors resulting from the limited bacterial cell thickness were reduced and, in turn, allowed to use Hertz model for calculation of the microorganisms mechanical properties (Azeloglu & Costa, 2011).

RESULTS

Detailed description of the bacteria morphology before and after treatment has allowed to estimate the effect of each of the cationic antimicrobial peptides. Figures 1A and 1C show representative AFM images of untreated *E. coli* and *B. cereus* bacteria. Intact gram-negative *E. coli* occur in AFM scans primarily as singly arranged elongated cells. In contrast, gram-positive *B. cereus* bacteria are larger, arranged in chains or singly. The average length, width, height and several calculated parameters of untreated cells are presented in Table 1. In Figures 1B and 1D, a magnification of the bacteria surface is shown, and it can be deduced that the *E. coli* membrane surface is rather structured. In contrast, the surface of *B. cereus* cells looks smoother.

However, all studied bacteria had no visible lesions. The average Young's modulus (E) of the intact cells was determined to be 2.56 ± 1.16 MPa for *E. coli* and 6.43 ± 1.28 MPa for *B. cereus* (Fig. 2).

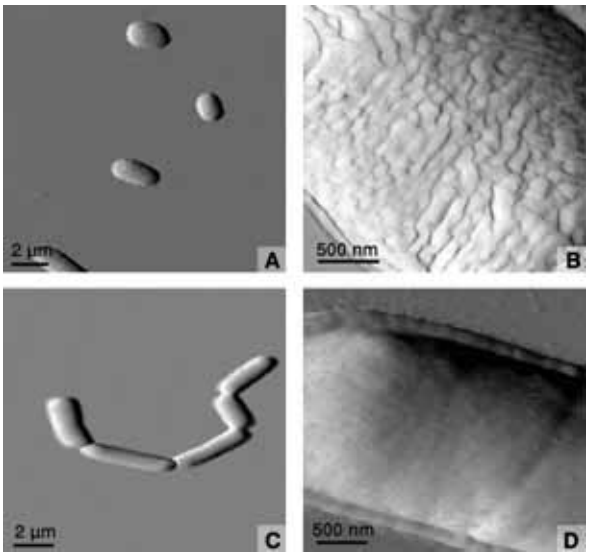

FIG. 1. AFM deflection images of untreated *Escherichia coli* K12 TG1 (A) and *Bacillus cereus* IP 5832 (C) cells; (B) and (D): magnitude images of *E. coli* and *B. cereus* surfaces, respectively.

The action of CAMP on E. coli cells

Figures 3A and 3C compare the effects of Mag 2 and HPE on *E. coli*. It is clearly visible that the effect of Mag 2 as well as HPE leads to morphological changes in most of the cells. A characteristic feature of damage induced by both CAMPs was the collapse of the cell structure at the apical ends. We have found that 76.19% of all visualized cells treated with Mag 2 were damaged, whereas the percentage of HPE treated cells having signs of lesions was 89.44%.

TABLE 1. Morphological characteristics of *E. coli* and *B. cereus* cells

Strain	Experimental groups	*Morphological characteristics*						
		Length (μm)	Width (μm)	Height (μm)	Perimeter section (μm)	Area section (μm^2)	Volume (μm^3)	Roughness (nm)
E. coli K12 TG1	Untreated	2.71 ± 0.67	1.33 ± 0.18	0.25 ± 0.03	2.47 ± 0.29	0.26 ± 0.05	0.70 ± 0.23	2.01 ± 0.25
	Treated with Mag 2	2.68 ± 0.40	1.48 ± 0.23**	0.20 ± 0.04**	2.65 ± 0.38*	0.24 ± 0.07	0.68 ± 0.21	5.03 ± 1.01**
	Treated with HPE	2.64 ± 0.66	1.38 ± 0.23	0.21 ± 0.04**	2.49 ± 0.37	0.22 ± 0.06*	0.59 ± 0.21**	5.73 ± 2.24**
B. cereus IP 5832	Untreated	3.65 ± 0.98	1.33 ± 0.19	0.34 ± 0.06	2.61 ± 0.31	0.35 ± 0.08	1.29 ± 0.44	0.98 ± 0.23
	Treated with Mag 2	3.66 ± 1.11	1.31 ± 0.30	0.27 ± 0.01**	2.48 ± 0.44	0.28 ± 0.12**	0.99 ± 0.50*	4.15 ± 1.78**
	Treated with HPE	3.59 ± 1.10	1.42 ± 0.24*	0.31 ± 0.14*	2.71 ± 0.33	0.33 ± 0.12	1.24 ± 0.70	4.88 ± 1.73**

* $p < 0.05$, ** $p < 0.01$ (Wilcoxon's signed-rank test)

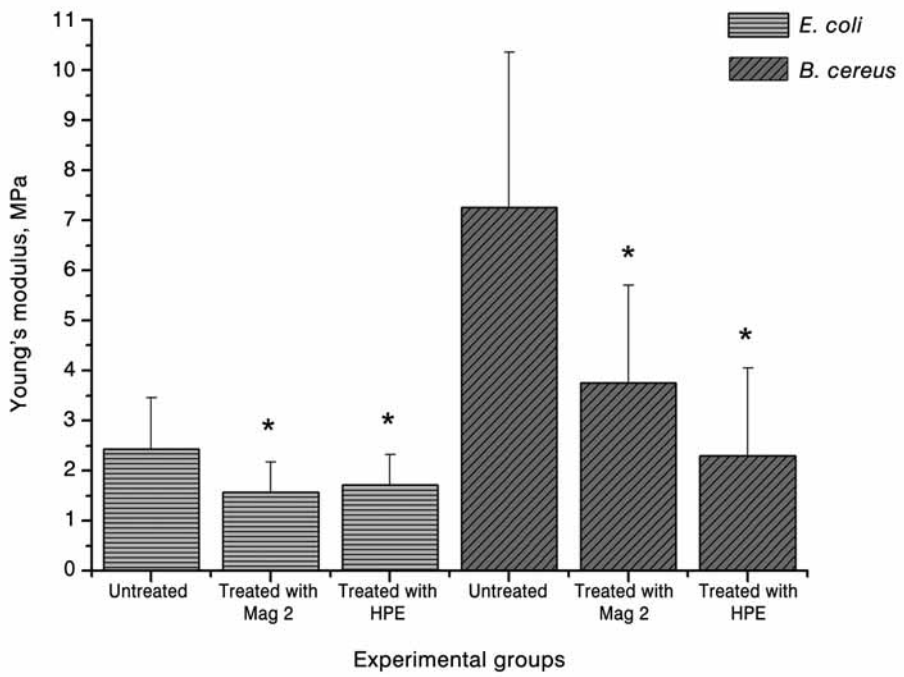

FIG. 2. Diagram of the Young's modulus distribution of *E. coli* and *B. cereus.* Asterisk denotes significant difference at $p < 0.01$ (Wilcoxon's signed-rank test).

In general, the effect of each evaluated CAMP did not lead to a change of rod-shaped form of *E. coli* cells. However, changing of cell dimensions has occurred. As follows from Table 1, action of Mag 2 resulted in increasing of width and perimeter section parameters and decreasing of *E. coli* height. At the same time cells treated with HPE had statistically significant reduction of height, area section and volume parameters.

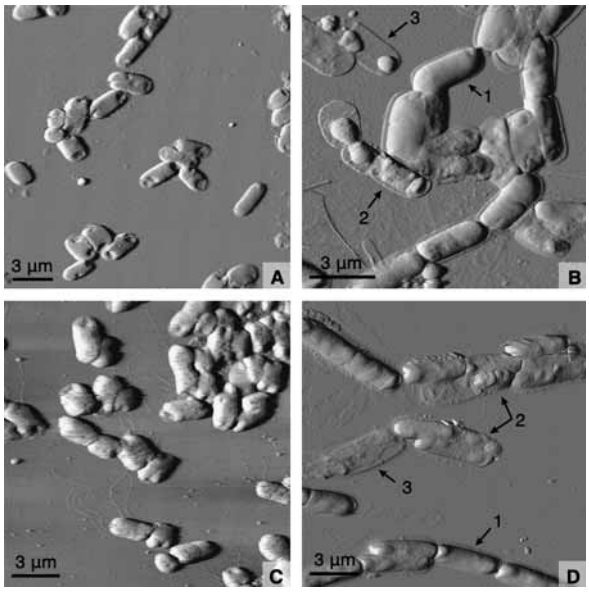

FIG. 3. AFM deflection images of *E. coli* (A, C) and *B. cereus* (B, D) treated with Mag 2 (A, B) and HPE (C, D). See the text for an explanation of arrows.

Detailed study of the *E. coli* surface (Figs 4A and 4D) has allowed to detect disorders of the structure of external membrane and formation of pore-like lesions. Figures 4B and 4E are a magnification of a part of the bacteria surface treated with Mag 2 and HPE, respectively. In both cases randomly distributed holes (dark areas) are clearly visible on cell surface. To describe differences between CAMP induced lesions, distribution of the pores by diameter was analyzed (see Figs 4C and 4F). It was established that diameter of the pores affected by HPE exposure significantly exceeds the respective of induced by Mag 2 (165.96 ± 62.75 nm against 39.74 ± 15.64 nm, respectively). In rare instances, structural damages of the cell wall with a diameter of 195.61 ± 106.40 nm were also observed in Mag 2 treated samples; these values were not taken into consideration for the calculation of mean values.

Besides these changes, the roughness and elasticity parameters of the cells were also affected. As can be seen from the Figure 2, the Young's modulus of each of treated bacterial cells was decreased. To Mag 2 - treated *E. coli* cells, decreasing value was 25.89% in comparison with intact cells. The *E* parameter of HPE-treated *E. coli* was comparable (25.50%). The

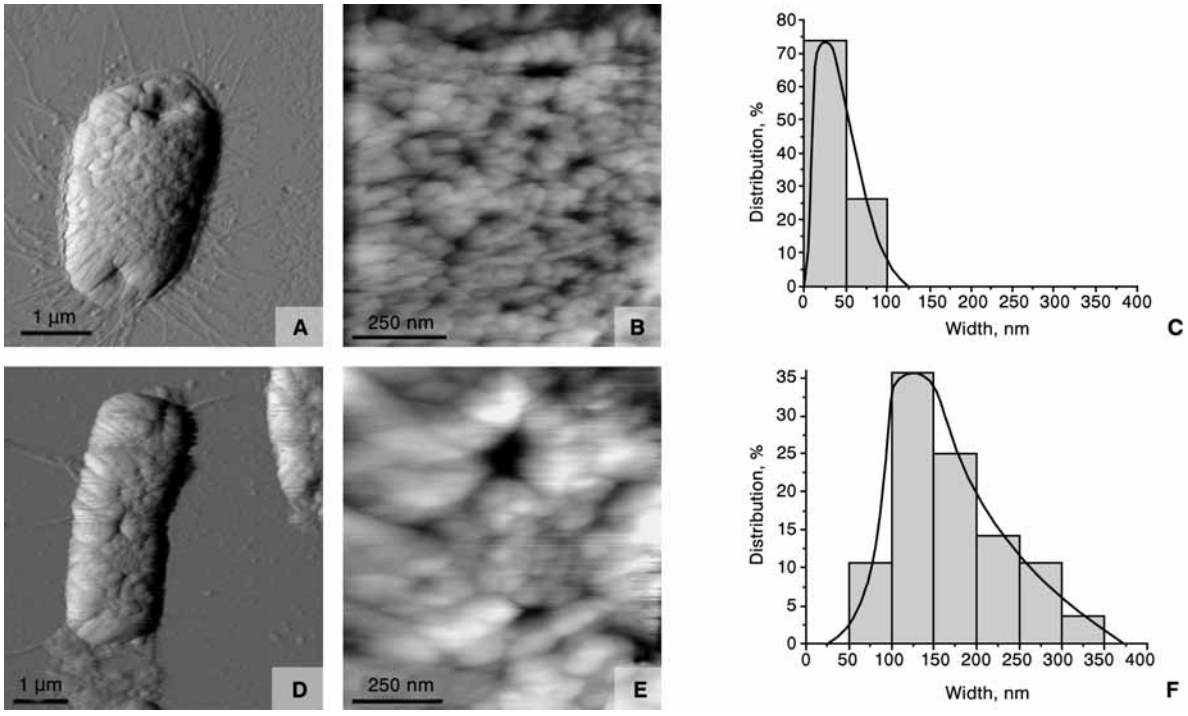

FIG. 4. AFM deflection images of the single *Escherichia coli* cells (A), magnitude height images of cell surface (B) and size-distribution diagrams of pore-like lesions as result of Mag 2 (C) and HPE (D, E, F) treatment.

surface roughness of the Mag 2 - treated cells increased by more than 2.5-fold from 2.01 ± 0.25 nm to 5.03 ± 1.01 nm (Table 1), which is comparable to that caused by HPE (R_q = 5.73 ± 2.24 nm, a 2.85-fold increase).

The action of CAMP on Bacillus cereus

Figures 3B and 3D show an example of the types of *B. cereus* damage due to peptide treatment. Using cell volume, roughness and cell height values as a criterion, three types of cells can be distinguished (see arrows on Figs 3B and 3D).

Cells labeled as "1" have no visible changes and are identical to intact cells according to morphological parameters. These bacteria account for 75.96% of the total amount of imaged cells treated with Mag 2 and 66.40% for cells treated with HPE. Cells of the second type ("2") could be characterized as having significant differences from intact cells. The frequency of the cell type occurrence is 18.82% and 29.60% for Mag 2 and HPE treated bacteria, respectively. Bacteria belonged to the group "3" had clearly visible disintegrated barrier structures that were apparently caused by the complete loss of intracellular contents. These bacteria are found in 5.22% cases for Mag 2 and 4.01% for HPE treated cells.

The morphological parameters of type "2" cells were compared in the Table 1. For *B. cereus* cells treated with Mag 2, statistically significant decrease of the height with synchronous increase of cell volume and area section were established. At the same time, the influence of HPE was reflected in a significant increase of the cell width and decrease of height when compared to untreated cells.

A loss of the treated cell surface homogeneity caused by folding of cell wall was observed. Quantitatively this was expressed by growth of the roughness more than four times for the cells treated with Mag 2 and five times for HPE treated bacteria (see Table 1). As in case of *E. coli* cells, *E* value of treated *B. cereus* bacteria was reduced (Fig. 2). The elasticity of the bacteria treated with Mag 2 and HPE was 41.44% and 64.18% down from the intact cells, respectively.

DISCUSSION

Cationic antimicrobial peptides are increasingly considered as a promising new group of pharmaceutical drugs with an antimicrobial activity. Initial electrostatic interaction with the outer barrier structures of bacterial cells is the feature of their mechanism of action. This determines the interest in the imaging of

bacterial cell lesions formed under the CAMP treatment. An atomic force microscopy is one of novel methods of visualization, that allows estimating the aftereffects of various CAMP action.

One of the well studied CAMPs is Mag 2 (Matsuzaki, 1998). Since the effect of Mag 2 on the *E. coli* cell envelope was studied in detail using AFM (Meincken *et al*., 2005), it was used as a model CAMP to compare with biological effects of poorly described HPE on gram-negative and gram-positive bacteria. We have found similar cell damages of *E. coli* caused by Mag 2, expressed in the collapse at the apical ends of cells, increasing of the surface roughness and deep lesions formation. The same types of damage are incidental not only for Mag 2, but also for other CAMPs, i.e. for apolipophorin III and cecropin B (Zdybicka-Barabas *et al*., 2011). Thus, the findings confirmed the idea that outer membrane of *E. coli* is a primary target for Mag 2. Used concentration of Mag 2 led to formation of visualized damages at 76.19% of the total number of cells in the experiment. At the same time, a substantially smaller activity of this CAMP concerning to *B. cereus* was shown. Only 24.04% of investigated gram-positive bacteria were affected at the same conditions. In this case, the results are in good agreement with the findings of Tang *et al*. (2002), Mohan *et al*. (2010) and Zdybicka-Barabas *et al*. (2011), where gram-negative bacteria exhibited higher sensitivity to CAMPs when compared to gram-positive ones.

Obtained results were used as a basis for comparison of insufficiently explored action of HPE on microorganisms. Platelets of mammals are the source of a wide range of substances with antimicrobial action. Action of antimicrobial peptides from human platelets (thrombocidins) (Krijgsveld *et al*., 2000) and rabbit platelets (thrombin-induced PMPs) are the most studied (Yeaman *et al*., 1998). Seven thrombin-releasable antimicrobial peptides from human platelets have been identified later. Five of them have been isolated by means of acid extraction (Tang *et al*., 2002). However, there are no AFM studies of the effect of substances extracted from platelets on bacterial cells.

In our study using AFM the nature of damage of *E. coli* and *B. cereus* cells treated with HPE for the first time was estimated, that allowed to reveal similarities and differences among analogous effects of Mag 2. One of the similar features of HPE action on *E. coli* cells consisted in collapse at the apical ends of the cells. This result corresponds well with the results from work of Meincken *et al.* (2005), where the possibility of trapping of a higher concentration of CAMPs at the apical ends is discussed. Another feature of studied peptides is the formation of pore-like lesions on the cell surface. Character of the damages conforms to the carpet model for the interaction of CAMPs with the bacterial membrane (Oren & Shai, 1998) that intends formation of transient pores at low concentration and severe membrane disruption at high values of peptides concentration (Gregory *et al*., 2009). In addition, the increasing of the cell surface roughness and the loss of cell rigidity occurred in the same range for both CAMPs. Apart from the similarities discussed above, the following differences were found; HPE formed the larger pores and caused greater damage on the *E. coli* cells. At the same time, the surface corrugations of the bacteria surface appeared less pronounced in the case of Mag 2 treatment.

As compared to Mag 2, HPE also showed the higher activity to *B. cereus* that was expressed in a greater number of damaged cells and a significant decrease of cell wall rigidity. However, populations of bacteria treated with both CAMPs were rather heterogeneous and included intact, partly destroyed and completely destroyed cells. Therefore, it can be concluded that the gram-positive *B. cereus* bacteria are more sensitive to the action of HPE than to Mag 2.

Thus, obtained results have allowed to ascertain the existence of certain similarity in action of the Mag 2 and HPE on *E. coli* cells consisting in the loss of rigidity of the outer membrane, roughness increasing and formation of pore complexes having however various quantitative morphological characteristics. On the other hand, the action of studied CAMPs on *B. cereus* cells appeared as significant changes of roughness and rigidity of the bacteria.

In a similar context, the result of the study once again confirms the efficiency of the AFM as a method for studying the nature of damage of various factors on the bacterial cells. At the same time, the findings form the basis for the further research of cationic peptides from mammalian platelets as a promising antimicrobial agent with a wide spectrum of biological activity.

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