BEHAVIOUR OF PLANKTONIC BACTERIA AND THOSE PRESENT IN THE STRUCTURE OF BIOFILMS IN RELATION TO THE ACTION OF SOME BIOCIDES

COMPORTAREA BACTERIILOR PLANCTONICE ȘI A CELOR PREZENTE ÎN STRUCTURA BIOFILMELOR FAȚĂ DE ACȚIUNEA UNOR BIOCIDE

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ABSTRACT | REZUMAT

Worldwide studies on bacterial behaviour in relation to biocides have shown that bacterial cells that have been attached to surfaces and are within the matrix of exopolysaccharides become more resistant to the action of antibiotics and disinfectants 10 to 100 times, compared to their planktonic counterparts.

The acquired biocide resistance of the bacteria occurs during an unpredictable event when one or more cells expressing resistance to one or more antibacterial substances appear in the population of a given bacteria species. The emergence of a strain resistant to a biocide occurs especially when the concentration of the latter one is inferior to the minimum inhibitory concentration.

Many of the commercial substances used to clean surfaces, instruments, medical devices or industrial machinery are corrosive to metals, cause irritation to the skin, mucous membranes or even show a toxic and carcinogenic effect. For these reasons, it has been necessary to find new solutions for combating or preventing the formation of biofilms on surfaces. In recent years, there has been a great deal of interest in the use of organic acids for this purpose. This group of substances exerts an antibacterial effect at certain pH values and acts by changes in the cell wall of the bacteria.

In the present paper, the efficacy of some organic acids (citric acid, malic acid and ADABLINE ALK product), on planktonic bacterial cells and those present in biofilm structures, on 3 bacterial species from the group of Gram negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) and a Gram-positive one (*Staphylococcus aureus*), was evaluated. For investigations,1% w/v and 2% w/v solution concentrations were dissolved into Tryptone soya broth with glucose (TSBG) in the determination of the minimum inhibitory concentration for planktonic cells and in physiological solution for the biocidal effect assessment on 5 days old, bacterial biofilm cells.

Keywords: organic acids, minimum inhibitory concentration, biocidal effect, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus*

Studiile referitoare la comportarea bacteriilor față de biocide, efectuate pe plan mondial, au demonstrat că celulele bacteriene care s-au fixat pe suprafețe și se află în interiorul matricei de exopolozaharide, devin mai rezistente la acțiunea antibioticelor și substanțelor dezinfectante de 10 până la 100 ori, comparativ cu omologele lor planctonice.

Rezistența dobândită a bacteriilor față de biocide apare în timpul unui eveniment imprevizibil, când, în populația unei specii bacteriene date, apare una sau mai multe celule care exprimă o rezistență față de una sau mai multe substanțe antibacteriene. Apariția unei tulpini rezistente față de un biocid, apare mai ales atunci când concentrația acestuia este inferioară concentrației minime inhibitorie.

Multe din substanțele comerciale, care se folosesc pentru igienizarea suprafețelor, a instrumentelor, a dispozitivelor medicale sau a utilajelor industriale sunt corozive pentru metale, produc iritații ale pielii, mucoaselor sau manifestă chiar efect toxic și cancerigen. Din aceste considerente a fost necesar să se găsească noi soluții pentru combaterea sau pentru împiedicarea formării biofilmelor pe suprafețe. În ultimii ani, a crescut mult interesul pentru utilizarea, în acest scop, a acizilor organici. Acest grup de substațe exercită efect antibacterian la anumite valori de pH și acționează prin modificări la nivelul învelișului celular al bacteriilor.

În prezenta lucrare a fost evaluată eficacitatea unor acizi organici (acid citric, acid malic și a produsului ADABLINE ALK), asupra celulelor bacteriene planctonice și a celor existente în structura biofilmelor, față de 3 specii bacteriene din grupa bacteriilor Gram negative (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) și una Gram pozitivă (*Staphylococcus aureus*).

Pentru realizarea investigațiilor s-au folosit soluții de 1% g/v și 2% g/v care au fost dizolvate în bulion triptonă soia cu glucoză, în cazul determinării concentrației minime inhibitorie, pentru celulele planctonice și în soluție fiziologică în cazul evaluării efectului biocid față de celulele bacteriene din structura biofilmelor, în vârstă de 5 zile.

Cuvinte cheie: acizi organici, concentrație minimă inhibitorie, efect biocid, *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus*

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Contamination of contact surfaces with pathogenic or conditional pathogenic microorganisms represents a worldwide significant and current issue, and it is demonstrated that after contamination of the surface bacteria manage to form biofilms shortly. (13, 27)

It is now accepted that biofilms represent, for about 99% of the known bacterial species, a normal or potential stage of their life cycle necessary to perform specific functions in correlation with the signals that component bacteria receive from their living environment. (1, 19, 27). In natural ecosystems it is considered that the planktonic existence of bacterial cells is only a transient stage, necessary for the formation of a new biofilm. (2, 7, 29)

Bacterial biofilms can have a negative impact on equipment and surfaces in most industrial sectors, but especially on human and animal health. (10, 12)

The main industrial sectors where bacterial biofilms can cause significant economic damage include: the maritime sector, the agro-food industry (the dairy industry, the meat industry, the crop products industry, the drinking water supply systems, etc.), in paper mills, in dentistry, in hospitals, etc. (12, 16, 22)

In the food industry, bacterial cells belonging to pathogenic or conditional pathogenic species, present in the biofilm on the surface of the equipment, can easily be transferred to the raw material or finished product and, after their consumption, they can cause food poisoning or infections in humans. (6, 17, 25). If bacterial cells in the biofilm are not pathogenic, after multiplying on the surface of the food, it causes the biological quality to be reduced or even altered. (3, 8)

In the medical and veterinary field, pathogenic bacteria from biofilms are involved in over 80% of cases of chronic mucosal and soft tissue diseases: chronic otitis, endocarditis, gastrointestinal ulcers, pulmonary infections in patients with cystic fibrosis, pharyngitis, laryngitis, conjunctivitis,sinusitis,etc. (5,16,23).

In hospitals, most nosocomial infections occur by transferring bacterial cells from existing biofilms to different surfaces (inadequately sterilized instruments and non-compliance with sanitation programs). (5,18)

Pathogenic or conditional pathogenic bacteria infections can also be caused by the use of prosthetic devices, on the surface of which the pathogenic bacteria (orotracheal probes, urethral probes, vaginal specula, contact lenses, valvular prostheses, etc.) can attach. (5, 6, 21)

Through the antigenic structure or the toxins that biofilm bacteria develop in the tissues where the bacterial film has been formed, the production of specific antibodies or specific marked cells is stimulated in the body, but they cannot penetrate inside the biofilm to stop the infectious process which will continue its evolution. (4, 9)

Clinical studies have shown that existing planktonic bacteria are much more sensitive to the action of antibiotics and biocides used in the treatment of bacterial infections, compared to those present in the biofilm structure, especially when they are made up of several bacterial species. (7, 20, 29)

Recent advances in fundamental research to combat infections caused by existing bacteria in biofilms recommend combining conventional treatments with the use of complementary methods to disintegrate the matrix in which bacterial cells are embedded, so that administered antibiotics or biocides can exert their bactericidal effect. (11, 14)

MATERIALS AND METHODS

Materials:

Biocides

The following biocides were used to assess the antimicrobial effect:

· Citric acid monohydrate;

· DL-malic acid;

· The ADABLINE ALK® product, manufactured by AMD INITIATIVE SRL (a mixture of organic acids, salts of organic acids and volatile natural oils).

Bacterial strains

4 bacterial strains were used: 3 belonged to Gram negative species (*Escherichia coli,* strain 720, *Klebsiella pneumoniae,* strain 1612 and *Pseudomonas aeruginosa,*strain 623) and a Gram-positive bacteria strain (*Staphylococcus aureus,* strain 1572).

Culture media

Tryptone soya broth, with 0.5% glucose (TSBG),
Tryptone soya agar with sheep blood.

WORKING TECHNIQUE

Working culture production.

In order to obtain working cultures, from each bacterial strain, kept on -20° C, in cryoprotective solutions, transplants are made with Tryptone soya broth. Sowed media were incubated at 37° C, for 24 hours. The culture production was controlled for purity by sowing on Tryptone soya agar with sheep blood and by bacterioscopic examination.

Technique of biofilm production.

The biofilms required to highlight the bactericidal

effect on the sessile cells embedded in the biofilm matrix were obtained on the walls of 16/120 mm glass tubes as follows.

· From the 24-hour old culture in TSBG, after incubation at 37° C of each bacterial strain, 10 ml were harvested, which were added to a flask containing 90 ml of TSBG. All seeded flasks were incubated at 37° C, for 24 hours, in a normal atmosphere to obtain working cultures.

· After checking the purity by bacterioscopic examination, 5 ml was harvested, distributed in 15 presterilized tubes. After individualization, all tubes were statically incubated at 37° C, for 120 hours, to form the biofilm.

Biocide solutions production.

The biocide solutions were prepared on the day when their antibacterial action was investigated, on the planktonic cells or the sessile cells from biofilm structure. To determine the minimum inhibitory concentration of the 3 antibacterial products, serial dilutions at rate 2, starting with a 4% w/v solution concentration of each antibacterial substance, were carried out up to a 0.062% w/v solution concentration, using as a diluent TSBG. In the determination of antibacterial action on cells of the biofilm structure, a 1% w/v and a 2% w/v solution concentration were prepared using sterile, distilled water as the diluent.

DETERMINATION OF INHIBITORY CONCENTRATION FOR PLANKTONIC CELLS

In each 4 series of serial dilutions at rate 2, made in TSBG, for each biocide, we have added 0.1 ml of the TSBG culture for each 24-hour old bacterial strain.

After gentle homogenization, the sown dilution sets are incubated for 24 hours, at 37° C in a normal atmosphere. The minimum inhibitory concentration was the last dilution at which the bacteria did not multiply. For confirmation, from the tubes in which the bacterial growth was inhibited, 0.1 ml was harvested and dispersed on the surface of a plate of Tryptone soya agar with glucose. If after incubation of the plaques on which the dispersions were made, colonies no longer appeared, the minimum inhibitory concentration was confirmed.

DETERMINATION OF ANTIMICROBIAL EFFECT ON BACTERIAL CELLS IN THE BIOFILM STRUCTURE

From the 60 tubes containing the mature biofilms, with a vacuum pump, to which a sterile 10 ml pipette was attached, it was carefully removed the culture medium, then into each 2 tubes, with the biofilm of each bacterial strain, was added 10 ml of 1% and 2% biocide solutions, respectively.

3 biofilm tubes were used to determine the number of colony-forming units (as a control group).

All tubes in which biocide solutions were added were inhibited at 37° C, for 24 hours, to inactivate bacterial cells in the biofilm structure. After incubation, the biocide was removed and there were added 9 ml of distilled, sterile water.

With the Labnet stirrer, each tube was homogenized for 5 minutes to release bacterial cells from the biofilm matrix. Finally, from each obtained suspension, the number of colony forming units was determined to provide the total number of cells that remained viable after controlling with the biocide solutions.

RESULTS AND DISCUSSIONS

Investigations of the antimicrobial action of citric acid, malic acid and the Adabline ALK product on the 4 bacterial species took place in two successive stages.

In the first stage the minimum inhibitory concentration (MIC) of each antimicrobial substance against the bacterial cells of *Escherichia coli* strains, *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* was determined and in the second stage the effect of the 3 substances on the bacterial cells in the biofilm structure was determined.

In Table 1, the minimum inhibitory concentrations of citric acid, malic acid and ALK product against the planktonic cells of the 4 bacterial strains used in the investigations are presented.

Worldwide studies on the mechanisms of action of organic acids on bacteria have shown that these substances show antimicrobial activity at certain pH values, but this group of substances primarily interferes with the structural proteins in the cell wall and cytoplasmic membranes, to which they destroy their links and the tertiary and quaternary structures. (4, 15, 24)

The detailed analysis of the data in Table 1 confirms that the 3 organic acids used show their antibacterial activity by the same mechanisms of action and their minimum inhibitory concentrations are the same, the value of this indicator being 0.25%.

Table 2, shows the data on the action of these antimicrobial substances for 1% w/v and 2% w/v solution concentration compared to bacterial cells present in the biofilm structure of the 4 bacterial species, after 120-hour static incubation, at 37° C in glass tubes.

Table 1

The minimum inhibitory concentration of the 3 antimicrobial agents to 4 bacterial strains

Antimicrobial agent	Dilution of the antimicrobial substance %	Bacterial species			
		Escherichia coli	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*
Citric acid	4	-	-	-	-
	2	-	-	-	-
	1	-	-	-	-
	0,50	-	-	-	-
	0,25	-	-	-	-
	0,125	+++	+++	+++	+++
	0,062	+++	+++	+++	+++
	Control group	+++	+++	+++	+++
Malic acid	4	-	-	-	-
	2	-	-	-	-
	1	-	-	-	-
	0,50	-	-	-	-
	0,25	-	-	-	-
	0,125	+++	+++	+++	+++
	0,062	+++	+++	+++	+++
	Control group	+++	+++	+++	+++
Adablinc ALK	4	-	-	-	-
	2	-	-	-	-
	1	-	-	-	-
	0,50	-	-	-	-
	0,25	-	-	-	-
	0,125	+++	+++	+++	+++
	0,062	+++	+++	+++	+++
	Control group	+++	+++	+++	+++

The obtained data show that the vegetative cells of *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains, existing in the biofilm structure, were completely inactivated after disinfectants' action in concentrations of 1% and 2%, after 24-hour contact, at 37° C. Moreover, the biofilms formed on the surface of the tube walls completely detached.

The vegetative cells of the strains of *Escherichia coli,* and *Klebsiella pneumonia,* in the 120-hour old biofilm structure, were not completely inactivated after a 24-hour contact with 1% or 2% citric acid, malic acid and ALK product solution concentration.

The comparative analysis of the data listed in Table 2 demonstrates that the number of viable bacterial cells expressed by the number of colony forming units (CFU), after a 24-hour contact, at 37° C with disinfectants, varies from one bacterial strain to another, depending on the type of antibacterial agent and the solution concentration.

We also note that ALK product is a complex mixture of organic acids, salts of organic acids and volatile natural oils, proved to be more active compared to ci-

Table 2

Antimicrobial activity of citric acid, malic acid and the Adabline ALK product on the vegetative cells present in the biofilm structure

Bacterial species	Control group	Antimicrobial substance					
		Citric acid		Malic acid		ALK	
		1%	2%	1%	2%	1%	2%
Escherichia coli	$9x10^8$	$4,8x10^4$	$12x10^2$	$9x10^5$	$7x10^3$	$6x10^3$	9x10
Klebsiella pneumoniae	$11x10^7$	$3,2x10^5$	$16x10^3$	$5x10^6$	$2x10^4$	$5x10^4$	2x10
Pseudomonas aeruginosa	$7x10^8$	0	0	0	0	0	0
Staphylococcus aureus	$12x10^9$	0	0	0	0	0	0

tric acid and malic acid. After the biofilms, formed by *Escherichia coli,* contact with solutions, prepared from the ALK product, 6x103 viable cells/ml was found for the 1% solution concentration and 9x10 viable cells/ml for the 2% solution concentration.

CONCLUSIONS

1. The minimum inhibitory concentration of citric acid, malic acid and the Adabline ALK product against the planktonic cells of the strains of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* was 0.25%.

2. The vegetative cells of *Pseudomonas aeruginosa* and *Staphylococcus aureus,* present in the 120-hour old biofilm structure, were killed by 1% and 2% citric acid, malic acid and Adabline ALK solution concentrations after 24-hour contact, at 37° C temperature.

3. The vegetative cells of the strains of *Escherichia coli* and *Klebsiella pneumoniae* in the 120-hour old biofilm structure were not completely inactivated after 24-hour contact, at 37° C with 1% or 2% solution concentration of the 3 disinfectants.

4. For the *Escherichia coli* strain, the number of viable vegetative cells, expressed as the number of colony forming units (CFU), after 24-hour contact with the 3 disinfectants in a 2% solution concentration was 12x102 for citric acid, 7x103 for malic acid and 9x10 CFU/ml for Adabline ALK product.

5. The number of CFU/ml in the case of the vegetative cells present in the 120-hour old biofilm structure of the *Klebsiella pneumoniae* strain after 24-hour contact, at 37° C with the 2% solution concentration of the 3 disinfectants was 16x103 for citric acid, 2x104 for malic acid and 2x10 for the Adabline ALK product.

6. The Adabline ALK product for the 2% solution concentration has been shown to be more active against the 2 bacterial species compared to malic acid and citric acid.

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