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Pseudomonas aeruginosa and pyocyanin negatively act on the establishment of *Enterobacteriaceae* biofilm on a ceramic surface

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ABSTRACT
The occurrence of biofilm on the porous surface of ceramic water filters and their candles may increase the risk of transmission of pathogens, especially enterobacteria. In addition, more than 90% of strains of *Pseudomonas aeruginosa* synthesize pyocyanin, a blue-green pigment, with antimicrobial activity against different organisms. The aim of this work was to verify the influence of *P. aeruginosa* and its pigment on the adhesion of four strains of *Enterobacteriaceae* to the surface of a ceramic coupon. Standard microbial suspensions ($\approx 10^8$CFU/mL) were transferred to reactors containing ceramic coupon with 100mm^2 of area, immersed in sterile mineral water, to which had been added 0.5 g/L of yeast extract. Static incubation occurred over 240h at 37°C. Three conditions of contact were evaluated: at equal times, after 48h of advantage for *P. aeruginosa* and in presence of a sub-inhibitory concentration of pyocyanin. Axenic cultures were tested for comparison. The doubling-time and the densities of the enterobacteria in the aqueous medium and on the coupon, were determined. Pyocyanin exerted a disturbance on the adhesion, limiting the development of enterobacteria on the coupon, but did not reduce its population in the aqueous medium, suggesting that other species-specific factors participate in the phenomenon of antagonism.

Keywords: Bacterial adhesion, *Enterobacter aerogenes*, *Escherichia coli*, Microbial antagonism, Natural phenazines.

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I. INTRODUCTION

Water is fundamental for the formation and maintenance of all living things and the quality of the water that is consumed has been a worldwide concern throughout history. Millions of people do not have access to drinking water. Different treatment options have been tried. Among these, ceramic gravity water filtration is considered the most efficient alternative to domestic filtration solutions, rather than sand or solar disinfection [1][2].

A ceramic water filter (CWF) consists basically of two compartments, one of affluent and the other of effluent, joined by a simple mechanism, containing a CWF candle with the function of retaining solid particles and biological material while a flow of water passes through a layer of activated carbon by gravity. It is estimated that millions of CWFs are in use throughout the world [3].

The filtration system is dynamic and the removal of particulates occurs as a function of porosity of the ceramic candle, whose properties result in a large surface area with high permeability and tortuosity, allowing the removal of 92-99% of bacteria and protozoa from water [4]. Sometimes, the microbial density in the treated effluent is greater than in the water source introduced in the upper compartment. This is due to the fact that the faucet in the lower compartment is not located close to the bottom. The remaining small volume of water concentrates a microbial load in this part, which, because it is not periodically cleaned, favors an environment for the accumulation and development of biofilms [5].

Biofilms are structures composed of one or more species living in close association, surrounded by an adhesive polymeric matrix. Often, biofilms are responsible for the increase of the microbial load in the water stored for human consumption, and the greatest risk is the presence of pathogenic organisms such as *Enterobacteriaceae* [6].

Competition for space and nutrients with other organisms in the aquatic environment, such as *Pseudomonas aeruginosa*, may limit the development

of enterobacteria. Under certain conditions, however, enterobacteria re-growth is favored, representing a health risk [7][8].

Pseudomonas aeruginosa is a Gram-negative non-fermentative rod found in various environments, preferably freshwater. The species has a specific metabolism, making it resistant to competition with other organisms or when exposed to the selective pressures exerted by the environment. Pyocyanin is an important molecule directly related to this phenomenon. The compound is a bright blue-green phenazine pigment (5-methylphenazine-1-one), synthesized by more than 90% of strains. Its diffusion constant is $0.5x10^{-9}$ m^2/s, which in solid media represents a diffusion time of 3 minutes between two points measuring 1 μm from each other [9].

Pyocyanin is thought to be antagonistic against other microbes by promoting the generation of reactive oxygen species. Antibiosis, however, may not be tied to one effect only, but rather associated with different mechanisms. These involve biological and abiotic factors that may promote death or biostatic effect on the susceptible microorganisms competing for space and nutrients with *P. aeruginosa* [10][11][12]. The aim of the present work was to evaluate the influence of pyocyanin and pyocyanin-producing *P. aeruginosa* strains on the adhesion of *Enterobacteriaceae* to a ceramic coupon surface, with the identical material as used in CWF candles.

II. EXPERIMENTAL MATERIALS AND METHODS

2.1. Microorganisms

A total of eight aquatic strains were used: four *Pseudomonas aeruginosa*, three of them pyocyanin-producing; and four enterobacteria: two *Escherichia coli* and two *Enterobacter aerogenes*.

2.2 Pyocyanin

A hydroalcoholic 10% pigment solution (Sigma-Alderich, St. Louis, USA) was prepared with ethanol according to the manufacturer's instructions, making a concentration of 23.78 mM and kept under refrigeration at 0 °C.

2.3 Evaluation of the antimicrobial activity of pyocyanin

The test determined the minimum inhibitory concentration (MIC) of the pigment on four enterobacteria using the microdilution technique [13]. The MWY solution, composed of non-carbonated hypothermal mineral water, supplemented with 0.5 g/L of yeast extract, previously autoclaved at 121 °C for 15 minutes, was prepared.

The bacterial suspensions were made in a sterile 0.85% NaCl solution, from a recent culture, standardized by tube No. 1 on the MacFarland scale. On microplates, 0.1 ml of the bacterial suspension ($\approx 10^8$ CFU/mL) were dispensed, and then 0.1 mL of the MWY solution was transferred so that pigment concentrations ranged from 0.380 to 0.0015 mM [14][15].

The system was incubated for 96h at 37 °C and at 24h intervals, aliquots of 2 μL were transferred to new wells containing nutrient broth (Sigma Aldrich, Rio de Janeiro, Brazil) and re-incubated under the same conditions in order to verify the formation of turbidity, indicative of cell viability. The control used only MWY. The test was performed in duplicate.

2.4. Biofilm formation tests

The assay was carried out using a ceramic coupon (Legato, Rio de Janeiro, Brazil) with a minimum contact area measuring 100 mm^2, immersed in a 50 mL capacity polyethylene reactor (Deskarplas, São Paulo, Brazil) filled with 10 mL of the MWY solution [16].

The pre-inoculum of each strain tested was prepared from fresh culture in a 0.85% sterile saline solution and a volume of this suspension was transferred to the system so that the initial population was established at $\approx 10^2$ CFU/mL. Then the reactor was statically incubated at 37 °C for 48h and at each 24h interval the coupon was aseptically removed and the material developed on the porous surface was transferred by scraping into a vessel containing 10 mL of 0.85% sterile saline. The *P. aeruginosa* strains were quantified by the pour plate technique on nutrient agar and the enterobacteria by the multi-tube technique in 3-tube series containing lactose broth [17]. The tests were performed in duplicate. The enterobacteria doubling-time, expressed in hours, was determined by counting the viable cells after incubation for 24h at 37 °C on nutrient agar [18].

2.5. Inhibitory activity test in liquid-state

The assay was conducted in duplicate to verify whether the presence of *P. aeruginosa* and/or pyocyanin disrupts the adhesion of *Enterobacteriaceae* to the surface of the coupon. In reactors containing the MWY solution, three conditions were tested under static incubation at 37°C for 240h: in the first, the contact between the microorganisms occurred in equal times. Under the second condition, a 48h growth advantage was allowed for *P. aeruginosa*, prior to inoculation of the enterobacteria, allowing pyocyanin to already be diffused in the medium. The third condition evaluated the participation of the sub-inhibitory concentration of pyocyanin on the formation and stability of enterobacteria biofilm. A non-pyocyanin-producing strain of *P. aeruginosa* was used for comparison purposes.

2.6. Statistical treatment

The statistical normality analysis was performed by the Kolmogorov-Smirnov test, followed by the t-test to evaluate the difference of the results in the presence and absence of pyocyanin in the medium.

III. RESULTS

3.1. Formation of biofilm by the strains as axenic cultures

The *P. aeruginosa* pyocyanin-producing strains demonstrated a subtle growth and exhibited pigment within 48 h. On the other hand, the cell density of the enterobacteria was identical in all tested strains. The quantifications of the cells developed on the coupon by the axenic cultures are presented in Table 1.

P. aeruginosa RX08, compared to the other pyocyanin-producing strains, TGC01 and TCG03, presented the shortest doubling-time, as well as a more intense blue color diffused in the medium. The enterobacteria exhibited identical values of cell density and approximate doubling-times.

Although under static conditions of incubation, the values of microbial population quantification were expressive, since all strains were fixed over a short time, starting from a low inoculum, suggesting that the first 24 hours were crucial to the process.

Tab. 1 Doubling-time (τ_g) and number of cells as axenic cultures on coupon surface.

Strains	τ_g (h)	Cell density (x10^4)	
		24h	48h
TGC01 (PA)	4.4±0.1	29.0±1.6 [a]	280.0±5.0 [a]
TGC03 (PA)	3.5±0.1	26.0±2.0 [a]	260.0±3.7 [a]
RX08 (PA)	2.7±0.1	31.0±4.5 [a]	430.0±4.3 [a]
AV08 (PA)*	3.5±0.1	16.0±4.0 [a]	110.0±1.6 [a]
AV01 (EC)	1.8±0.1	0.4[b]	2.3[b]
AV02 (EC)	1.5±0.1	0.4[b]	2.3[b]
AV13 (EA)	1.5±0.1	0.4[b]	2.3[b]
AV14 (EA)	1.9±0.1	0.4[b]	2.3[b]

*****non-pyocyanin producing strain
[a]CFU/cm^2, [b]MPN/mL/cm^2, PA- *P. aeruginosa*, EA – *E. aerogenes*, EC – *E. coli*)

3.2 Evaluation of the antimicrobial activity of pyocyanin

Among four strains of enterobacteria, pyocyanin produced an antagonistic effect on two of them, AV01 and AV14. In Table 2 the results are summarized, where the MIC of the pigment was observed from the detection of viable cells over the 96h.

Tab 2. Minimum inhibitory concentration of pyocyanin on *Enterobacteriaceae*

Concentration (mM)	AV01 / AV14			
	24	48	72	96
0,380	− / −	− / −	− / −	− / −
0,190	− / −	− / +	− / +	− / +
0,090	+ / −	+ / +	+ / +	+ / +
0,048	+ / −	+ / +	+ / +	+ / +
0,024	+ / −	+ / +	+ / +	+ / +

(+) indicates cell viability

AV01 growth at 0.09 mM concentration was observed from the beginning of the test, suggesting that the concentrations of 0.380 and 0.190 mM had a biocidal effect on the bacteria in up to 96 hours. On the other hand, pyocyanin exhibited an antimicrobial effect on AV14, initially at the highest concentration, 0.38 mM. Microbial growth, however, was verified in the following interval, starting from the concentration at 0.190 mM, and remaining thus until the end of 96h. In the AV02 and AV13 strains, no biocidal effect was detected.

3.3. Inhibitory activity test in liquid-state assay between *Pseudomonas aeruginosa* and enterobacteria in equal times

Table 3 shows the cell densities of AV01 and AV14 developed in the biofilm. Regardless of whether they were in competition, the pyocyanin-producing strains, took the same amount of time, when axenically cultured, to display the pigment.

Tab 3. MPN/cm^2 of enterobacteria strains* (values obtained among two repetitions)

Strains	Time and type of cultivation			
	Mixed		Axenic	
	24h	48h	24h	48h
AV01 x TGC01	0.9	0.7	0.4	2.3
AV01 x TGC03	0.9	0,9	0.4	2.3
AV01 x RX08	0.9	0.3	0.4	2.3
AV01 x AV08*	0.4	0.9	0.4	2.3
AV14 x TGC01	0.0	0.0	0.4	2.3
AV14 x TGC03	0.4	0.4	0.4	2.3
AV14 x RX08	1.5	0.0	0.4	2.3
AV14 x AV08*	0.4	0.9	0.4	2.3

*****non-pyocyanin producing strain

The presence of *P. aeruginosa* reflected disturbances in the adhesion of the enterobacteria to the surface of the coupon. We made the premise that the first 24h were crucial for the adhesion of the AV01 and AV14 strains, competing with *P. aeruginosa* without the pyocyanin being dissolved in the medium. We observed three different responses: inhibition of adhesion, reduction of the microbial density in the biofilm and stasis of the microbial population. The RX08 strain promoted the highest inhibition, followed by TGC01 and TGC03. The AV14 strain was the most sensitive

Enterobactericaea, compared to AV01. On the other hand, competing with a non-pyocyanin-producing strain, both enterobacteria exhibited the same MPN obtained when cultivated axenicaly, suggesting that other *P. aeruginosa* factors besides pigment may participate in the antagonistic phenomenon.

3.4 Inhibitory activity test in liquid-state assay with a 48-hour growth advantage of *Pseudomonas aeruginosa*

The 48-hour advantage given to the *P. aeruginosa* strains ensured that pyocyanin was already widespread enough to be able to significantly disturb the adhesion of isolates AV01 and AV14. Table 4 shows the MPN values, comparing them with the growth in axenic cultures.

The presence of the pigment diffused in the medium, associated with the adhesion of *P. aeruginosa* to the ceramic coupon as well as with an increased doubling-time exhibited by enterobacteria AV01 (4.4h) and AV14 (1.5h), produced a negative effect on the development of these strains. Two different responses were observed: antibiosis with biocidal action and disturbance in the maintenance of the enterobacteria population, resulting in marked reductions in the number of cells adhering to the ceramic coupon. The highest inhibitions were promoted by TGC03, followed by RX08 and TGC01. On the other hand, in the absence of pyocyanin, after 48h, the result was similar to that obtained in the inhibitory activity test in liquid-state assay for equal time, reinforcing the hypothesis that other species-specific factors of *P. aeruginosa* may also participate in the phenomenon of inhibition.

Tab 4. MPN/cm^2 of *Enterobacteriacea* strains adhered on coupons* (values obtained among two repetitions)

Strains	**Time and type of cultivation**			
	Mixed		**Axenic**	
	24h	**48h**	**24h**	**48h**
AV01 x TGC01	0.4	0.0	0.4	2.3
AV01 x TGC03	0.0	0.0	0.4	2.3
AV01 x RX08	1.4	0.0	0.4	2.3
AV01 x AV08*	1.4	0.9	0.4	2.3
AV14 x TGC01	0.4	0.3	0.4	2.3
AV14 x TGC03	0.0	0.0	0.4	2.3
AV14 x RX08	0.0	0.0	0.4	2.3
AV14 x AV08*	1.4	0.9	0.4	2.3

*****non-pyocyanin producing strain

3.5 Evaluation of biofilm formation in the presence of pyocyanin

An increase of test time made it possible to measure the degree of perturbation in the dynamics of biofilm formation by the enterobacteria. The sub-inhibitory concentrations of pyocyanin, respectively 0.09 and 0.190 mM, on the strains AV01 and AV14 (Fig. 1) promoted a significant negative effect, where cell density was gradually reduced and could not be detected after 240h of incubation. In order to verify if the reduction of the enterobacteria population in the coupon occurred due to a biocidal effect caused by the pyocyanin dissolved in the medium, the cell density of AV01 and AV14 was also quantified in the MWY solution (Fig. 2). Both remained viable throughout 240h, suggesting that, under sub-inhibitory concentrations, the pigment participates in some mechanism of inhibition involving adhesion factors.

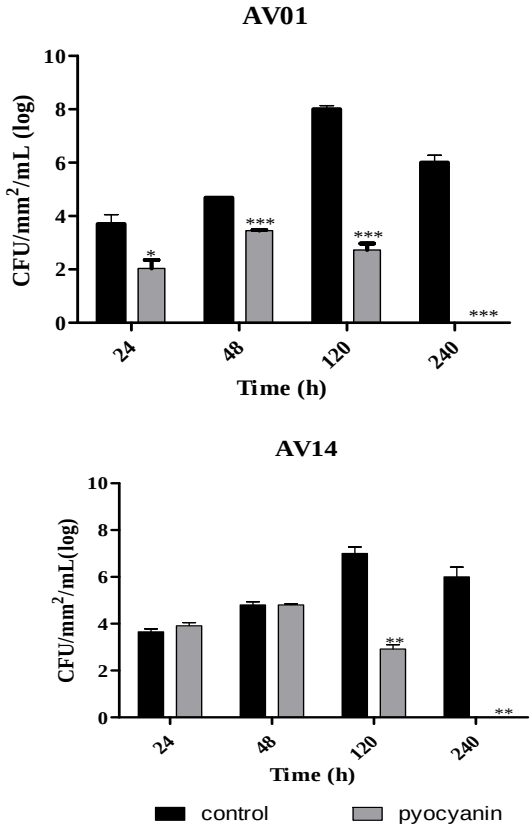

Fig. 1. Number of enterobacteria cells on coupon (*$p < 0.05$; ** $p < 0.01$; ***$p < 0{,}001$)

IV. DISCUSSION

From the ecological point of view, the aquatic environment is a medium characterized by a competitive relation between the organisms that coexist with each other. This relationship is associated to the fact that the nutrients are diluted, as well as to the selective pressures that are exerted by the medium in different ways. Thus, some strains may become more prevalent than others, due to their metabolic constitution, such as *P. aeruginosa* if compared to enterobacteria. Hypothetically, enterobacteria are more sensitive than other aquatic

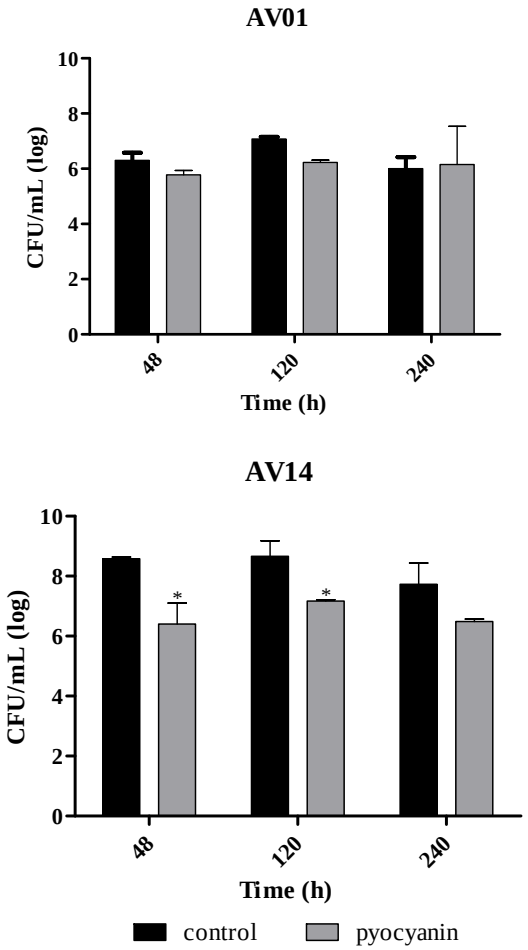

Fig. 2. Number of enterobacteria cells in MWY medium (*$p < 0.05$)

bacilli and their re-growth depends on different factors that favor their permanence in the medium, especially those factors related to adhesion and colonization on surfaces [19][20]. An advantage of enterobacteria over *P. aeruginosa* in aquatic environments is based on the differences in doubling-time between the two microorganisms. While *E. coli* for example may multiply up to three times within one hour, *P. aeruginosa* may require an average of 3-6 hours to double its population only once [21].

The doubling-time exhibited by the enterobacteria used in this study were significantly different from the parameters described in the literature [22][23]. Although strains AV01 and AV14 showed increased doubling-times, the valuesobtained were still lower than those determined with the *P. aeruginosa*, TGC01, TGC03, RX08 and AV08 strains. Unlike growth conditions in an enriched media, longer periods for the microbial population to double in number may occur frequently under conditions similar to those applied in this study are employed, i.e., a culture medium with low nutrient contents. In these cases, the limited concentrations of organic carbon promote pressures and force the microbiota to employ other metabolic paths to establish and remain viable, which include the synthesis of exopolysaccharides [24][25]. Thus, as they multiply, some individuals can overcome initial disturbances caused by negative interactions between two distinct microorganisms that coexist and compete for nutrients and space.

Amensalism is a common ecological phenomenon that occurs between two competing organisms. While one of them produces substances of various natures, such as enzymes, organic acids, toxins and pigments, among others, the second has its development impaired, either by a biocidal effect or by a biostatic effect [26][27]. In the aquatic environment, several factors facilitate the occurrence of amensalism, among them: temperature, pH, nutrient level, oxygen presence and microbial density.

P. aeruginosa is a rod known to develop in an aquatic environment with low levels of dissolved organic matter [28]. It is believed that pyocyanin, a phenazine compound prevalent in more than 90% of bacterial strains, plays a key role in antibiosis through the generation of reactive oxygen species [10][29]. Under certain conditions, the survival potential of pathogenic enterobacteria organized in biofilms is more limited and data from the literature confirm that *P. aeruginosa* has an advantage over *E. coli* and *E. aerogenes* [11][30].

Pyocyanin in an aquatic environment is synthesized during the end of the log phase and the beginning of the stationary phase, which may vary between 48 and 72 hours [31]. All strains used in this study in some tests produced pyocyanin between 24-48h, possibly due to the low organic carbon content present in the medium. Under conditions of nutrient deficiency, pigment production is favored so that the *P. aeruginosa* becomes more successful in eventual competition with other microorganisms present [32][33][34]. It should be noted that a low carbon content was used to simulate conditions of possible organic matter accumulation in a CWF, and in this scenario, *P. aeruginosa* would have a better chance of surveillance, compared to other microorganisms, including *Enterobacteriaceae*.

On the other hand, the availability of organic matter was more in the MWY solution than in the coupon. It is suggested that given the static conditions of the test, simulating a CWF compartment, a possible sedimentation of the organic matter, over time could explain the increase of the population of AV01 and AV14 on the surface of the coupon, when pyocyanin was not present.

Although other microorganisms exhibit antagonistic relationships to enterobacteria, for example *Aeromonas hydrophila* and *Lactobacillus acidophilus*, *P. aeruginosa* is still considered one of

the most significant bacteria in the event [31], especially as a result of pyocyanin occurrence [11][35]. Previous works observed different antagonistic relationships in water involving *P. aeruginosa* strains against several pathogens such as *Escherichia coli*, *Enterobacter aerogenes*, *Salmonella paratyphi* and *Klebsiella pneumoniae* [34][35]. About 90-95% of the observations were correlated to the synthesis of pyocyanin and/or associated with the presence of pyoverdine, the second most important pigment synthesized by *P. aeruginosa*.

The first report of antimicrobial activity of pyocyanin occurred during World War II in a study involving inhibition of *E. coli* growth [38]. Near the end of the last century, the biocidal effect of pyocyanin was found to be concentration-dependent [39]. It is now believed that this activity involves different mechanisms, so that *P. aeruginosa* benefits by disrupting stability or eliminating potential competitors [40]. In recent years theories are that the probable antimicrobial mechanism of pyocyanin occurs at the level of the respiratory chain and disruption of the mechanism of active transport by the membrane. It is also believed that the pigment can cause a decrease in the oxygen supply to the cells, leading to the accumulation of superoxide and hydrogen peroxide. In addition, pyocyanin may degrade the flow of electrons, promoting a toxic effect on the affected cells [36]. Even under sub-inhibitory concentrations, important characteristics of enterobacteria can be altered, such as growth kinetics, biochemical profile, mechanisms against oxidative stress, motility and biofilm production [41].

The results of this study also indirectly alert to the risk that the presence of *P. aeruginosa* represents when coexisting with *E. coli* or *E. aerogenes* in an aqueous medium. These pathogens are responsible for serious gastrointestinal disorders. Under this scenario, *P. aeruginosa* can be indicated as a possible non-fecal indicator in colimetric analyzes of water for human consumption. Even if *P. aeruginosa* prevents the adhesion of enterobacteria to surfaces, an underestimated population of this group, when exposed to the conditions that favor its growth, can be maintained later by organic matter available in the medium. In the case of strains that are more resistant to the inhibitory effects of *P. aeruginosa* or its pigment, the risk is magnified. The origin of these strains may reveal the nature of this resistance [42]. Strains AV02 and AV13 were tolerant of different concentrations of pyocyanin. They were isolated from drains of sinks from beauty salons, through which had passed effluents loaded with stress-promoting compounds, such metals, preservatives, and oxidizing agents.

Finally, it is important to note that the metabolic versatility of *P. aeruginosa* goes beyond the synthesis of pyocyanin, guaranteeing growth advantage with subsequent predominance in competition scenarios, as could be observed in the non-pyocyanin producing strain.

Although inhibition of biofilm formation was shown, it should be emphasized that cell density in the MWY medium was high and reduction of the microbial density in the coupon may illustrate a possible specific effect on the adhesion of enterobacterial cells by *P. aeruginosa* during the establishment of the biofilm. This needs further investigation to better understand the cellular and molecular events involved.

V. CONCLUSIONS

Pyocyanin produced a concentration-dependent inhibitory effect on the studied strains of enterobacteria, subsequently interfering with the adhesion of two of them to the surface of a ceramic coupon. However, it did not disturb viability of these bacteria in an aqueous medium. The differences observed in the density of enterobacteria in the MWY medium and on the coupon when in contact with the pyocyanin suggest that other species-specific factors of *P. aeruginosa*, besides the pigment, act in the kinetics of antagonism.

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