Antagonism between clinical and environmental isolates of *Pseudomonas aeruginosa* against coliforms

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ABSTRACT

Water is essential to life, yet many people worldwide do not have access to clean drinking water and suffer or die from preventable diseases caused by unsafe water. Microbiological water analysis is based on coliform bacteria, but these microorganisms can have their growth affected by others, such as *Pseudomonas aeruginosa*. A total of 60 isolates of *P. aeruginosa* was investigated in this study to obtain better knowledge about the relationship between *P. aeruginosa* pigments and the effect of antagonism against coliforms. Of these, 40 isolates were isolated from environmental samples (drinking water and soil) and 20 from clinical patients. Three environmental coliform isolates from water samples, two *Escherichia coli* and one *Klebsiella* pneumoniae, and an *Enterobacter aerogenes* were used in antagonism tests. The results have demonstrated that these bacteria were inhibited by *P. aeruginosa* by an effect known as antagonism. The inhibitory action of *P. aeruginosa* against coliforms was more effective when *P. aeruginosa* produced pyocyanin and/or pyoverdin.

Key words | antagonism, coliforms, environmental strains, *Pseudomonas aeruginosa*, pyoverdin

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INTRODUCTION

The improvement of global access to clean drinking water is a cheap and effective way to improve global health. More than 1.1 billion people lack access to improved drinking water, and as a consequence, millions suffer from preventable illnesses and die every year (Montgomery & Elimelech 2007). Testing for the presence of coliforms is an analysis that guarantees the quality of the water. *Escherichia coli* and thermotolerant coliforms (*Klebsiella*, *Enterobacter*, *Citrobacter*) are the most important indicators of contamination in water (Foppen & Schijven 2006), but several factors can be responsible for failure in drinking water analysis, including interaction with other organisms, such as *Pseudomonas aeruginosa* (Rompré *et al.* 2002;Vaconcelos *et al.* 2010). When this type of error happens, the water might be considered as safe and potable, when in fact, it can be contaminated by fecal coliforms and other pathogens, which can cause human diseases.

P. aeruginosa is a bacterium distributed in nature and has received the definition of ubiquitous bacteria (Shao *et al.* 2010). It possesses the capacity to metabolize independently of the supplement of nutrients and has a great armory of survival strategies, ranging from pigments to the production of biofilm (Klausen *et al.* 2003; Kipnis *et al.* 2006). This bacterium can secrete a variety of pigments, including pyocyanin (blue-green) and pyoverdin (yellow-green and fluorescent) (Kipnis *et al.* 2006; Ganter *et al.* 2009).

Pyocyanin (PCN) is a zwitterion that can easily penetrate biological membranes. It can be recovered from the sputum of cystic fibrosis (CF) patients and ear secretions infected by *P. aeruginosa* (Lau *et al.* 2004; Liu & Nizet 2009). However, besides its role in the infections caused by *P. aeruginosa* strains, pyocyanin has been related to an antagonistic phenomenon against other microorganisms, especially against coliform bacteria, through the generation

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of reactive oxygen species, which could be the major factor of *P. aeruginosa* antimicrobial activity (Vaconcelos *et al.* 2010).

Pyoverdin (PVD), which is considered a virulence factor (Musthafa *et al.* 2011), is a siderophore excreted in large amounts by *P. aeruginosa*, which has a strong iron requirement (ÓMay *et al.* 2009). It is a powerful iron chelating agent that helps in the transport of iron from the extracellular environment via outer membrane proteins with a specific receptor (Hannauer *et al.* 2010).

The aim of this study was to obtain further knowledge about how the pigment production by *P. aeruginosa* can affect coliform growth. For this, two experiments were carried out. In the first experiment, with a total of 60 isolates of *P. aeruginosa* isolated from drinking water, soil and clinical sources, the action of the pigments was tested using the agar disk experiment, in which the pigment(s) is diffused into the solid medium, without bacteria interaction. In the second experiment, the interaction between *P. aeruginosa* and coliform bacteria in a liquid medium containing a low amount of nutritional resources was tested to discover whether the pigment-producing bacteria have an advantage over those which do not produce pigment.

MATERIALS AND METHODS

Isolation and Identification

In total 20 *P. aeruginosa* clinical isolates were obtained from patients with CF (Table 1) who attended the School Hospital of Campinas State University (UNICAMP), Brazil, between April 1996 and January 1998 (Ethical Commission Process number no. 045/98 CEP/FCM from 05/27/98).

For isolation of 20 *P. aeruginosa* isolates from water, samples were collected from aquatic habitats (Table 1), from three different Brazilian states, into sterile flasks. For the isolation of bacterial colonies of *P. aeruginosa*, 100 mL aliquots were filtered through sterile 0.45 mm pore membrane filters (Millipore), 47 mm in diameter, with the aid of a vacuum pump. Membranes aseptically removed from the filtration equipment were placed on the surface of cetrimide agar (Difco) plates and incubated at 37 °C for 48–72 h.

Isolation of 20 *P. aeruginosa* isolates from soil was performed as described previously by Mukherjee *et al.* (2011). The soil samples were collected from plantation areas of different crops and also from sand (Table 1). The samples were collected 5 cm below the land surface in sterile containers. One gram of each soil sample was dispersed in 9 mL of 0.85% NaCl into sterile test tubes and a series of dilutions from 10^{-2} to 10^{-10} were prepared in 0.85% NaCl. A 0.2 ml aliquot of the appropriate dilution was spread aseptically onto cetrimide agar and incubated at 37 °C for 48–72 h.

Typical *P. aeruginosa* colonies were confirmed by oxidase test, pigmentation, growth at 42 °C, and biochemical tests, e.g. citrate assimilation (+), carbohydrate fermentation (−), indol (−), and lysine decarboxylase (−) (Casanovas-Massana *et al.* 2010; Murray *et al.* 2011), and also confirmed by PCR using specific primers to amplify the open reading frame of the oprL gene (De Vos *et al.* 1997; Zanetti *et al.* 2013). The primers (Invitrogen, Brazil) had the following sequences: PAL1, 5′-ATGGAAATGCTGAAATTCGGC-3′ (a 21-mer corresponding to the beginning of the open reading frame of oprL); and PAL2, 5′-CTTCTTCAGCTCGACGCGACG-3′ (a 21-mer corresponding to the end of the open reading frame of oprL). PCR products separated by 1.8% agarose gel electrophoresis were visualized by staining with ethidium bromide. *P. aeruginosa* ATCC 27853 served as a positive control.

Coliform isolates were isolated from water samples as described above and were identified by using MacConkey agar (Difco) and eosin-methylene-blue agar (Difco) at 37 °C for 24–48 h of incubation. Biochemical tests were performed by Enterobacterial Panel (Probac do Brasil).

Antagonism tests in a solid medium

The antagonism tests in a solid medium were conducted as described by Ichikawa *et al.* (1971). The Muller-Hinton (MH) agar (Difco) disks were prepared with a sterilized barren cylinder of 8 mm, after which 30 μL of a 0.5 McFarland *P. aeruginosa* solution were inoculated onto the surface of the disks. After incubation at 37 °C for 48 h, these disks, containing growths of *P. aeruginosa,* were transferred onto the agar surface, where coliforms were spread on and incubated at 37 °C overnight. The inhibition zones formed were measured. A greater inhibition zone diameter indicated more coliform susceptibility to *P. aeruginosa*. The antagonism test was performed with all 60 isolates of *P. aeruginosa*.

Table 1 | Results of the presence of antagonism between *P. aeruginosa* and coliform bacteria related to pigment production and sources of strains

Strain	Source	Sample	Pigment	Ec1	Ec2	Kp1	Ea1
Pa01	Clinical	Patient with CF	PVD	−	+	−	+
Pa02	Clinical	Patient with CF	PVD	−	+	−	+
Pa03	Clinical	Patient with CF	PCN	+	+	+	+
Pa04	Clinical	Patient with CF	PCN and PVD	+	+	+	+
Pa05	Clinical	Patient with CF	PCN and PVD	+	+	+	+
Pa06	Clinical	Patient with CF	PCN	+	+	+	+
Pa07	Clinical	Patient with CF	PCN	+	+	+	+
Pa08	Clinical	Patient with CF	PCN and PVD	+	+	+	+
Pa09	Clinical	Patient without CF	PCN	+	+	+	+
Pa10	Clinical	Patient without CF	PVD	−	−	−	+
Pa11	Clinical	Patient without CF	PVD	−	−	−	+
Pa12	Clinical	Patient with CF	PVD	−	−	−	+
Pa13	Clinical	Patient with CF	PVD	−	+	−	−
Pa14	Clinical	Patient with CF	PCN	−	+	−	+
Pa15	Clinical	Patient with CF	None	−	−	−	−
Pa16	Clinical	Patient without CF	PVD	−	−	−	+
Pa17	Clinical	Patient with CF	PCN	+	+	+	+
Pa18	Clinical	Patient with CF	PCN	+	+	+	+
Pa19	Clinical	Patient with CF	PCN	+	+	+	+
Pa20	Clinical	Patient with CF	PVD	+	+	+	+
Pa21	Water	Residence	PCN	−	+	+	+
Pa22	Water	Residence	PCN	−	+	+	+
Pa23	Water	Residence	None	−	−	−	−
Pa24	Water	Residence	None	−	−	−	−
Pa25	Water	Residence	None	−	−	−	−
Pa26	Water	Residence	None	−	−	−	−
Pa27	Water	Residence	PVD	−	+	−	−
Pa28	Water	School	PVD	−	+	−	−
Pa29	Water	School	PVD	−	−	−	−
Pa30	Water	Well	PVD	−	−	+	−
Pa31	Water	Well	PCN and PVD	+	+	+	+
Pa32	Water	Well	PVD	−	−	−	−
Pa33	Water	Well	PVD	−	−	−	−
Pa34	Water	Well	PVD	−	−	−	−
Pa35	Water	Well	PVD	−	−	−	−
Pa36	Water	Well	PVD	−	−	−	−
Pa37	Water	Well	PVD	−	−	−	−
Pa38	Water	Well	PVD	−	−	−	−
Pa39	Water	Well	PVD	−	−	−	−
Pa40	Water	Well	PVD	−	−	−	−

(*continued*)

Table 1 | continued

Strain	Source	Sample	Pigment	Ec1	Ec2	Kp1	Ea1
Pa41	Soil	Lettuce	None	–	–	–	–
Pa42	Soil	Sand	None	–	–	–	–
Pa43	Soil	Chilli	None	–	–	–	–
Pa44	Soil	Sugar Cane	None	–	–	–	–
Pa45	Soil	Sugar Cane	None	–	–	–	–
Pa46	Soil	Orange	None	–	–	–	–
Pa47	Soil	Orange	None	–	–	–	–
Pa48	Soil	Orange	None	–	–	–	–
Pa49	Soil	Chrysanthemums	None	–	–	–	–
Pa50	Soil	Chrysanthemums	None	–	–	–	–
Pa51	Soil	Chrysanthemums	PVD	–	–	–	–
Pa52	Soil	Chrysanthemums	None	–	–	–	–
Pa53	Soil	Garden	PCN and PVD	–	+	–	+
Pa54	Soil	Garden	None	–	–	–	–
Pa55	Soil	Garden	None	–	–	–	–
Pa56	Soil	Garden	PVD	–	–	–	–
Pa57	Soil	Garden	None	–	–	–	–
Pa58	Soil	Garden	None	–	–	–	–
Pa59	Soil	Soya	None	–	–	–	–
Pa60	Soil	Soya	PVD	–	–	–	–

Antagonism tests in a liquid medium

The antagonism tests in a liquid medium were performed as described by Vaconcelos *et al.* (2010) using an *E. coli* isolate (Ec2) and 10 *P. aeruginosa* isolates. McFarland solutions (0.5) were prepared from a 24 h growth after which the CFU mL^{-1} was determined by the Most Probable Number (MPN) method. After this, the necessary volumes from both bacteria to achieve a final concentration of 100 CFU mL^{-1} in a 100 mL solution of diluted MH (0.5% $m.m^{-1}$) were calculated. After 24, 48 and 72 h of incubation in a shaker (37 °C and 80 rpm), the *E. coli* CFU mL^{-1} was determined by the MPN method (US Food and Drug Administration 2010).

Statistical analysis

Fisher's exact test was done to verify and confirm if the production of pigment is related to antagonism. The determination of the MPN was done using the MPN calculation program distributed by Jarvis *et al.* (2010). Analysis of variance (ANOVA) tests were performed to determine whether there exists a difference between the *E. coli* (Ec2) growth when in contact with different isolates of *P. aeruginosa* after 24, 48 and 72 h, in the antagonism test in the liquid medium.

RESULTS AND DISCUSSION

The antagonism test in the solid medium was performed with two *E. coli* isolates (Ec1 and Ec2), a *K.* pneumoniae (Kp1) and an *Enterobacteraerogenes* (Ea1), all of them being isolated from well water. Ec1 and Kp1 were isolated from a sample in which *P. aeruginosa* was isolated concomitantly and Ec2 and Ea1 from a sample without *P. aeruginosa* isolation. At the end of the experiment the *P. aeruginosa* isolates were analyzed to determine if they showed antagonism or not by measuring the inhibition halo. The results are given in Table 1.

From the clinical isolates ($n = 20$), 40% (8 isolates) produced only pyocyanin, 40% (8 isolates) produced only

pyoverdin and 15% (3 isolates) produced both pigments. The bacteria that produced both pigments presented antagonism against all coliforms tested. From the pyocyanin producers, all showed antagonism against Ec2 and Ea1, whereas just one did not show antagonism against Ec1 and Kp1. From the pyoverdin producers, 12.5% (1 isolate) presented antagonism against Ec1 and Kp1, 50% (4 isolates) against Ec2 and 87.5% (7 isolates) against Ea1. The isolate without pigment did not present antagonism.

From the water isolates ($n = 20$), 10% (2 isolates) produced only pyocyanin, 75% (15 isolates) produced pyoverdin and 5% (1 isolate) produced both pigments. The isolate which produced both pigments showed antagonism against all coliforms. The pyocyanin producers presented antagonism against Ec2, Kp1 and Ea1. From the pyoverdin producers, 13.3% (2 isolates) presented antagonism against Ec2, 6.67% (1 isolate) against Kp1 and none against Ec1 and Ea1. The bacteria with no pigment production did not present antagonism.

From the soil isolates ($n = 20$), 15% (3 isolates) produced pyoverdin, 5% (1 isolate) produced both pigments and 80% (16 isolates) did not produce any pigment. The isolate that produced both pigments showed antagonism only against Ec2 and Ea1. The other *P. aeruginosa* isolates which produced pyoverdin or no pigment did not present antagonism.

Using Fisher's exact test (H_0 = pigment production and antagonism are independent), it was confirmed that the pigment production and antagonism are dependent for all tested coliforms ($P < 0.05$) (Table 2). The frequencies of inhibiting the growth of coliform bacteria tested are shown in Table 2. The data indicate that the production of pyocyanin showed a more inhibitory power against coliform bacteria, when compared with pyoverdin.

Table 2 | *P*-value for Fisher's exact test and frequency (%) of occurrence of antagonism between isolates of producing and non-producing pigment *P. aeruginosa* and coliforms

Pigment	Ec1 (%)	Ec2 (%)	Kp1 (%)	Ea1 (%)
Pyocyanin ($n = 10$)	70	100	90	100
Pyoverdin ($n = 24$)	4.2	25	8.3	29
Both ($n = 5$)	80	100	80	100
None ($n = 21$)	0	0	0	0
P-value	0.0049	< 0.0001	0.0005	< 0.0001

In the antagonism experiment on solid medium performed by Vasconcelos *et al.* (2006), *E. aerogenes* and *E. coli* suffered antagonism against *P. aeruginosa*, with *E. aerogenes* being more sensitive. Our results show the same pattern where Ea1 ($n = 22$) was the most sensitive followed by Ec2 ($n = 21$). Ec1 ($n = 12$) and Kp1 ($n = 15$) were less susceptible against *P. aeruginosa*. These results show that coliform isolates from a sample without *P. aeruginosa* presence were more sensitive than isolates from a sample with *P. aeruginosa*, suggesting a resistance acquisition by these bacteria.

All bacteria that produced both pigments and only pyocyanin ($n = 15$) showed antagonism against Ec2 but not with Ec1, which was isolated from a sample in which *P. aeruginosa* was present. This suggests that *E. coli* can become resistant to pyocyanin when it comes in contact with *P. aeruginosa* in the environment. Hassett *et al.* (1992) investigated how *P. aeruginosa* protects itself against pyocyanin, comparing how *P. aeruginosa* and *E. coli* act when pyocyanin is present. This resistance was attributed to several factors, including enzymes (catalase and superoxide dismutase (SOD)) production and the penetration of the pyocyanin through the cell membrane. Battistoni *et al.* (1998) demonstrated that the over expression of SOD in *E. coli* enhanced its resistance to macrophages, a phagocyte that reduces oxygen to hydrogen peroxide, superoxide and a hydroxyl radical as an antimicrobial defense. An unexpected mutation that allows *E. coli* to produce more catalase and/or SOD is a possible explanation for this resistance to pyocyanin, a molecule that can generate radical species.

It was possible to observe that 4.2% of only pyoverdin producers ($n = 24$) showed antagonism against Ec1, 25% against Ec2, 8.3% against Kp1 and 29% against Ea1 (Table 2). One reason why it was not possible to observe antagonism in all pyoverdin producers relates to the quantity of the pigment produced, as it was possible to visualize that those which produced a lot of pigment showed antagonism, and the others which produced less pigment, did not present antagonism (results not shown). West & Buckling (2003) correlated that the amount of siderophore produced is directly proportional to the virulence of the microorganism.

The antagonism test in the liquid medium was performed with 10 isolates, among them, one pyocyanin producer, three pyoverdin producers, one that produced both pigments and five bacteria without pigment production. The diluted medium, containing only the necessary amount of nutrients, was used to stimulate the competition for nutrients. This test was performed against Ec2, which was the most sensitive *E. coli* in the antagonism test in the solid medium.

According to Figure 1, after the interaction between the bacteria for a period of 72 h, the results confirmed the experiment carried out on the solid medium where the bacteria which produced pigment were the most effective ($P_{ANOVA,24h} = 0.0010$; $P_{ANOVA,48h} < 0.0001$; $P_{ANOVA,72h} < 0.0001$), especially after 48 h from the initiation of the experiment, when some results showed a difference of about four orders of magnitude, when compared with the strains that showed no pigment production, which achieved a maximum difference of two orders of magnitude.

Thus, while we revealed a significant positive correlation between the presence of pigments and antagonism, we also found cases in which strains of *P. aeruginosa* were lacking pigments, but still behaved antagonistically.

In a scenario proposed by Nicholson (1954), competition for a limited resource can be categorized into two groups: contest and scramble. Contest competition happens when direct and antagonistic interactions between competitors occur, with the 'champion' appropriating the resources. Scramble competition happens when competitors without direct interaction utilize the limited resources rapidly. Applying this simple scenario, *P. aeruginosa* with pigment(s) production is using contest competition to survive, whereas *P. aeruginosa* without pigment production is using scramble competition. In the first 24 h, apart from clinical isolates Pa23 and Pa01, it was not possible to detect a big difference in the growth between *P. aeruginosa* with and without pigment production. But after 48 h, bacteria growth with pigment production was larger, showing that the contest competition is more successful when compared to scramble competition. In other words, the production of pyocyanin (antibiotic) and pyoverdin (siderophore) made *P. aeruginosa*

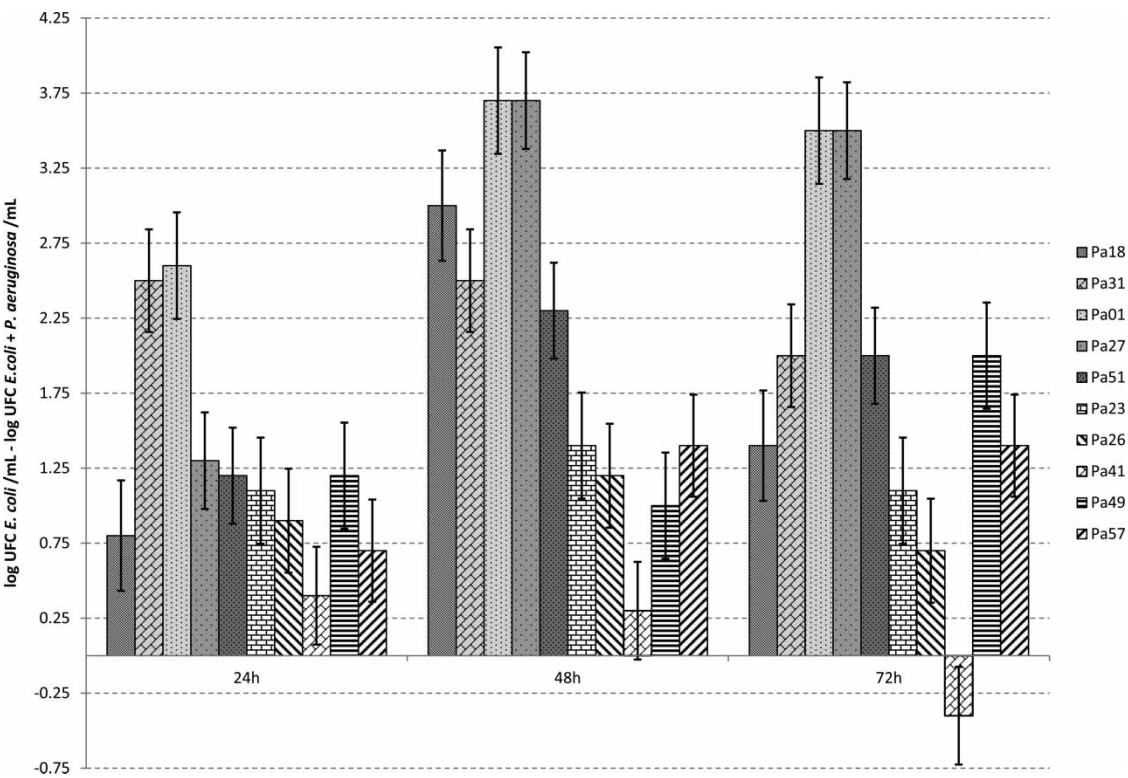

Figure 1 | Results of the antagonism test in liquid medium against Ec2 (PCN = Pa18; PCN and PVD = Pa 31; PVD = Pa01, Pa27 and Pa51; No pigment = Pa23, Pa26, Pa41, Pa49 and Pa57).

'stronger'. But if we look deep into the 'microbial jungle', the way a microorganism interacts and fights for nutrients is more complex. Hibbing *et al.* (2010) wrote a review about these ways of survival, discussing that they can range from antibiotics and siderophore production to stock of nutrients and *quorum sensing*.

The comparison between the antagonistic effect of pyoverdin and pyocyanin in the solid medium showed that there is a tendency for pyocyanin to be more important than pyoverdine for antagonism, as observed in Table 2. In this test, although the production of pigment was not quantified, it was observed visually that different quantities of pigment were produced. In the liquid medium, however, the correlation between antagonism and the type of pigment produced is not possible.

These results show that when *P. aeruginosa* is present in water samples, it can influence the growth of coliform bacteria, and during water analysis it can cause a misreading of the result, allowing in some cases for a water sample to be considered as safe to drink when actually it is unsafe and dangerous. Vaconcelos *et al.* (2010) emphasized that the presence of *P. aeruginosa* in a water sample is a problem and suggested that its presence should be investigated, together with the presence of fecal coliforms, improving the quality of water analysis. Our results support the Vaconcelos *et al.* (2010) statement.

The Australian Government published the 'Review of Coliforms as Microbial Indicators of Drinking Water Quality' (National Health and Medical Research Council 2003), in which it recommends to change the use of coliforms as microbial indicators of drinking water quality. In this review, some issues are discussed that the current analysis presents, such as the demonstration of whether the water treatment process is working effectively. To verify the success of the treatment after chlorination, the absence of total coliforms and *E. coli* are verified, but the problem is that coliforms are more sensitive to chlorination when compared with protozoan pathogens. Shrivastava *et al.* (2004) showed that in river samples containing *P. aeruginosa*, *E. coli*, *Klebsiella* spp., *Enterobacter* spp. and other bacterial species, after suboptimal chlorine treatment, all bacteria were killed except the multidrug-resistant *P. aeruginosa*. This illustrates a great problem because *P. aeruginosa* is an important nosocomial pathogen, so it can cause diseases, and it can present antagonism against *E. coli* and coliforms, causing an error in the microbial analysis.

CONCLUSIONS

This study shows that production of pyocyanin and pyoverdin by *P. aeruginosa* enhanced its antagonistic effect against coliform bacteria when compared with *P. aeruginosa* without pigment production. This ability can cause a mistake in water sample analysis, making it possible to consider water clear and safe when, actually, it is not.

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