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Artigo

COFFEE BREAKS THE JOURNEY OF PSEUDOMONADS: A PAUSE FOR A REFLECTION

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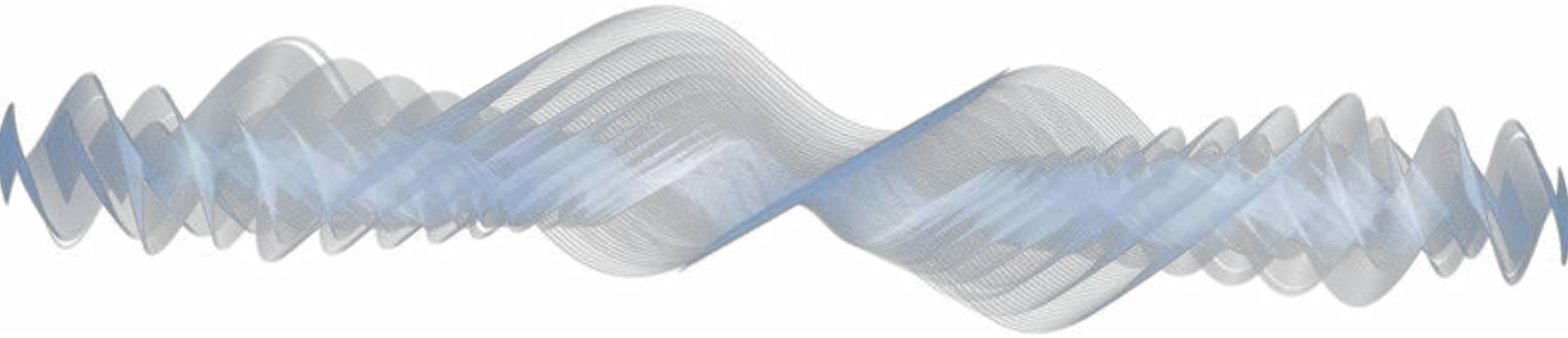

ABSTRACT

Pseudomonas aeruginosa is a critical pathogen according to the WHO list of priority bacteria. It is nutritionally versatile and expresses different virulence factors, which contribute to its resilience to environmental stresses as well as its tolerance and resistance to antimicrobials. These demands the search for strategies to combat microbial growth, a scenario in which natural bioactive compounds are being widely investigated. In this work, the activity of caffeine on the motility and biofilm adhesion to glass and plastic surfaces were tested with two strains of *P. aeruginosa*: TGC-04 (wild type) and ATCC 9027 (clinical strain). Both strains adhered weakly to the tested surfaces. The clinical strain showed a reduction in adhesion to plastic and glass of 46.9 and 65.0%, respectively, while the wild type strain was unaffected by caffeine (1024 µg/mL). In addition, there was inhibition in swarming and twitching motilities, and swimming motility was not affected. The results suggest that the strain origin may be an important factor in the susceptibility of *P. aeruginosa* to caffeine.

KEYWORDS: *Pseudomonas aeruginosa*. Caffeine. Motility. biofilm.

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RESUMO

Pseudomonas aeruginosa é um patógeno crítico de acordo com a lista de bactérias prioritárias da OMS. Ele é nutricionalmente versátil e expressa diferentes fatores de virulência, que contribuem para sua resiliência a estresses ambientais, bem como sua tolerância e resistência a antimicrobianos. Isso demanda a busca de estratégias para combater o crescimento microbiano, cenário em que compostos bioativos naturais estão sendo amplamente investigados. Neste trabalho, a atividade da cafeína sobre a motilidade e adesão de biofilme a superfícies de vidro e plástico foram testadas com duas linhagens de *P. aeruginosa*: TGC-04 (selvagem) e ATCC 9027 (padrão). Ambas aderiram fracamente às superfícies testadas. A linhagem padrão apresentou redução na adesão ao plástico e ao vidro de 46,9 e 65,0%, respectivamente, enquanto a linhagem selvagem não foi afetada pela cafeína (1024 µg/mL). Além disso, houve inibição em dois de três tipos de ensaios de motilidade testadas. Os resultados sugerem que a origem da linhagem pode ser um fator importante na suscetibilidade de *P. aeruginosa* à cafeína.

KEYWORDS: *Pseudomonas aeruginosa*. Cafeína. Motilidade. Biofilme.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, non-fermenting rod most prevalent in hospital environments, affecting bedridden patients. It is associated with several types of infections [1], as well as increased morbidity in patients affected by cystic fibrosis and immune system suppression [2]. *P. aeruginosa* also presents natural resistance to certain antimicrobials. This resistance can also be acquired or adaptive [3] due to its flexible metabolic capacity and the genome that encode a series of resistance genes [4]. In addition, *P. aeruginosa* is nutritionally versatile and can grow on humid surfaces [5].

The search for compounds with an anti-biofilm effect may be the key to new antibiotic therapies [6]. Natural biodegradable compounds are noted for being eco-friendly and sustainable [7]. While new compounds have been discovered, interest has turned to the past, bringing back to the scene some bioactive compounds prescribed in old antimicrobial therapies, however through other perspectives [8].

Caffeine (1,3,7-trimethylxanthine) is a natural alkaloid, present in seeds, leaves and fruits of several plant species [9-11]. It is used in beverages, foods and medicines [12]. Caffeine exhibits antibacterial [13], antifungal [14] and insecticidal [15] activities.

Concentrations ranging between 2000 and 8000 µg/mL of caffeine demonstrated action against enterobacteria [16], as well as other Gram-negative bacteria, including *Pseudomonas* spp. [17]. The mechanism of caffeine against Gram-negative bacteria occurs by changes in the permeability of the membrane, leading to cell lysis [18-20]. It is assumed that the mechanism of action of caffeine is by interference in the quorum sensing (QS) systems of pseudomonads, either by modifying the structure of AHL synthetases; by competitive/non-competitive binding the site of action of AHL synthetases, or by blocking receptor proteins [21-22]. When QS systems are inhibited or blocked, many virulence factors are not expressed [23]. The present work aimed to evaluate the antibiofilm and antimotility potential of caffeine against two strains of *P. aeruginosa* distinguished by origin.

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MATERIAL AND METHODS

Caffeine

Anhydrous caffeine, 99.9% pure, was used (Jilin Shulan Synthetic Pharmaceutical, Co. LTD, China). The standard solution was dissolved in sterilized distilled water, whose final concentration was 4128 µg/mL in order to obtain 2064 µg/mL in the first wheel to determine the Minimum Inhibitory Concentration (MIC) text [25].

Microorganisms

Two strains of *P. aeruginosa* were used: TGC-04, recovered from a soil contaminated by petroleum hydrocarbons (registration in SisGen nº A6D0C2F) and ATCC 9027, from clinical origin.

Determination of the antimicrobial activity of caffeine

Tests were carried out in triplicate to determine the caffeine Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), by using the microdilution technique (serial dilutions 1:2), in Müeller-Hinton broth. Caffeine concentrations ranged from 2064 to 8 µg/mL. The inoculum was prepared in a 0.85% NaCl solution, with turbidity standardized by tube No. 2 on the MacFarland scale and corresponded to 10% of the wells' volume [24]. After 24 h of incubation at 36±1 ºC, the MIC was defined as the lowest concentration of caffeine that inhibited microbial growth, verified by visual inspection of turbidity, compared to the control [25].

For MBC determination, drops of 1% resazurin solution were transferred into all wells and incubated for 2 h at room temperature (25±1 ºC). MBC was the lowest concentration of caffeine that caused the death of the inoculum, verified by the maintenance of the blue color. Otherwise, the blue solution changed to pink or colorless, indicating the presence of viable cells [26].

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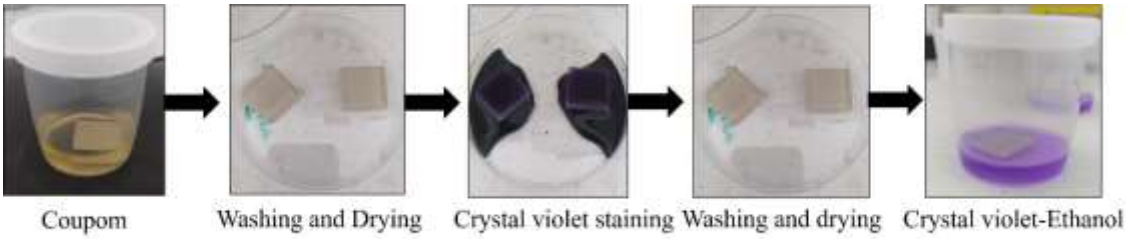
In vitro assays of biofilm formation

The test was performed in triplicate using the crystal violet technique [27]. Microcosms were prepared containing Müeller-Hinton broth, the caffeine solution (MIC and sub-MIC) and inoculum; in a ratio of 9:1:0.1 (Fig. 1). Two types of surfaces were tested by immersing 1 cm^2 polypropylene or glass coupons into the broth. The microcosms were incubated for 96 h at 28±1 ºC.

The percentage of biofilm inhibition was calculated by the difference between the mean absorbance of the crystal violet solution (control and treatment), divided by the absorbance of the control, with the result multiplied by 100. Cell adherence was classified as weak (≤ 40.00%), moderate (40.01-79.99%) or strong (≥ 80.00%) [28]. The absorbance at 590 nm of Müeller-Hinton broth was used to determine the cutoff value, demonstrating that absorbances three times this value indicated a cell with capacity to colonize biofilm [29].

Figure 1 – Crystal violet assay: the coupons are soaked in Müeller-Hinton broth, cleaned, stained, dried then placed in an ethanolic solution to determine absorbance



Motility Tests

Swimming, swarming, and twitching motility tests were performed as described by Rashid and Kornberg [30]. BHI broth and agar to which caffeine (MIC) had been added were used. The plates were incubated for 24-48 h at 28±1 ºC and then the diameter of the displacement zone was measured from the point of inoculation (cm).

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RESULTS

Inhibitory and bactericidal activity

The MIC and MBC concentrations in caffeine against both *P. aeruginosa* strains TGC-04 and ATCC 9027 was 2064 µg/mL.

In vitro assay of biofilm formation

Only the sub-MIC (1032 µg/mL) exerted activity on the biofilm formation of *P. aeruginosa* TGC-04 and ATCC 9027, which reduced the formation of the biofilm by 46.9±0.7 and 65.0±1.0%, respectively. Cells were moderately adhered (Table 2). The cutoff value was 0.533 (OD_{590}= 0.177). This meant that the cells did not lose their ability to form a biofilm.

Table 1 summarizes the test results with both plastic and glass coupons at the subinhibitory concentration (1024 µg/mL) of caffeine. On the polypropylene coupon, the biofilm was formed in the first 24 h of incubation, and in the subsequent 24 h, there was a significant reduction in the adherence of *P. aeruginosa* TGC-04 (≈ 65%). At 72 h up to 96 h, however, the adhered cellular content was increased (≈37%). As for the strain *P. aeruginosa* ATCC 9027, the inhibition lasted up to 72 h, when the biofilm grew again.

In the test using glass coupons, cell adherence increase was observed up to 96 h for *P. aeruginosa* TGC-04 strain (OD590 from ≈0.8 up to ≈2.0). Compared with the polypropylene coupon. This result suggested that the *P. aeruginosa* TGC-04's cellular wall was more hydrophilic than *P. aeruginosa* ATCC 9027 because glass is a hydrophilic material and suitable for the adhesion of hydrophilic cell walls, than plastic, a hydrophobic material. In addition, there was pyocyanin production, verified by the blue color diffused in the microcosms with which the wild type strain was incubated. On the other hand, *P. aeruginosa* ATCC 9027 suffered reduction in cell adhesion within 48 h (≈30%), with subsequent regrowth, as seen with TGC-04. The average reduction in cell adhesion, however, was around 26.8±0.2% and practically all the cells were weakly adhered throughout the test (Table 1). The cutoff in the glass coupon test was

0.864 (OD_{590}= 0.288), suggesting a possible hydrophobic nature of the *P. aeruginosa* ATCC 9027 cell wall.

Table 1 – Optical density (OD_{590}) and biofilm inhibition percentage of *P. aeruginosa* strains on plastic surface at 1024 µg/mL of caffeine (mean±standard error)

Strains	**Incubation (h)**				**%reduction**
	24	**48**	**72**	**96**	**(24-96h)**
Plastic					
TGC-04	2.179±0.061	0.767±0.047	0.726±0.007	1.156±0.119	46.900±0.700
Control	2.192±0.107	0.984±0.156	0.839±0.025	1.432±0.103	34.700±0.100
ATCC9027	2.076±0.044	1.212±0.581	0.631±0.180	0.727±0.461	65.000±1.000
Control	2.051±0.072	1.088±0.790	1.355±0.493	0.865±0.182	57.800±0.400
Glass					
TGC-04	0.828±0.001	0.868±0.171	1.814±0.126	1.940±0.042	---
Control	1.341±0.322	1.067±0.041	1.868±0.031	.810±0.143	---
ATCC9227	1.023±0.005	0.723±0.071	0.809±0.028	0.749±0.210	26.800±0.200
Control	0.574±0.150	0.538±0.021	0.639±0.055	0.701±0.029	---

Weakly adherent cells (≤40.000%) and moderately adherent cells (40.001-79.999%)

Although *P. aeruginosa* TGC-04 is suggested to be more hydrophilic than *P. aeruginosa* ATCC 9027, both strains were observed as hydrophobic in terms of the cell wall, because both adhered better to a plastic surface than to glass, a hydrophilic material. This may be seen in Table 2, where the values of the percentual of adherence are higher on the plastic surface than on the glass surface.

Motility tests

Table 3 summarizes the results of the motility tests. Figure 2 illustrates macroscopic aspects of some colonies during the motility test. The exposure to caffeine at MIC (2064 µg/mL) contributed to a 23.5% reduction in the swimming motility

of the *P. aeruginosa* TGC-04, however it did not inhibit and increased the flagellar movement of *P. aeruginosa* ATCC 9027.

For the swarming motility, there was an opposite result. Caffeine did not produce an inhibitory effect on flagellar movement in *P. aeruginosa* TGC-04, and a subtle reduction of 12.8% was observed in *P. aeruginosa* ATCC 9027. In addition, the percentages of reduction in the twitching motility were 27.3 and 8.0%, respectively, to TGC-04 and ATCC 9027 strains.

Table 2 – Percentage of adherence of strains to different substrates

Strains	**Time (h)**		
	24	**48**	**72**
Plastic			
TGC-04	0.6	13.5	22.1
ATCC 9027	1.3	10.2	53.4
Glass			
TGC-04	38.3	18.7	3.0
ATCC 9227	44.0	26.0	21.0

Table 3 – Motility zones (cm) of *Pseudomonas aeruginosa* TGC-04 and ATCC 9027 exposed to 2064 µg/mL of caffeine

Tests	**Control (TGC-04)**	**Treatment (TGC-04)**	**Control (ATCC 9027)**	**Treatment (ATCC 9027)**
Swimming	1.7±0.2	1.3±0.6	2.0±0.6	4.9±0.8
Swarming	1.3±0.3	4.0±1.0	4.7±1.2	4.1±1.7
Twitching	3.3±1.0	2.4±0.8	5.0±0.9	4.6±1.6

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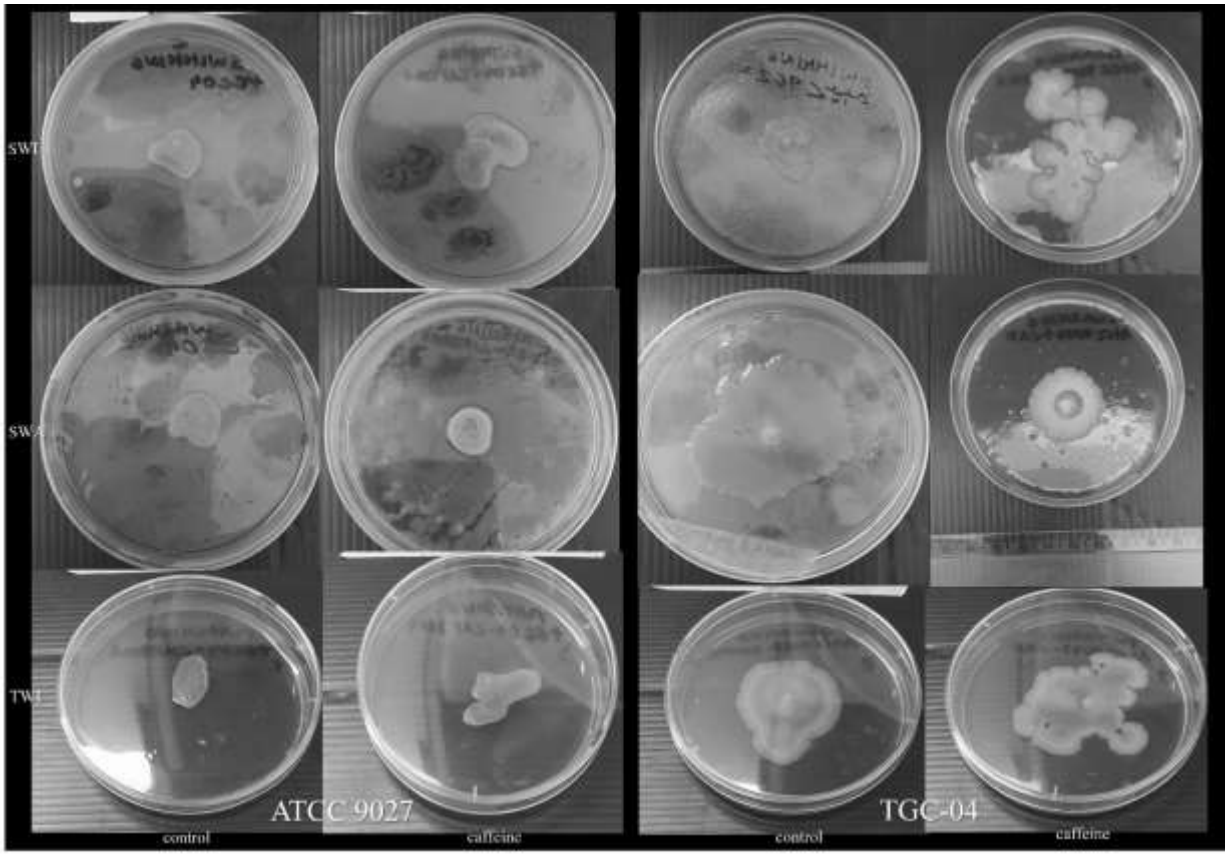
Figure 2 – Diameter of motility zones from *P. aeruginosa* strains in the presence of caffeine (left) and in the control (right)



SWI – swimming; SWA – swarming; TWI – twitching motilities

DISCUSSION

Activity on planktonic and sessile cells

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The present study aimed to identify whether caffeine has any inhibitory effect in two specimens of *P. aeruginosa*, both in planktonic and sessile state. In addition, the study assessed the effect of caffeine on the motility of these specimens. The inhibition of *P. aeruginosa* biofilms mediated by botanical extracts containing caffeine has already been observed prior to this study. Jadeja et al. (2020) [31] observed that 1700 µg/mL extracted from green tea (*Camellia sinensis*), inhibited the synthesis of pyocyanin, aiding the reduction of biofilm formation. *P. aeruginosa* ATCC 9027 was also sensitive when exposed to *Psidium cattlejanum* and *Butia odorata* leaf extracts, exhibiting MIC of 1560 and 3120 µg/mL respectively [32-33].

P. aeruginosa has some characteristics that confer greater resistance to antimicrobial agents, such as the formation of biofilms [34]. These responses are promoted through cell-to-cell communication [35], and virulence factors such as: adhesins, *pili*, flagella, and rhamnolipid synthesis [36]. In addition, it is not uncommon to find bacteria resistant to caffeine [37], which includes caffeine degraders [38].

Additionally, resistance to caffeine may also be related to the site of origin of any microbe exposed to an environment under highly recurrent stress, which favors gene exchange [39]. This premise has led to the use of the two specimens in this work, representing both, a wild and a clinical isolate, respectively. *P. aeruginosa* TGC-04 was recovered from a Brazilian gas station soil, contaminated by a highly concentration of fossil fuels [40]; and *P. aeruginosa* ATCC 9027 was isolated from an ear infection in the 1980s in the United States. This strain also produces low levels of virulence factors and is widely used in standardization tests [41]. Planktonic cells exposed to caffeine showed very high MIC values, agreeing with what has been previously reported regarding the action of caffeine against *P. aeruginosa* [17, 42].

Cell adhesion depends on characteristics related to the hydrophilicity and/or hydrophobicity of the material as well as the cell wall. Hydrophilic cell surfaces are characterized by a high C:N ratio, while hydrophobic surfaces have a high C:O ratio. Depending on the bacterial species, the stage of development and the characteristics of the environment, hydrophobicity is prevalent [43-44]. Furthermore, low concentrations of rhamnolipids increase the hydrophobicity of the cell wall in *P.*

aeruginosa, causing a release of lipopolysaccharide (LPS) from the cell surface [5]. In addition, caffeine is preferentially located in the hydrophobic moiety of the membrane [45]. This characteristic may disturb the adhesion process because caffeine molecules may give a more hydrophilic property to the cell.

Zhong [46] identified the *P. aeruginosa* ATCC 9027 strain as hydrophobic. Gram-negative bacteria are more hydrophobic microbes due to the presence of the lipid bilayer in the outer membrane and the lipid A core of LPS. They favor attachment, particularly on hydrophobic surfaces [47]. The surface of glass is acknowledged as more hydrophilic than plastic [48]. This may explain the fact that greater adhesion was observed on the plastic coupons. However, it is emphasized that moderate and weak adherence provide an ecological advantage for *P. aeruginosa*. By adhering in this way, *P. aeruginosa* may be successful in competition with other microorganisms, as it is able to migrate more easily after detaching from the biofilm [49].

Based on the cutoff values of the two strains, it is suggested that the cells did not lose the ability to form biofilm during the assays, indicating a temporary inhibitory effect of caffeine. Detachment of cells from the biofilm and migration to the aqueous medium may have happened during the test, given the chemical stress; this hypothesis was not confirmed, however, because viable planktonic cells were not quantified. Furthermore, the cells were confined in a static aqueous system, which may explain the return to the sessile state observed with increased cell adhesion, especially to the glass coupon.

Activity on motility

The motility of *P. aeruginosa* strains may vary according to the environment of origin. The study by Souza et al. (2019) [50] evaluated this feature in specimens isolated from clinical and wild environments. The authors verified that swimming motility is the type that varied the most varied; no difference was identified in swarming and there was a less significant difference in twitching motilities.

Swimming motility is associated with chemotaxis and hydrodynamic strength. Over certain surfaces, *P. aeruginosa* consolidates adherence using swarming and twitching motilities to bind. Swarming motility involves the initiation of adhesion and requires flagellar action, type IV *pili*, rhamnolipids, as well as specific bacterial density and nutrient availability [51]. Twitching motility is crucial for initiation of attachment because the type IV *pili* of planktonic cells interact with the surface in response to environmental signals, allowing aggregation [52].

Rossi et al. [53], investigating the motility capacity in 74 strains of *Pseudomonas* spp. isolated from dairy products, have verified that the microbe run, and tumble were not greater than 1 cm, relating this finding to the temperature used in the test, 30 °C, as well as to the polar monotrichous flagellum, present in *P. aeruginosa*. In contrast to what those authors observed, in the present study, the flagellar movements of the strains were significantly greater, especially for *P. aeruginosa* TGC-04, which is more adapted to environmental stress conditions, compared to *P. aeruginosa* ATCC 9027, previously described as less virulent.

The action of caffeine on the inhibition of motility is unclear. However, it is believed that the QS systems may be involved because the inhibition of QS systems regulation may interfere with motility, as well as cell-cell aggregation during adhesion [54]. To avoid cell damage, *P. aeruginosa* induces its virulence system in a multifactorial mean, involving the action of cAMP and mutations in regulatory genes, in a process that is still unclear [55]. This confers some independence in regulation among the three modalities of bacterial motility, while implying that the fewer the changes in twitching motility, the greater virulence the strain demonstrates [56].

CONCLUSION

Under the conditions employed in this study, caffeine was shown as an important bioactive compound, in terms of antimicrobial activity, cell adhesion and motility inhibition, however particular and specific phenotypes seem to influence the

response to stress caused by caffeine. Future studies may clarify the mechanisms by which these events may occur and be correlated to the origin of *P. aeruginosa* isolates.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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