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Biofilm Formation in *Bacillus cereus*, *B. licheniformis* and *B. pumilus*: An Alternative for Survival in Impacted Environments

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Resumo: *Bacillus* é um gênero de bactérias Gram-positivas que se apresentam em forma de bastonetes, podendo ser aeróbios facultativos e resistentes a condições de estresse no meio ambiente. A diversidade fisiológica das formas vegetativas desse gênero faz com que as espécies sejam consideradas micro-organismos ubíquos, podendo ser isoladas do solo, de água doce e marinha, bem como de alimentos. No presente estudo, isolados de três espécies (*B. cereus, B. licheniformis* e *B. pumilus*), obtidos de um ambiente impactado (Riacho do Cavouco, Recife, Brasil), foram investigados quanto ao perfil de susceptibilidade/resistência a antibióticos por meio de antibiograma (difusão em disco), hidrofobicidade celular pelo método de ligação a hidrocarboneto e capacidade de formação de biofilme em diferentes meios de cultura pelo método cristal violeta. *B. cereus* foi a espécie que apresentou maior resistência (nove antimicrobianos), seguido de *B. pumilus* (dois) e *B. licheniformis* (um). Os isolados foram formadores de biofilme com maior formação nos meios suplementados com glicose a 1% e todos foram hidrofóbicos. Este trabalho é um indicativo de que *B. cereus, B. licheniformis* e *B. pumilus* parecem possuir mecanismos de resistência distintos e não diretamente relacionados à capacidade de formação de biofilme. Estudos adicionais são necessários para melhor compreender a dinâmica de sobrevivência dessas espécies em ambientes impactados.

Palavras-chave: suscetibilidade, hidrofobicidade celular, Gram-positivos

Abstract: *Bacillus* is a genus of Gram-positive and rod-shaped bacteria that may be facultative anaerobes and resistant to stress conditions in the environment. In view of the physiological diversity of the vegetative forms of this genus, the species are considered as ubiquitous microorganisms, being isolated from soil, freshwater and seawater, as well as food. In the present study, isolates of three species (*B. cereus, B. licheniformis* and *B. pumilus*), obtained from an impacted environment (Cavouco stream, Recife, Brazil), were investigated for the susceptibility/resistance profile toward antibiotics by antibiogram (disk diffusion), hydrophobicity by the hydrocarbon-binding method, and ability to form biofilm in different culture media by the crystal violet method. *B. cereus* was the species with the highest resistance (nine antimicrobials), followed by *B. pumilus* (two) and *B. licheniformis* (one). The isolates were biofilm formers, with higher formation in media supplemented with 1% glucose, and all were hydrophobic. This work is an indication that *B. cereus, B. licheniformis* and *B. pumilus* appear to possess distinct resistance mechanisms that are not directly related to biofilm formation ability. Further studies are needed to understand better the dynamics of survival of these species in impacted environments.

Keywords: susceptibility, cellular hydrophobicity, Gram-positive

1. Introduction

Water is considered an essential natural resource for the maintenance of life. However, the inadequate disposal of large amounts of polluting waste in water bodies has contributed to the scarcity of this resource and has compromised the relationships between living organisms. The urban dejects that are launched in aquatic environments attract decomposing

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microorganisms, causing ecological imbalances by reducing the oxygen present in the water as well as changes in the dynamics of these ecosystems (Freitas and Gaudio, 2015).

The contamination of water bodies by resistant bacteria pathogenic to humans and other animals is another problem that affects these environments (Nascimento and Araújo, 2013; Berglund, 2015). According to the World Health Organization (2016), approximately a half of the population living at the developing world will be affected by infections directly related to water that is out of quality standards and due to inadequate or non-existent sanitation.

The Cavouco stream is a tributary of the Capibaribe River, which is one of the main rivers in the Pernambuco state, Brazil. Some works have been carried out in this environment in order to diagnose and monitor the environmental impact. According to Araújo and Oliveira (2013), this stream receives polluting loads of chemical and domestic waste, which compromises the quality of its water. Purificação-Junior et al. (2017), analyzing the microbiota of this environment, detected the presence of pathogenic Gram-positive and Gram-negative isolates, resulting a risk of disease transmission.

One of these isolates previously identified corresponds to the genus *Bacillus*. Aerobic or facultative anaerobic, catalase-positive and spore-producing Gram-positive bacteria represent this genus, which may be present in both aquatic and terrestrial environments. It includes species of ecological importance, such as *B. thuringiensis* (Raymond and Federici, 2017) and medical relevance, for example, *B. antracis* (Banada et al., 2017) and *B. cereus* (Owusu-Kwarteng et al., 2017). This genus also includes *B. pumilus* and *B. licheniformis*, being the last increasingly recognized as a human pathogen, causing serious infections in immunocompromised patients, food poisoning and ocular infections (Mohapatra and La Dut, 2012). For *B. pumilus*, there are some reports of its action as a human pathogen (Shivamurthy et al., 2016), being reported in cases of sepsis of newborns and immunocompromised individuals, in central venous catheters and causing cutaneous infections (Kimouli, 2012).

The present work aimed to investigate the antibiotic susceptibility/resistance profile and biofilm formation ability of isolates belonging to three species of the genus *Bacillus* collected from the Cavouco stream, an impacted aquatic environment.

2. Methodology

2.1 Biological Material and Culture Conditions

Three Gram-positive isolates of the genus *Bacillus* (*B. cereus*, *B. licheniformis* and *B. pumilus*), previously obtained and identified by Purificação-Júnior et al. (2017), were investigated. The isolates were kept in glycerol (15%) at -80 °C and reactivated for the assays in Brain Heart Infusion (BHI) medium at 37 °C for 24 h. An ATCC strain of *Staphylococcus aureus* (UFPEDA 02) was used as reference control.

2.2 Susceptibility/Resistance Profile

The isolates were tested for susceptibility to 18 antimicrobial agents: Penicillins (Ampicillin - AMP - 10 µg, Oxacillin - OXA - 1 µg), Cephalosporins (Cefazolin - CFZ - 30 µg, Cephalotin - CFU - 30 µg, Cefoxitin - CFO - 30 µg, Cefuroxime - CRX - 30 µg, Cefotaxime - CTX 30 µg, Cefepime - CPM - 30 µg), Carbapenems (Imipenem - IPM - 10 µg, Meropenem - MER - 10 µg); Aminoglicosides (Gentamycin - GEN - 10 µg, Amicacin - AMI - 30 µg); Lincosamides (Clindamycin - CLI - 2 µg); Fluoroquinolones (Ciprofloxacin - CIP - 5 µg); Afenicols (Chloramphenicol - CLO - 30 µg); Tetracyclines (Tetracycline - TET - 30 µg) and others (Nitrofurantoin - NIT - 300 µg, Trimethoprim - TRI - 5 µg). The profiles were determined by the disk diffusion method according to the recommendations of the Clinical and Laboratory Standards Institute - CLSI 2015).

2.3 Biofilm Formation

To evaluate the potential for biofilm formation, we used the using the crystal violet method described by Stepanovic et al. (2007), performed in microtiter plates. Three culture media were evaluated: Luria Bertani Miller (LB Miller), Tryptose Soy Broth (TSB) and BHI, all supplemented or not with 1% (w/v) glucose. From the readings of optical density (OD), the mean absorbance values of each sample (OD_s) were determined in comparison with the absorbance of the sterility control (OD_c). The isolates were classified as strong ($4 \times OD_c < OD_s$), moderate ($2 \times OD_c < OD_s \leq 4 \times OD_c$) or weak ($OD_c < OD_s \leq 2 \times OD_c$) biofilm formers. The isolates presenting values of absorbance equal to or less than the control were classified as non-biofilm producers.

2.4 Cellular Hydrophobicity Profile

Cell surface hydrophobicity (CSH) was determined based on the hydrocarbon-binding method described by Tendolkar et al. (2004) with adaptations. The bacterial isolates grown in BHI broth at 37 °C for 18 h were transferred to microtubes and centrifuged for 10 min at 7,000 rpm. The supernatant was discarded and the pellet resuspended in PUM buffer (Trihydrate and Monobasic Potassium Phosphate, Urea and Heptahydrate Magnesium Sulphate), adjusted to 0.5

and the initial reading (OD_i) obtained at 600 nm. Subsequently, the hydrocarbon para-xylene was added to the bacterial suspensions, followed by vortexing for 2 min. After separation of the phases at room temperature, the final reading at 600 nm (OD_f) of the lower phase of each microtube was performed. To determine CSH (%), the following formula was used: CSH (%) = (OD_i - ODf) / OD_i × 100. Bacteria that had CSH (%) lower than 30% were considered hydrophilic and CSH higher than 70% were hydrophobic. Samples that presented CSH between 30% and 70% were classified as moderately hydrophobic.

3. Results and Discussion

The antibiotic resistance/susceptibility profiles of the three isolates of the genus *Bacillus* were evaluated and are shown in Table 1. *B. cereus* was resistant to nine antimicrobials (ampicillin, oxacillin, cefazolin, cephalothin, cefoxitin, cefuroxime, cefotaxime, cefepime and trimethoprim) from 18 tested. *B. lichenformis* presented resistance only to cefotaxime and *B. pumilus* to cefotaxime, in addition to intermediate susceptibility to cefepime. The ATCC was sensitive to all antimicrobials. The high resistance index observed for *B. cereus* has been also reported in the literature (Owusu-Kwarteng et al., 2017). However, for *B. licheniformes* and *B. pumilus*, low pathogenic potential and antimicrobial resistance are reported (Celandroni et al., 2016).

All isolates were resistant to cefotaxime, a third generation cephalosporin of the beta-lactam group. Resistance to this group of antimicrobials has been reported frequently in recent years (Chong et al., 2015; Ghafourian et al., 2015). For Gram-negative bacteria, the resistance is due to the synthesis of several enzymes such as extended spectrum beta-lactamases (ESBLs), which are capable of breaking the beta-lactam ring (Ghafourian et al., 2015). Recent studies have demonstrated the resistance of Gram-positive isolates such as *Enterococcus faecium* and *E. faecalis* to different classes of antimicrobials (Adesida et al., 2017). Similar results were reported for *S. aureus* isolates, which showed increasing resistance to penicillins, cephalosporins, aminoglycosides, tetracyclines and clindamycin (Lobova et al., 2015; Nicolau and Silberg, 2017).

In the present study, *Bacillus cereus* isolate stood out because it was resistant to all tested penicillins and cephalosporins, including third and fourth generation. This result is worrying because indicates resistance even to the last generations antibiotics, which are usually prescribed in the case of infections caused by resistant strains Gram-negative. In addition to resistance to two classes of beta-lactam antibiotics, this isolate also showed resistance to trimethoprim, a folic acid antagonist that prevents bacterial replication; thus, it can be considered a multi-resistant microorganism (Fernandes et al., 2013; Guimarães; Momesso and Pupo, 2010). However, it is worth mentioning that resistance to different classes of antibiotics depends on the strain obtained and the environment from which the microorganism was obtained (Bottone et al., 2010). Another point that deserves attention is the mechanism of sporulation that the species of the genus *Bacillus* present. This mechanism, together with other factors such as biofilm formation, may contribute to the permanence and dissemination of these bacteria in different environments, increasing their survival capacity (Logan et al., 2009).

Table 1: General characterization of the *B. cereus*, *B. licheniformis* and *B. pumilus* isolates.

Isolates	Resistance profile	Hydrophobicity	Biofilm	
			Without glucose	With glucose
ATCC *S. aureus*	-	Hydrophilic	MO/ST former	ST former
B. cereus	AMP, OXA, CFZ, CFO,CFL, CTX, CRX CPM, TRI	Hydrophobic	MO/ST former	ST former
B. licheniformis	CTX	MO Hydrophobic	MO former	MO/ST former
B. pumilus	CTX, CPM	MO Hydrophobic	MO former	MO/ST former

MO: moderate. ST: strong.

The cellular hydrophobicity profile and biofilm formation ability were also evaluated. *B. cereus* isolate presented a hydrophobicity index equal to 70%, and thus was classified as hydrophobic. The isolates of the other species were moderately hydrophobic except the ATCC, which was hydrophilic (Figure 1). Hydrophobicity is one of the physicochemical factors that influence the process of microbial adhesion on different substrates. In general, the currently existing methods can measure the

interaction between cells and a hydrophobic or hydrophilic material (Wang; Lee and Dykes, 2014). In this study, *B. cereus* presented a hydrophobic cell surface and strongly produced biofilm, corroborating with other studies showing that as higher the hydrophobicity index of the bacterial cell greater is the capacity for biofilm formation (Rodrigues et al., 2009; Trentin et al., 2014). However, the ATCC was hydrophilic and formed biofilm. Czerwonka et al. (2016), when investigating *Proteus mirabilis* isolates, demonstrated that those with high biofilm production capacity were hydrophilic. Thus, there seems to be no direct correlation between cell surface hydrophobicity and biofilm formation, being these characteristics specific for each isolate.

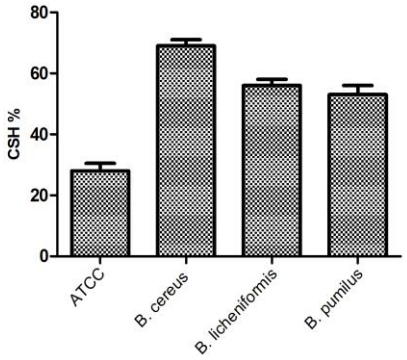

Figure 1: Hydrophobicity profile of *B. cereus, B. licheniformis, B. pumilus* isolates and ATCC.

Concerning biofilm formation, it was possible to verify that all the species formed biofilm in the different media tested, with better results for those supplemented with glucose (Figure 2).

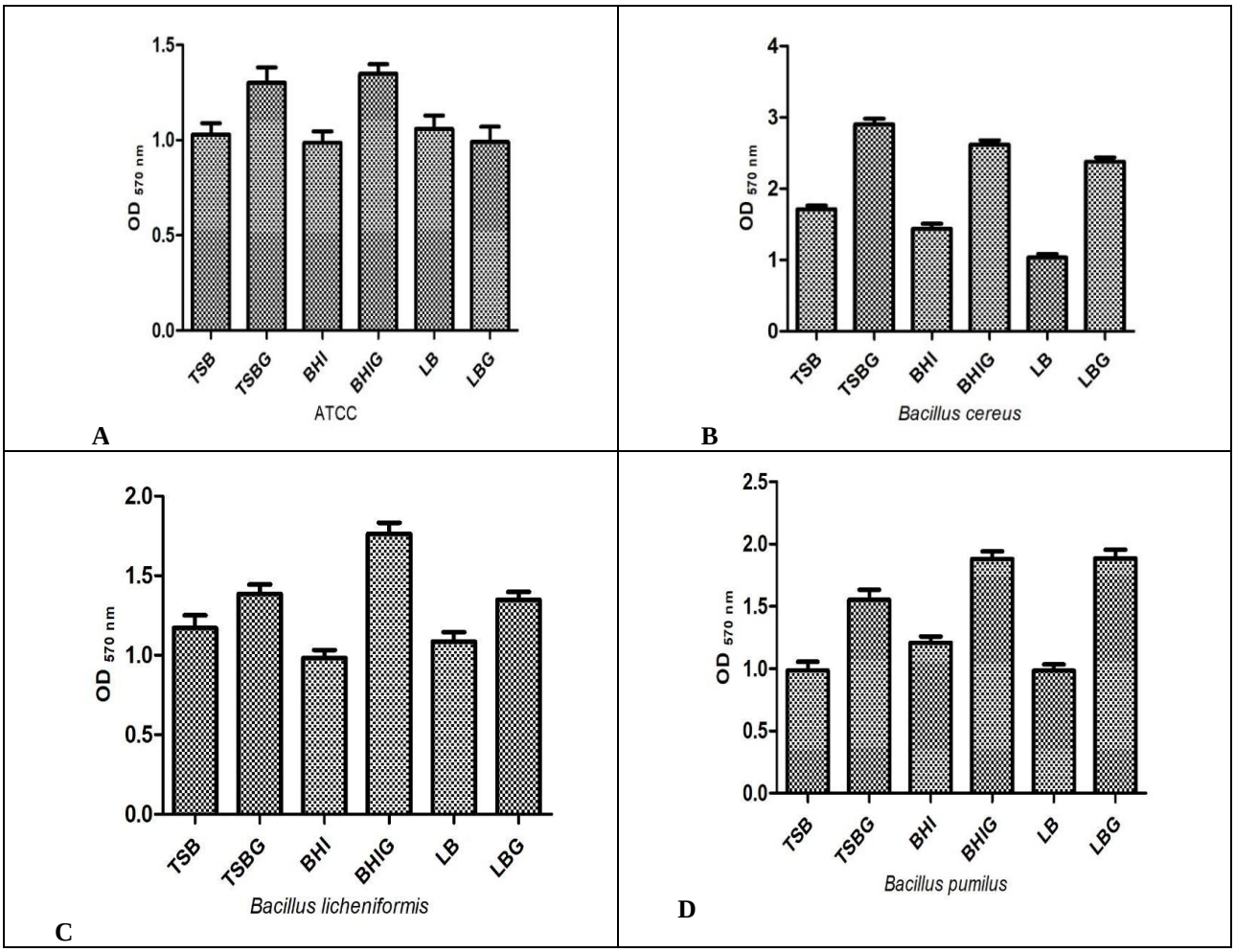

Figure 2: Biofilm forming ability of *B. cereus*, *B. licheniformis*, *B. pumilus* isolates and ATCC in different culture media: Luria Bertani Miller (LB), Tryptose Soy Broth (TSB) and Brain Heart Infusion (BHI), without or with 1% (w/v) glucose (G).

The adhesion of bacteria to a surface – biotic or abiotic – is the first step of biofilm formation. Some culture media such as TSB and BHI, supplemented or not, are preferred for evaluation of biofilm formation. Rodrigues et al. (2009) in a study with *Salmonella* spp evaluated the ability of biofilm formation in TSB medium without and with addition of glucose at 0.5%, 1.0% and 1.5% and observed that the addition of this sugar did not increase the biofilm formation. Similarly, Moliva et al. (2017), evaluating the biofilm formation ability of *Streptococcus uberis* also in TSB medium supplemented with 0.5% glucose and 0.5% or 5% lactose, also did not notice an increase in the formation. Although supplementation of culture media was apparently not a determining factor for biofilm formation in *Salmonella* and *Streptococcus*, this supplementation was favorable in the genus *Bacillus*. In the present study, all species investigated had a higher biofilm formation capacity in the supplemented media.

The ability of adhesion on immersed abiotic surfaces or even on living tissue as well as the biofilm formation ability may contribute to colonization of *B. cereus* in different environments. The spores produced by this bacterium confer tolerance to various kind of stresses and a high adhesive capacity on several substrates, including materials composed of steel, which are widely used in the food industry (Majed et al., 2016). In aquatic surfaces, isolates from this species showed less adhesion power but were strong biofilm formers (Kurinčić, et al., 2016). In the present work, the isolates investigated were collected from aquatic environment and presented both good adhesion and biofilm formation abilities, showing that these characteristics are specific for each isolate.

For *B. licheniformis*, a study performed by Zain et al. (2017) detected biofilm formation in TSB supplemented with lactose and artificial minerals at 1%, 5% and 20%, demonstrating better adhesion and thermo-resistance at higher temperatures on metal surfaces. For *B. pumilus*, Bae et al. (2014) evaluated isolates obtained from food industry equipment and found a moderate biofilm formation in moderately hydrophobic isolates.

In the present study, *B. pumilus* and *B. licheniformis* were biofilm-forming agents in media supplemented or not with glucose and these species were moderately hydrophobic. These data demonstrate that these species, as well as *B. cereus*, share common strategies for survival and permanence in impacted aquatic environments despite developing distinct resistance mechanisms.

4. **Conclusion**

The present study demonstrates the specificities of the species investigated in relation to the resistance profile and indicates the influence of the mechanisms of sporulation and biofilm formation in the survival and permanence of these microorganisms under adverse conditions.

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