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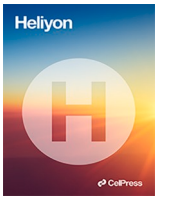

Research article

Assessment of antimicrobial resistance, biofilm formation, and surface modification potential in hospital strains of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

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A B S T R A C T

The occurrence of healthcare-associated infections is a multifactorial phenomenon related to hospital space contamination by bacteria. The ESKAPE group, specifically *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, play a relevant role in the occurrence of these infections. Therefore, comprehensive research is needed to identify characteristics that justify the prevalence of these species in the healthcare environment. In this line, the study aimed to determine the antimicrobial resistance, biofilm formation, and the potential for polymer degradation in a collection of 33 *P. aeruginosa* strains and 2 *K. pneumoniae* strains sampled from various equipment and non-critical surfaces in a Portuguese hospital. Antimicrobial susceptibility tests revealed that none of the strains was categorized as multidrug-resistant (non-MDR). An assessment of their biofilm-forming capabilities indicated that 97 % of the strains exhibited biofilm-producing characteristics. Notably, within this group, the majority of *P. aeruginosa* and half of *K. pneumoniae* strains were classified as strong biofilm producers. Furthermore, the strains were evaluated for their potential to cause damage or change medical devices, namely infusion sets, nasal cannula, and urinary catheters. Three *P. aeruginosa* strains, two strong and one moderate biofilm producers, showed the highest ability to modify surfaces of the nasal cannula and infusion sets. Additionally, the Chi-square test revealed a statistically significant relationship between the presence of *P. aeruginosa* strains and the water accession spots. In conclusion, this work suggests that bacteria from this group hold a significant ability to grow in the healthcare environment through the degradation of non-critical materials. This suggests a potential concern for the persistence and proliferation of these organisms in hospital environments, emphasizing the importance of robust infection control measures to mitigate the risks associated with bacterial growth on such surfaces.

1. Introduction

Pseudomonas aeruginosa and *Klebsiella pneumoniae* belong to the ESKAPE pathogens group, which includes *Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species. These bacteria are recognized as the main agents behind nosocomial infections [1–3]. *P. aeruginosa* is an aerobic gram-negative bacterium commonly found in the environment, responsible for severe respiratory infections, especially in immunocompromised patients [3–9]. *K. pneumoniae* strains can cause pneumonia, urinary tract infections, and bloodstream infections [3,10–12]. In Portugal, in 2020, approximately 11 % of P. *aeruginosa* strains and 24 % of *K. pneumoniae* strains exhibited a multidrug resistance profile [13]. Infections caused by these microbial agents can be attributed to both exogenous and endogenous sources. These microorganisms are often transmitted through direct or indirect contact involving patients, healthcare personnel, contaminated objects, visitors, and even several environmental reservoirs [2].

Of particular concern, inanimate surfaces within the hospital environment play a critical role in the emergence and propagation of

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nosocomial infections [14]. Studies have reported the significant presence of bacteria on surfaces such as bed rails, floors, gloves, and faucet handles in patient rooms [15,16]. Furthermore, water has been identified as a reservoir for environmental bacteria, particularly *P. aeruginosa* strains [17]. Consequently, the extensive surface area of faucets and pipes is recognized as an ideal environment for the growth of microorganisms, the development of biofilms, and, consequently, the potential harbouring of pathogens like *P. aeruginosa* [18].

The colonization of hospital space by strains of *P. aeruginosa* and *K. pneumoniae* involves a multitude of factors. Among these, their antibiotic resistance and biofilm-producing capabilities are essential contributors to their persistence on non-critical surfaces within the hospital environment. This persistence, in turn, makes the action of biocides ineffective [19], facilitating ongoing cross-contaminations via the hands of healthcare staff [20].

The main objective of this work was to study a group of *P. aeruginosa* and *K. pneumoniae* strains previously isolated from hospital surfaces and non-critical materials. The study was designed to evaluate these strains for their antibiotic resistance profiles, ability to produce biofilms, and potential to degrade the polymeric materials used to produce medical devices, including infusion sets, nasal cannulas, and urinary catheters. Knowing the bacterial antibiotic resistance and the ability to use polymeric materials to grow will contribute to the understanding of the persistence of these pathogenic bacteria in healthcare environments, which is essential for assessing their potential threat to human health.

2. Materials and methods

2.1. Strains used in this study

A total of 35 bacterial strains were used in the present study, 33 *P. aeruginosa* and 2 *K. pneumoniae* strains (Table 1). These bacterial strains were isolated from surfaces and equipment of 3 different wards of a hospital in the Center of Portugal. These strains correspond to the totality of *P. aeruginosa* and *K. pneumoniae* strains recovered from the sampling works that occurred monthly from October 2019 to January 2020 included in a survey for the prevalence of ESKAPE group organisms in the hospital environment. The isolates obtained from specific culture media designed for the isolation of *Pseudomonas* and *Klebsiella* strains, *Pseudomonas* Isolation Agar (PIA) and *Klebsiella* Selective Agar (KSA), respectively, were distinguished based on their Random Amplified Polymorphic DNA Polymerase Chain Reaction (RAPD-PCR) profiles. Isolates displaying unique band patterns were subsequently selected for further identification by

Table 1
Selected strains used in the present study.

STRAIN	SAMPLING PLACE	NURSING
***P. aeruginosa* 118Pa**	WC shower inner part	cardiology
***P. aeruginosa* 133 Pb**	WC shower inner part	pneumology
***P. aeruginosa* 140Ka**	WC hose inner part	pneumology
***P. aeruginosa* 140Kd**		
***P. aeruginosa* 140P bf**		
***P. aeruginosa* 140b pol**		
***P. aeruginosa* 152 pol**	WC hose inner part	hematology
***P. aeruginosa* 218P pol**	WC hose inner part	cardiology
***P. aeruginosa* 227P pol**	WC shower inner part	cardiology
***P. aeruginosa* 244Pa**	WC shower inner part	pneumology
***P. aeruginosa* 244P pol**		
***P. aeruginosa* 247P pol**	WC hose inner part	pneumology
***P. aeruginosa* 266 Pb pol**	WC hose inner part	hematology
***P. aeruginosa* 266Pi′**		
***P. aeruginosa* 266Pa pol**		
***P. aeruginosa* 266Pa′**		
***P. aeruginosa* 326Pa**	WC wall	hematology
***P. aeruginosa* 335Pa bf**	WC shower inner part	pneumology
***P. aeruginosa* 337Ka**	nursing bench	pneumology
***P. aeruginosa* 349Pa bf**	WC shower inner part	hematology
***P. aeruginosa* 364Pa**	WC hose inner part	hematology
***P. aeruginosa* 364pol**		
***P. aeruginosa* 410 Pb**	WC hose inner part	cardiology
***P. aeruginosa* 410Pa**		
***P. aeruginosa* 417P**	WC shower inner part	cardiology
***P. aeruginosa* 417Ppol**		
***P. aeruginosa* 427Pa**	WC sink's drain	cardiology
***P. aeruginosa* 440Pa**	WC shower inner part	pneumology
***P. aeruginosa* 440Pi**		
***P. aeruginosa* 440P pol**		
***P. aeruginosa* 443P**	WC shower	pneumology
***P. aeruginosa* 454Pa**	WC shower inner part	hematology
***P. aeruginosa* 462P pol**	WC hose inner part	hematology
***K. pneumoniae* 104Ke**	WC sink's drain	cardiology
***K. pneumoniae* 337Ka**	WC chair	hematology

16S rRNA gene sequencing. These strains were cryopreserved in the Bacterial Culture Collection of the University of Coimbra (UCCCB) at −80 °C in Luria Bertani (LB) medium with 15 % glycerol.

2.2. Antimicrobial susceptibility tests

Following standard operating procedures, antimicrobial susceptibility tests were performed using the Kirby-Bauer disk diffusion method [21,22]. Briefly, bacterial suspensions of each tested strain were prepared in 5 mL of 0.85 % saline solution at 0.5 of the McFarland scale, corresponding to $1.4x10^8$ cells/ml. A volume of 0.1 mL from these suspensions was spread onto Mueller-Hinton agar (Oxoid, Hampshire, England). On the medium surface were placed disks of the antibiotics, levofloxacin (5 μg), ciprofloxacin (5 μg), ofloxacin (5 μg), amikacin (30 μg), tobramycin (10 μg), gentamicin (10 μg), netilmicin (10 μg), imipenem (10 μg), meropenem (10 μg), cefepime (30 μg), ceftazidime (10 μg), polymyxin B (50 μg). While ofloxacin was exclusively used in the assay with *K. pneumoniae* strains, netilmicin and polymyxin B were used to assay *P. aeruginosa* strains [6,21]. Petri dishes were incubated at 37 °C for 24 h. Then, the bacterial inhibition areas were measured, and the results were interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [21] and the Clinical and Laboratory Standards Institute (CLSI) [22]. *P. aeruginosa* DSM 1117 and *Escherichia coli* ATCC 25922 were used as control group reference strains for *P. aeruginosa* and *K. pneumoniae* [21], respectively.

2.3. Biofilm production assay

The capacity of *P. aeruginosa* and *K. pneumoniae* strains to form biofilm was evaluated using a microtiter plate assay [6,23]. Cells of *P. aeruginosa* and *K. pneumoniae* grown on LB solid medium were used to inoculate 3 ml of phosphate-buffered saline (PBS, pH 7.4) solution at 0.5 of McFarland scale, corresponding to $1.4x10^8$ cells/ml. From each bacterial suspension, a tenfold dilution was performed using tryptone soy broth medium (TSB).

The suspensions were placed into 96-well microtiter plates with a volume of 0.2 ml per well and incubated at 37 °C for 48 h. TSB medium without cells was used as a negative control. After incubation, the optical density (OD_{600nm}) of assays was measured using a spectrophotometer plate reader (TECAN, Infinite M200).

To quantify the biofilm formation, the bacterial growths were discarded without perturbing the biofilm present in the wells. Biofilm was washed three times with 200 μl of deionized water to remove planktonic cells, not adhered to plate wells. Then, 200 μl of 0.1 % crystal violet solution (CV) was added to stain the cells. After 15 min of staining, the CV was discarded, and the wells were washed three times with 200 μl of deionized water. The well content was resuspended using 250 μl of acetic acid 30 % for 15 min. Finally, the absorbance at 570 nm was measured with a microtiter plate reader [6,23,24]. Biofilm assays for each strain were conducted with a minimum of two biological replicates and each one was performed at least in six technical replicates.

The results were interpreted considering the cut-off point between the ODs averages of negative control (ODc) and the strains tested (OD). The strains were classified as non-biofilm producers ($OD \leq ODc$), weak ($ODc < OD \leq 2xODc$), moderate ($2xODc < OD \leq 4xODc$) and strong biofilm producers ($4xODc < OD$) [2].

2.4. Polymeric surface modification assay

The potential of strains to degrade or modify polymeric material used in medical devices was assessed by analysing bacterial growth in the presence of different polymeric materials used as a sole carbon source. Specifically, three commonly used medical devices in hospital settings were selected for assay: infusion set, nasal cannula, and urinary catheter. These devices are composed of distinct materials as follows: i) infusion set is primarily constructed from polyvinyl chloride (PVC); ii) nasal cannula is a composite of PVC and di(2-ethylhexyl) phthalate (DEHP); iii) urinary catheter is constructed from latex and coated with silicone. Samples of each of these materials measuring 0.8 cm^2 were prepared, washed in 70 % alcohol for 10 min and air-dried for 30 min at room temperature [25]. This meticulous preparation process was intended to eliminate any potential contaminants and ensure the suitability of the materials for further testing.

The carbon-free basal medium was prepared by dissolving in 1 L of deionized water, 0.06 g Tris (pH 7,2–7,3), 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2 g $MgCl_2.6H_2O$, 0.03 g $CaCl_2.2H_2O$, 0.23 g $Na_2HPO_4.12H_2O$. For the inoculum, bacterial suspensions were thoughtfully prepared using a 0.85 % saline solution. The growth assays were carried out in 12 multiwell plates. Each well contained 3 ml of the carbon-free basal medium, along with two pieces of polymeric material. The initial bacterial OD_{600nm} was adjusted to 0.08, corresponding to a cellular concentration of $1.5x10^8$ cells/ml, for consistency and accuracy in the experiments [26]. Additionally, wells only containing carbon-free medium with each type of polymeric material (negative control), and inoculated media without the presence of materials were also prepared as a positive control. Each assay was performed at least in duplicate. Subsequently, the multiwell plates were placed in an incubator, with stirring at 130 rpm, and maintained at a temperature of 37 °C for 48 h. Optical density measurements at 600 nm were taken at two time points: 24 h and 48 h into the incubation period. These readings were used to assess the bacteria's ability to utilize the carbon provided by the medical material for their growth, thereby allowing for an in-depth analysis of their carbon source utilization.

The strains exhibiting the highest ODs in the previous test were chosen for further validation of their potential in polymer biodegradation or modification. In these subsequent assays, the inoculum from each selected strain was distributed in duplicate into 50 ml conical tubes, each containing 10 ml of the carbon-free basal medium and pieces of the respective polymeric material. As before, both negative and positive controls were performed to ensure the integrity of the results. The tubes were then placed in an incubator at 130 rpm, with continuous stirring, and maintained at a temperature of 37 °C for 360 h. Throughout this incubation period, the OD_{600nm}

was regularly measured at specific intervals, including 96, 168, 264, and 360 h.

2.5. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM, Veja3 from Tescan GmbH) was employed to confirm the colonization of the surface of the tested medical devices by *P. aeruginosa* strains and to identify potential surface modifications caused by bacterial activity, which could be indicative of the initial stages of polymer degradation. Thus, the polymeric materials, from the *P. aeruginosa* growth assays that have revealed the highest ODs, were fixed with glutaraldehyde 2.5 % for 15 min, dehydrated with ethanol solutions 70–100 % and dried at room temperature [27]. Before observations, the samples were sputtered coated with a thin film of 10 nm of gold to eliminate the charging effect. Each sample was observed in three different areas with an accelerating voltage of 20 keV, in the secondary electron mode [28].

2.6. Statistic data analysis

The Chi-square test was employed to evaluate the significance level of the tested *P. aeruginosa* and *K. pneumoniae* strains concerning their association with specific points within the water access network. These points represent locations in the network where the water flows before coming into direct contact with the hands of healthcare professionals, patients, or visitors. The test consisted of determining the difference between each set of observed and expected values for *P. aeruginosa* and *K. pneumoniae* strains, squared and divided by their respective expected values [29]. Additionally, this test was used to assess whether there exists a significant association between the production of biofilms and antibiotic resistance [30,31].

To analyze the polymer assay data, we employed statistical analyses to assess differences between the chosen group of *P. aeruginosa* strains when grown with and without polymers. These analyses were conducted utilizing GraphPad Prism 6 software (Graph-Pad Software in San Diego, California, USA). Comparisons over time for the same strain were also carried out using this software. The significance of the observed differences was evaluated through p-values, calculated via an independent samples *t*-test, after analysis by Shapiro-Wilt. The *t*-test was used to compare the actions of each strain on the polymer with those observed in the positive control group.

3. Results

3.1. Antimicrobial Susceptibility tests

The antimicrobial susceptibility profiles of bacterial strains are shown in Table 2. The results reveal that *P. aeruginosa* and *K. pneumoniae* strains exhibited a susceptibility profile to the majority of the tested antimicrobial agents. It is noteworthy that all bacterial strains displayed susceptibility to amikacin, gentamicin, and meropenem. Moreover, the results obtained from the positive controls for both *P. aeruginosa* and *K. pneumoniae* were entirely consistent with the established indicators provided by the EUCAST [21,32].

The susceptibility profiles of the *K. pneumoni*ae strains revealed susceptibility to a range of antibiotics, including levofloxacin, ciprofloxacin, tobramycin, imipenem, cefepime, and ceftazidime. In the case of *P. aeruginosa* strains, they exhibited an intermediate susceptibility profile (meaning susceptibility at high doses) to levofloxacin, ciprofloxacin, and imipenem.

Specifically, for *P. aeruginosa* strains, a resistance profile was quantified for tobramycin (6.6 %), cefepime (9.7 %), and ceftazidime (3.2 %). Polymyxin B was tested exclusively for *P. aeruginosa* strains, and the results indicated 100 % susceptibility to this antibiotic.

Table 2
Resistance results of the *P. aeruginosa* and *K. pneumoniae* tested strains to antimicrobial agents.

Antibiotics	*P. aeruginosa* (%)			*K. pneumoniae* (%)		
	S (%)	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**
Levofloxacin		33 (100 %)		2 (100 %)	1 (50 %)	
Ciprofloxacin		33 (100 %)		2 (100 %)		
Ofloxacin[b]				1 (50 %)		
Amikacin	31 (93,9 %)		2 (6,1 %)[c]	2 (100 %)		
Tobramycin	31 (93,9 %)		2 (6,1 %)	2 (100 %)		
Gentamicin	33 (100 %)			2 (100 %)		
Netilmicin[a]	33 (100 %)					
Imipenem	33 (100 %)	33 (100 %)		2 (100 %)		
Meropenem				2 (100 %)		
Cefepime		30 (90,9 %)	3 (9,1 %)	2 (100 %)		
Ceftazidime		32 (97 %)	1 (3 %)	2 (100 %)		
Polymyxin B[a]	33 (100 %)					

S: sensitive; I: intermediate (susceptible, increased exposure – EUCAST); R: resistant.
[a] antibiotic tested only in *P. aeruginosa* strains.
[b] antibiotic tested only in *K. pneumoniae* strains.
[c] if resistant to Tobramycin and sensitive to Gentamicin, related Amikacin resistant (Leclerc et al., 2013).

On the other hand, the antibiotic ofloxacin was exclusively tested on *K. pneumoniae* strains. The results indicated that 50 % of these strains exhibited an intermediate resistance pattern, which means they became more susceptible when exposed to higher doses of the antibiotic.

In light of the criteria used to define multidrug resistance (MDR), which typically classifies isolates as multidrug-resistant if they display resistance to three or more classes of antibiotics with anti-*Pseudomonas* activity, it is relevant that the tested strains do not meet this criterion, being considered non-MDR strains [6,23] (Table 3).

3.2. Biofilm production assay

Out of the 35 strains subjected to biofilm production testing, the results revealed that a significant majority, 23 (65.7 %), were categorized as strong biofilm producers. Additionally, 8 (22.9 %) strains showed a moderate biofilm production capacity, 3 (8.6 %) demonstrated weak biofilm production, and only 1 (2.9 %) was considered a non-biofilm producer (Fig. 1).

In the subset of *P. aeruginosa* strains assessed, 22 (66.7 %) were identified as strong biofilm producers, while 7 (21.2 %) showed a moderate capacity for biofilm production. Moreover, 3 (9.1 %) of the *P. aeruginosa* strains showed a weak ability to form biofilms, and 1 (3 %) strain did not produce biofilm.

Regarding the *K. pneumoniae* strains, one strain was characterized as a strong biofilm producer, while the other strain was categorized as a moderate biofilm producer.

The Chi-square test showed high significance ($X^2 = 10.36$, df = 1, p-value = 0.0014) between the water accession points and the presence of *P. aeruginosa* strains (Table 4). Out of the 33 strains of *P. aeruginosa* selected, 97 % were recovered from wet areas (Table 1), namely, the inner part of the shower hose (48.6 %), inner part of the shower (39.4 %), sink drain (3 %), bathroom shower (3 %), toilet wall (3 %) and nursing room bench (3 %). Thus, approximately 93 % originated from the access points of the water distribution network. This study considered the water distribution network's access points and hospital equipment that came into contact with the network but not staff, patients, or visitors.

3.3. Polymeric surface modification assay

An initial screening was conducted during 48h of incubation to identify bacteria with a high potential for modifying or degrading polymeric material. In Table 5, a "+" sign denotes that the OD_{600nm} measured in the test assay was 1.5 fold higher than that in the control assay (without polymeric material), while a "–" sign signifies that the measured ODs were comparable to those in the control assays.

The strains that demonstrated the remarkable highest ODs for all three medical devices tested in this screening process were *P. aeruginosa* 244Pa, 266Pa pol, 427Pa, 440Pa, 440Pi, and 462P pol (Table 4). This initial screening was aimed at identifying organisms with the ability to thrive in a medium devoid of carbon supplementation, which can indicate their potential to extract carbon from polymeric material. To further validate their capacity for material degradation, these six strains were selected for additional growth assays.

After a 7-day incubation period, the growth of *P. aeruginosa* strains 440Pa, 440Pi, and 244Pa demonstrated the highest ODs, as shown in Fig. 2. Notably, except for strain *P. aeruginosa* 244Pa with the urinary catheter, these three strains exhibited statistically significant increases in growth in the presence of the tested polymeric materials compared to the control assay. However, there were cases with other tested strains where non-significant differences were observed. For instance, the strain *P. aeruginosa* 266Pa pol with the urinary catheter and the infusion set; strain *P. aeruginosa* 462 pol at 96 h for the infusion set and the urinary catheter; and strain *P. aeruginosa* 427Pa in the case of the infusion set and the nasal cannula, as depicted in Fig. 2. These results provide valuable insights into the specific strains and conditions under which significant growth differences were observed when interacting with the polymeric materials.

The medical devices employed in the assays of *P. aeruginosa* strains 440Pa, 440Pi, and 244Pa were subjected to visualization by SEM. This analysis was conducted to assess the bacterial colonization capacity on the device surfaces and to evaluate potential biodegradation effects resulting from bacterial actions (Figs. 3 and 4).

All three strains of *P. aeruginosa* exhibited high colonization on the three tested surfaces (Fig. 3). The surfaces of both the infusion set and the nasal cannula exhibited deformations and cracks, as illustrated in Fig. 4. When compared with the control assay, it becomes evident that the infusion set in particular, exhibited the most prominent fractures in the material. These visual observations suggest that the bacterial actions on these materials led to noticeable surface alterations, which could be indicative of biodegradation or

Table 3
Categorization of the isolates resistant to antibiotics with anti-pseudomonas activity.

Strain	Antimicrobial agent	Classes of antibiotics	Classification
*P. aeruginosa*_266Pi'	tobramycin	aminoglycosides	non-MDR
*P. aeruginosa*_247Pol	cefepime	cephalosporins	non -MDR
*P. aeruginosa*_427Pa	cefepime	cephalosporins	non -MDR
	tobramycin	aminoglycosides	
	cefepime	cephalosporins	
	ceftazidime	cephalosporins	

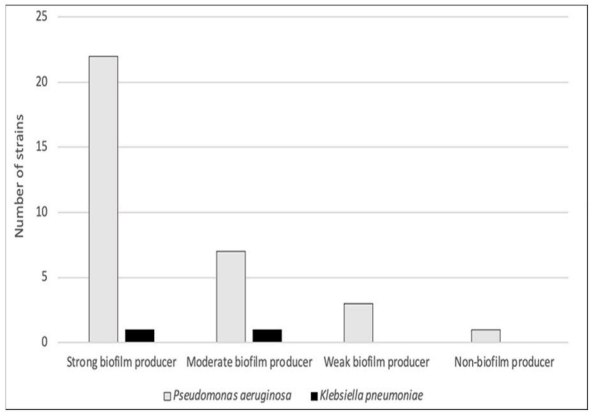

Fig. 1. Classification of strains based on biofilm production capacity evaluated by cristal violet assay.

Table 4
Correlation between *P. aeruginosa* and *K. pneumoniae* strains and the water distribution system.

	Water distribution system – N. (%)	(no) Water distribution system – N. (%)	Total
P. aeruginosa	29 (82.9 %)	4 (11.4 %)	33 (94.3 %)
K. pneumoniae	0 (0 %)	2 (5.7 %)	2 (5.7 %)
Total	29 (82.9 %)	6 (17.1 %)	35 (100 %)
p-value	0.0014		

Table 5
Bacterial growth results in the presence of three different polymeric materials as carbon sources.

STRAIN	MEDICAL DEVICES		
	INFUSION SET	URINARY CATHETER	NASAL CANNULA
***P. aeruginosa* 118Pa**	–	–	–
***P. aeruginosa* 133 Pb**	+	+	–
***P. aeruginosa* 140Ka**	–	–	–
***P. aeruginosa* 140Kd**	–	–	–
***P. aeruginosa* 140P bf**	–	–	–
***P. aeruginosa* 140b pol**	–	–	–
***P. aeruginosa* 152 pol**	–	+	–
***P. aeruginosa* 218P pol**	–	–	–
***P. aeruginosa* 227P pol**	–	–	–
***P. aeruginosa* 244Pa**	+	+	+
***P. aeruginosa* 244P pol**	+	+	–
***P. aeruginosa* 247P pol**	–	–	–
***P. aeruginosa* 266 Pb pol**	–	–	–
***P. aeruginosa* 266Pi′**	–	–	–
***P. aeruginosa* 266Pa pol**	+	+	+
***P. aeruginosa* 266Pa′**	–	–	–
***P. aeruginosa* 326Pa**	–	–	–
***P. aeruginosa* 349Pa bf**	–	–	–
***P. aeruginosa* 364Pa**	–	+	–
***P. aeruginosa* 364pol**	–	–	–
***P. aeruginosa* 410 Pb**	–	–	–
***P. aeruginosa* 410Pa**	–	–	–
***P. aeruginosa* 417P**	–	–	–
***P. aeruginosa* 417Ppol**	–	+	–
***P. aeruginosa* 427Pa**	+	+	+
***P. aeruginosa* 440Pa**	+	+	+
***P. aeruginosa* 440Pi**	+	+	+
***P. aeruginosa* 440P pol**	–	–	–
***P. aeruginosa* 443P**	–	–	–
***P. aeruginosa* 454Pa**	–	–	–
***P. aeruginosa* 462P pol**	+	+	+

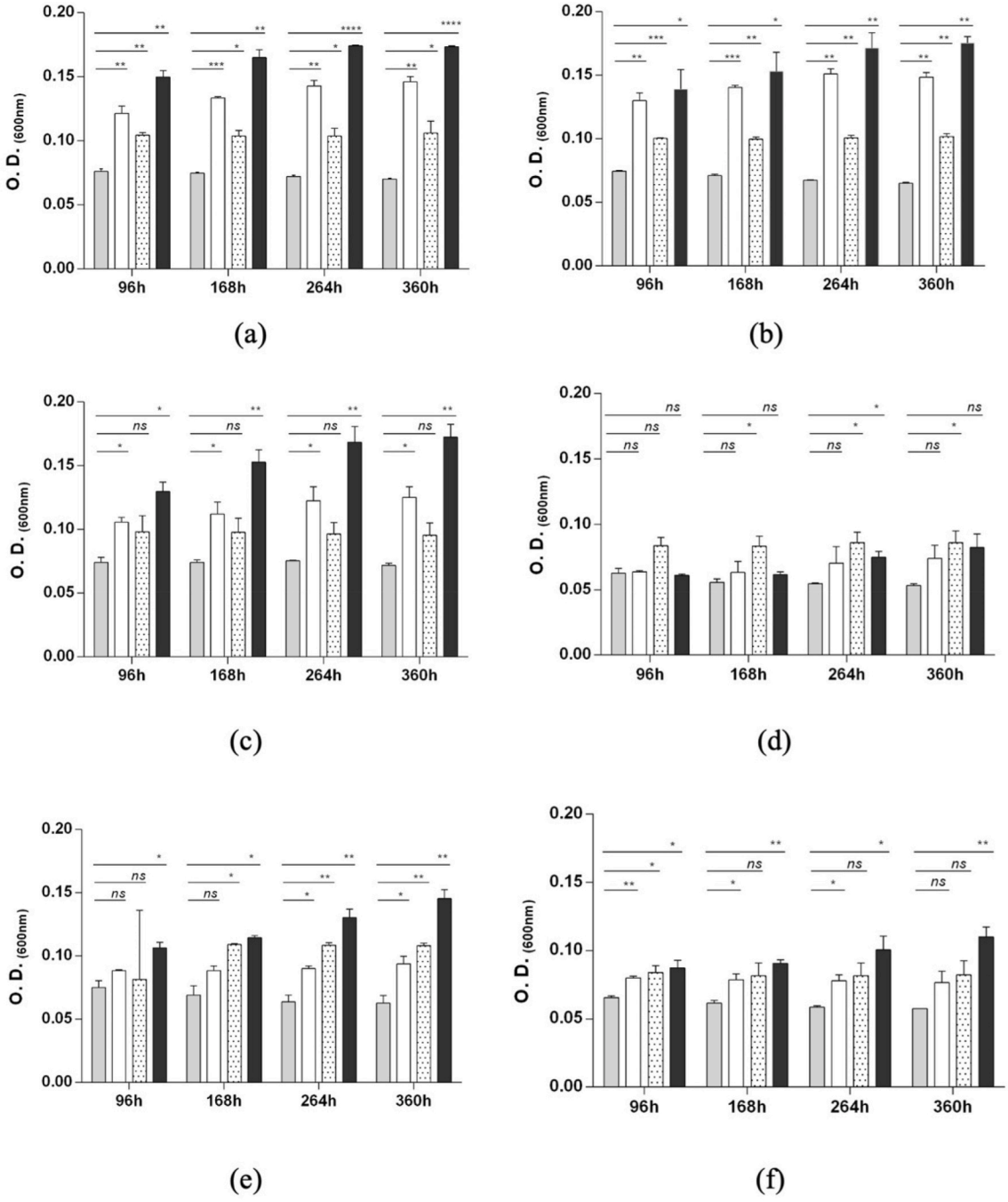

Fig. 2. Growth of different *P. aeruginosa* strains in the presence of different polymeric materials as unique carbon source at 37 °C. The bacterial growth was evaluated measuring ODs at different time intervals, during 360h. The tested *P. aeruginosa* strains were: 440Pa (a), 440Pi (b), 244Pa (c), 427Pa (d), 462Pol (e), 266Pa pol (f). Assays were conducted in a carbon-free basal medium under 4 different conditions: without any medical devices (grey bars); with infusion set (white bars), with urinary catheter (dotted bars), and with nasal cannula (black bars). The data represent the mean values (±standard deviation) obtained from at least two replicates. Ns – no significant difference; *, **, ***, **** - statistical sign of significant difference compared to control, in significance levels $p < 0.04$, $p < 0.009$, $p < 0.0004$ and $p < 0.0001$ respectively.

modification.

4. Discussion

The presence of *P. aeruginosa* and *K. pneumoniae* strains has provided insights into the identification of potential microbial

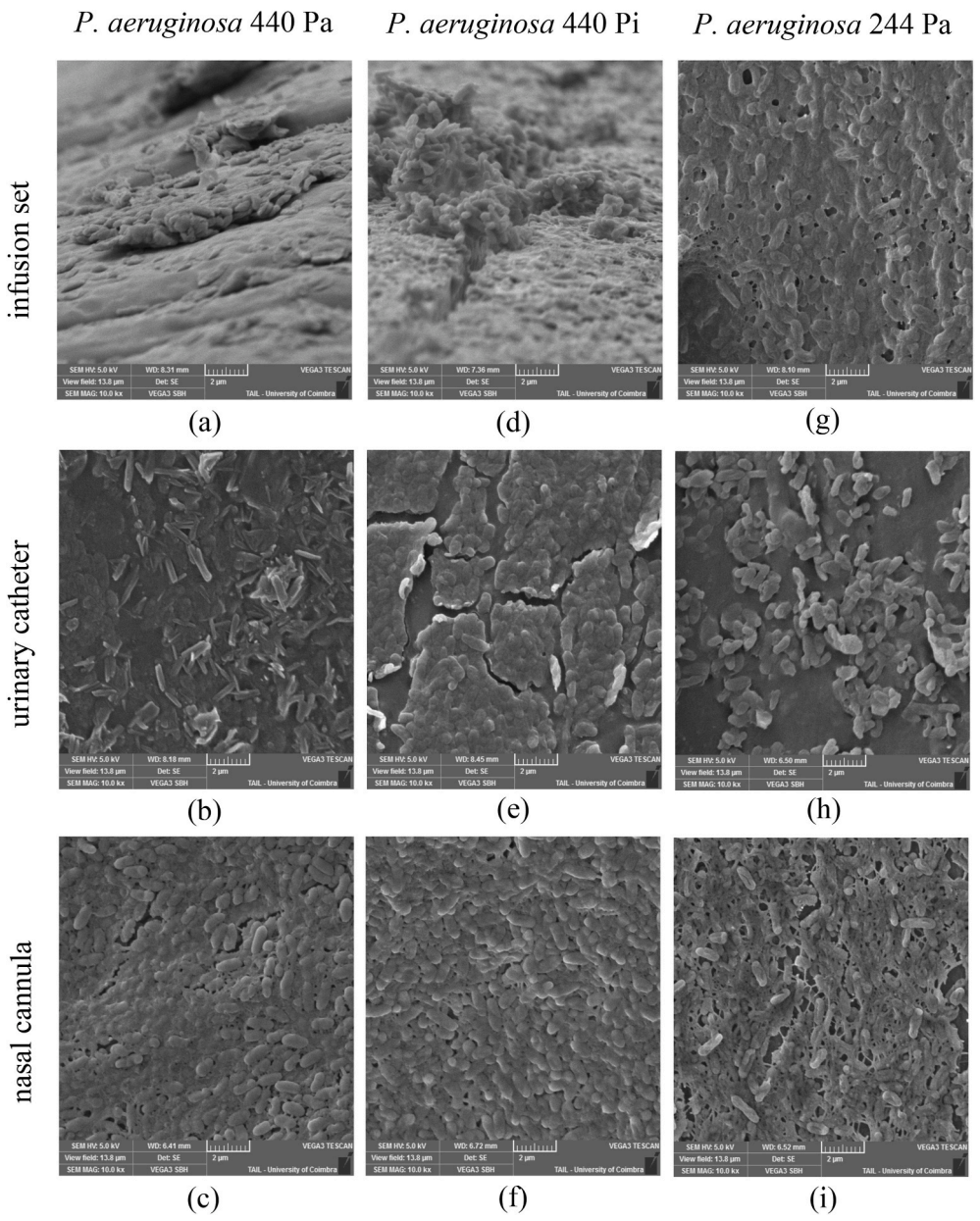

Fig. 3. SEM of three different device surfaces after 360h of incubation with selected *P. aeruginosa* strains to show the bacterial surface colonization. a) infusion set with 440Pa; b) urinary catheter with 440Pa; c) nasal cannula with 440Pa; d) infusion set with 440Pi; e) urinary catheter with 440Pi; f) nasal cannula with 440Pi; g) infusion set with 244Pa; h) urinary catheter with 244Pa; i) nasal cannula with 244Pa. Images were obtained with a microscope magnification of 10,000×.

reservoirs within the hospital environment and the dynamics of this ecosystem. It is noteworthy that the highest rates of *P. aeruginosa* isolation were observed in wet or moist areas, which aligns with findings from prior research studies [5,9,33–35]. This information highlights the significance of understanding and monitoring microbial patterns in hospital settings, particularly in areas where moisture plays a crucial role in microbial proliferation.

The characterization of the antimicrobial susceptibility of *P. aeruginosa* and *K. pneumoniae* environmental strains was performed by testing antibiotics of clinical relevance. Surprisingly, the tested strains showed susceptibility to the majority of the antibiotics, which differs from the results reported in other studies [14,36–39]. In this context, it is plausible to hypothesize that the points of access to drinking water within the hospital environment, such as taps, showers, and sinks, could serve as critical entry points for external environmental bacteria introduction into the hospital environment. These transient waterborne strains might not yet carry resistance to many clinical antibiotics, which could explain their susceptibility. The Chi-square test reinforces the idea that the presence of *P. aeruginosa* is linked to the points of access within the water distribution network. These findings align with the established

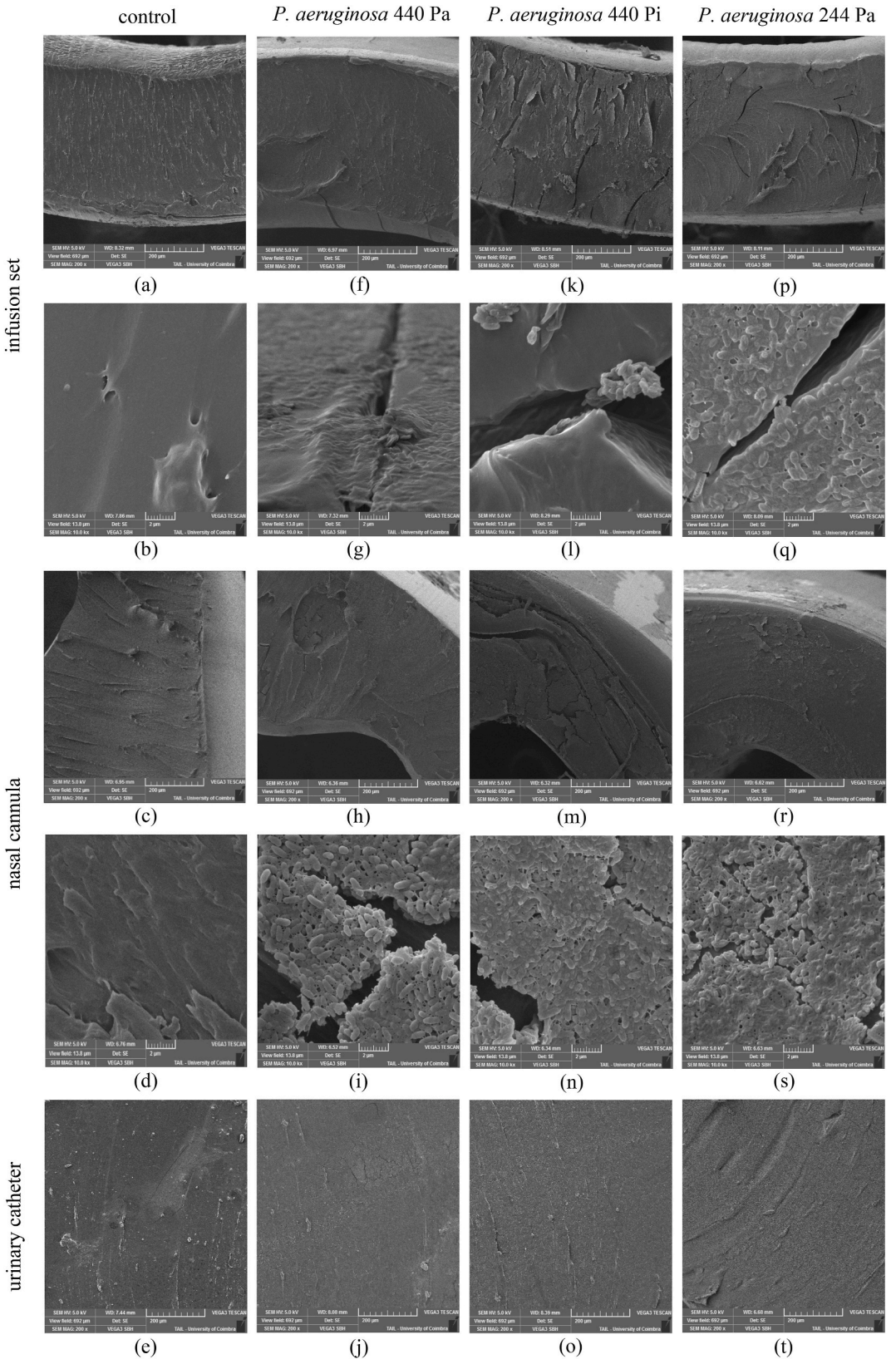

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Fig. 4. SEM of the three tested devices in the absence (control assay) and in the presence of selected *P. aeruginosa* strains, to reveal the surface modifications resulting from the bacterial growth. a) and b) infusion set control; c) and d) nasal cannula control; e) urinary catheter control; f) and g) infusion set with 440Pa; h) and i) nasal cannula with 440Pa; j) urinary catheter with 440Pa; k) and l) infusion set with 440Pi; m) and n) nasal cannula with 440Pi; o) urinary catheter with 440Pi; p) and q) infusion set with 244Pa; r) and s) nasal cannula with 244Pa; t) urinary catheter with 244Pa. Images a), c), e), f), h), j), k), m), o), p), r) and t) were obtained with a microscope magnification of 200×. Images b), d), g), i), l), n), q), s) were obtained with a microscope magnification of 10,000×.

understanding that water reservoirs are frequently identified as environmental sources of *P. aeruginosa* [5,7]. These bacteria can potentially be introduced into the hospital environment either through direct contact with water or indirectly through the hands of healthcare staff [5,7,14,40,41]. Environmental *P. aeruginosa* strains have been detected in very low amounts, typically 2 % or less, within drinking water samples [17]. Nevertheless, their prevalence in the hospital environment can be significantly higher due to their ability to produce biofilms. This bacterial capacity is crucial for ensuring their survival in challenging and nutrient-poor environments [5,6,8]. When considering the distribution of *P. aeruginosa* within a healthcare setting, the risk of transmission arises when patients are exposed to the bacteria during the bath and through contact with contaminated medical devices [5,7].

Detecting and quantifying pathogenic microorganisms within a hospital environment is of utmost importance. Equally essential is assessing the features that allow these organisms to persist in such settings. In this context, this study evaluated the potential relationship between antibiotic resistance and biofilm formation. Within our collection of strains, a significant proportion of *P. aeruginosa* (~67 %) and 50 % of *K. pneumoniae* strains were identified as strong biofilm producers and non-MDR. Other research studies have also attempted to establish correlations between antibiotic resistance and biofilm production [6,14,30,36–39,42]. For example, a study involving 302 *P. aeruginosa* strains isolated from various sources found that approximately 59.3 % of them were strong biofilm producers, spanning both MDR and non-MDR groups [6]. These findings underscore the complex relationship between antibiotic resistance and biofilm formation in pathogenic organisms, which can vary across different strains and clinical settings.

It is important to note that all of these strains were submitted to antimicrobial susceptibility tests as planktonic cells and not as a biofilm. The bacteria community growing as biofilms referred to be able to express different genes that hamper antibiotic action [43–46]. Consequently, once established and maintained in the hospital environment, bacterial biofilms may exhibit reduced susceptibility to antimicrobial agents compared to genetically identical planktonic cells [34,43–45,47,48].

The colonizing capacity of *P. aeruginosa* strains on medical devices can serve as a valuable indicator of their potential to biodegrade these materials. Usually, biofilm formation precedes the process of polymer biodegradation [49]. During the initial bacterial attachment phase to the surface of the polymer, bacteria have the capacity to release extracellular enzymes that are capable of capturing carbon [49,50]. All three tested devices are constructed from materials that serve as carbon sources for bacteria. Polyvinyl chloride, a thermoplastic polymer, is primarily composed of carbon, hydrogen, and chloride elements, denoted by its chemical formula $(C_2H_3Cl)n$ [51–53]. Di-ethylhexyl phthalate (DEHP) is used as a plasticizer to impart flexibility and softness to PVC without causing adhesion issues [54,55]. DEHP is comprised of carbon, hydrogen, and oxygen ($C_{24}H_{38}O_4$) [54,55]. In the case of latex, it is composed of approximately 35 % hydrocarbons, with a chemical formula of $(C_5H_8)n$ [56]. *P. aeruginosa* strains 440Pi, 440Pa, and 244Pa demonstrated the most promising potential for modifying polymeric materials, as they exhibited the best growth in a basal medium without additional carbon supplements. This result suggests that these strains can extract the necessary carbon for their growth from the tested medical materials. Of the several medical devices used in these assays, it was observed that the nasal cannula yielded the highest ODs for the strains *P. aeruginosa* 244Pa, 440Pa, and 440Pi. This was closely followed by the infusion set, which resulted in notable ODs for strains *P. aeruginosa* 440Pa and 440Pi. While the ODs of the growths with the urinary catheter were relatively consistent, it is worth mentioning that they consistently remained higher than those in the control group. Furthermore, the strains *P. aeruginosa* 266Pa pol, 462Pol, and 427Pa consistently showed the lowest recorded ODs. However, it is also visible that even these strains exhibited higher growth values in the presence of the tested materials when compared to the control group.

SEM images revealed that control samples showed rough surfaces devoid of any bacterial growth. In contrast, these surfaces underwent visible alterations in the assays with the presence of *P. aeruginosa* cells. Notably, among all the materials, nasal cannula and infusion set were the devices that exhibited the most significant surface modifications as a result of bacterial colonization. Overall, these observations highlight the differential interactions of the bacterial strains with distinct medical devices, emphasizing the significance of the material-specific responses in their growth dynamics.

Both the infusion set and nasal cannula are primarily composed of PVC, a thermoplastic material characterized by a structure consisting solely of carbon atoms, which makes it highly resistant to degradation [50,52,57]. However, it is susceptible to colonization by biofilm-forming bacteria [58]. The presence of DEHP, a plasticizer commonly used in PVC products, appears to facilitate the modification of the nasal cannula by *P. aeruginosa*. In a previous study, *P. aeruginosa* was employed in two slurry-phase reactors containing DEHP-contaminated soil. One reactor contained only *P. aeruginosa*, while the other introduced *P. aeruginosa* into the native community of an effluent treatment [59]. In both cases, the presence of *P. aeruginosa* significantly contributed to the degradation of DEHP.

The urinary catheter is composed of latex coated with silicone, and degradation of this material has been under study for more than a century [60–62]. Numerous publications focused on rubber biodegradation that both bacteria and fungi possess the capability to degrade rubber, but this process is extremely slow [60–62]. One strain, *P. aeruginosa* AL98, obtained from samples of dirty water from deteriorated car tires, demonstrated the capacity to degrade both natural and vulcanized rubber [59].

In conclusion, this study reveals a significant association between the distribution of *P. aeruginosa* strains within the hospital environment and the proximity to drinking water access points. These strains showed a sensitivity profile to the majority of the

antimicrobial agents tested (>90 %) and were categorized as strong biofilm producers, with a rate of 66.7 %. Despite the current study has evaluated antibiotic resistance profiles and biofilm-forming abilities of the studied strains, it does not delve into the underlying genetic mechanisms driving these traits. Future research will provide deeper insights into the genetic basis of antibiotic resistance and biofilm formation that will help to understand the persistence and spread of these pathogens in healthcare environments.

Medical devices were employed to assess their susceptibility to colonization and biodegradation by hospital isolates. All three tested materials exhibited notable biofilm colonization. Particularly, *P. aeruginosa* strain 440Pi demonstrated the highest capability for modifying polymeric surfaces, implying that this strain may possess the ability to extract carbon from medical devices, potentially leading to material degradation. However, additional tests might be contemplated to provide more evidence of surface deterioration by cells, such as transmission electron microscopy (TEM) that observe the ultrathin cross-section of polymeric samples. While the study does not investigate the mechanisms underlying material degradation, it opens the door for further work to elucidate these intricate processes and enhance our understanding of the interactions between bacteria and medical device surfaces.

Data availability statement

Data associated with this study was not deposited into a publicly available repository because all data is included in the article.

CRediT authorship contribution statement

Roberta Lordelo: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Rita Branco:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Conceptualization. **Fernando Gama:** Writing – review & editing, Visualization, Resources. **Paula V. Morais:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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