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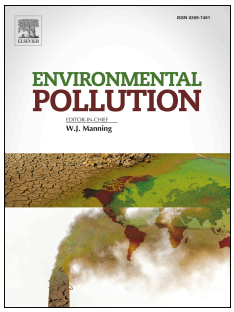

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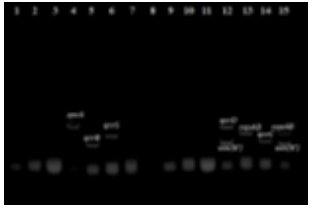
Bioaugmentation strategies

Environmental Biotechnology

A non-pathogenic bacterium that lacks clinically relevant antimicrobial resistance

***Pseudomonas* sp. P26**

In vitro safety testing



In vivo safety testing

Galleria mellonella

Safety attributes of *Pseudomonas* sp. P26, an environmental microorganism with potential application in contaminated environments

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Abstract

Currently, the selection of non-pathogenic microorganisms that lack clinically relevant antimicrobial resistance is crucial to bioaugmentation strategies. *Pseudomonas* sp. P26 (P26) is an environmental bacterium of interest due to its ability to remove aromatic compounds from petroleum, but its safety characteristics are still unknown. The study aimed to: a) determine P26 sensitivity to antimicrobials, b) investigate the presence of quinolone and β-lactam resistance genes, c) determine the presence of virulence factors, and d) evaluate the effect of P26 on the viability of *Galleria mellonella* (an invertebrate animal model). P26 antimicrobial sensitivity was determined *in vitro* using the Kirby-Bauer agar diffusion method and the VITEK 2 automated system (BioMerieux®). Polymerase Chain Reaction was employed for the investigation of genes associated with quinolone resistance, extended-spectrum β-lactamases, and carbapenemases. Hemolysin and protease production was determined in human blood agar and skimmed-milk agar, respectively. In the *in vivo* assay, different doses of P26 were injected into *Galleria mellonella* larvae and their survival was monitored daily. Control larvae injected with *Pseudomonas putida* KT2440 (a strain considered as safe) and *Pseudomonas aeruginosa* PA14 (a pathogenic strain) were included. *Pseudomonas* sp. P26 was susceptible to most evaluated antimicrobials, except for trimethoprim-sulfamethoxazole. No epidemiologically relevant genes associated with quinolone and β-lactam resistance were identified. Hemolysin and protease production was only evidenced in the virulent strain (PA14). Furthermore, the results obtained in the *in vivo* experiment demonstrated that inocula less than 10^8 CFU/mL of P26 and *P. putida* KT2440 did not significantly affect larval survival, whereas larvae injected with the lowest dose of the pathogenic strain *P. aeruginosa* PA14 experienced instant mortality. The results suggest that *Pseudomonas* sp. P26 is a safe strain for its application in environmental bioremediation processes.

Additional studies will be conducted to ensure the safety of this bacterium against other organisms.

Keywords: Environmental bacteria; Antimicrobial resistance; Infective capacity; *Galleria mellonella.*

Introduction

Numerous microorganisms offer eco-friendly solutions for remediating petroleum-contaminated sites by adjusting their catabolic activities to use toxic organic pollutants as growth substrates (Hossain et al., 2022). However, in areas contaminated with crude oil derivatives, the indigenous microbiota is often inhibited by the contaminants' toxicity or lack the ability to fully degrade them (Li et al., 2023). Introducing specially prepared microorganisms (bioaugmentation) can significantly improve the efficiency and rate of oil degradation (Sarkar et al., 2020). Nonetheless, it is crucial to assess any potential health risks that these microorganisms may pose to humans, animals, and plants (Ferreira et al., 2019).

In this sense, using non-pathogenic microorganisms is highly recommended, often preferred, and sometimes mandatory (Ferreira et al., 2019). Currently, it is essential to select non-pathogenic microorganisms that lack clinically relevant antimicrobial resistance for bioaugmentation strategies (Rajkumari et al., 2021). In fact, the transfer of genetic material containing antimicrobial resistance genes among microorganisms through mobile genetic elements, plasmids, or transposons is a significant global concern (Delgado-Blas et al., 2022). For this reason, identifying the presence of quinolone and β-lactam resistance genes is of critical importance. These genes facilitate the selection and spread of β-lactam and quinolone-resistant bacterial strains rapidly and pose a greater threat to public health (Strahilevitz et al., 2009). Moreover, these genes and their combinations must be investigated because they can be associated with a bacterial fitness reduction (Dahlberg and Chao, 2003). While the application of beneficial microorganisms for plant-growth promoting is currently undergoing safety regulatory processes (Vílchez et al., 2016), there are very few studies addressing the evaluation of safety characteristics in strains employed for environmental bioremediation (Poszytek et al., 2018).

Pseudomonas sp. P26 is an environmentally interesting bacterium due to its ability to remove aromatic compounds from petroleum (Isaac et al., 2013, 2015; Lobo et al., 2023). However, its safety characteristics are still unknown. The *Pseudomonas* genus is known for its ecological significance and production of industrially relevant secondary metabolites (Anayo et al., 2019). Despite these benefits, some *Pseudomonas* strains are also notorious for their pathogenicity (Jurado-Martín et al., 2021; Oh et al., 2019; Ruiz-Roldán et al., 2020) and multidrug resistance (Rodulfo et al., 2019; Urbanowicz et al., 2022). The genus's adaptability and pathogenic potential are partly attributed to quorum-sensing systems, which regulate both beneficial traits and virulence factors, including hemolysins and proteases (Saqr et al., 2021). This complexity underscores the importance of thorough safety evaluations for *Pseudomonas* strains intended for bioremediation.

Considering that the expression of virulence factors differs between *in vitro* growth, including media that mimics the host's environment, and in animals or humans, various invertebrate models are available and utilized for the study of host-microorganism interactions (Anju et al., 2020; Elizalde-Bielsa et al., 2023). *Galleria mellonella* (wax moth larvae) is an invertebrate model for studying host-microorganism interactions (Elizalde-Bielsa et al., 2023; Ménard et al., 2021; Pereira et al., 2020). Due to the lack of nociceptors and insensitivity to pain in larvae, ethical restrictions are less stringent compared to vertebrates (Ménard et al., 2021). Despite the absence of an adaptive immune system, wax moth larvae share a substantial number of orthologous genes responsible for general functions with vertebrates. This similarity in their innate immune systems makes *Galleria mellonella* a suitable model for investigating microorganisms affecting humans (Cutuli et al., 2019).

In this study, the hypothesis was that *Pseudomonas* sp. P26 (P26) exhibits an appropriate safety profile concerning antimicrobial resistance and harmlessness,

supporting its potential application in bioremediation processes. The objectives of the present work were to assess the sensitivity of P26 to antimicrobials and to identify the presence of quinolone and β-lactam resistance genes. Furthermore, there is a proposed investigation into virulence factors, such as hemolysin and protease production, and examination of the impact of P26 on the viability of *Galleria mellonella*.

Materials and methods

Bacterial strains

In this study, *Pseudomonas* sp. P26 (GenBank Acc. Num. HE798531) was primarily examined. The Instituto Nacional de Enfermedades Infecciosas (INEI)-ANLIS "Dr. Carlos G. Malbrán" provided the reference strains for genetic analysis, including *Escherichia coli* 7932 and 49-1, *Klebsiella pneumoniae* 48-3, 55-4, 1153, 1803, 54-2, 52-3, and 51-2, *Proteus mirabilis* 11452 and 54-2, and *Providencia stuartii* 51-1. Specifically, *E. coli* 7932, *K. pneumoniae* 48-3, and *P. mirabilis* 11452 served as positive controls for *qnrA*, *qnrB*, and *qnrC* genes, respectively, in fluoroquinolone resistance gene studies. Additional controls included *K. pneumoniae* 55-4 for *aac(6')* and *oqxAB*, *K. pneumoniae* 1153 for *qnrS* and *oqxAB*, and *P. mirabilis* 54-2 for *qnrD* and *aac(6')* genes. *K. pneumoniae* 1803 was a positive control for extended-spectrum β-lactamases genes (*bla*$_{PER}$, *bla*$_{CTX-M}$, and *bla*$_{CMY}$). Strains *K. pneumoniae* 54-2, 52-3, *E. coli* 49-1, *P. stuartii* 51-1, and *K. pneumoniae* 51-2 were positive controls for carbapenemase genes (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-48-like}$, *bla*$_{KPC}$). *Pseudomonas putida* KT2440 and *Pseudomonas aeruginosa* PA14 were risk group 1 (Kampers et al., 2019) and 2 (Song et al., 2023) representatives, respectively, used in assessing P26 virulence.

These microorganisms were individually stored at -20°C in modified Luria Bertani (LBm) broth (% w v^{-1}: NaCl, 1; yeast extract, 0.5; meat peptone, 1; pH 7.0) added with 20% (v v^{-1}) glycerol.

Bacterial identification and antimicrobial susceptibility testing

Pseudomonas sp. P26 was analyzed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS Biotyper). To obtain the spectrum, a single colony from blood agar and Tripticase soy agar (TSA) agar plate (incubated at 35°C) was directly spotted on the target slide, followed by the addition of the matrix (70% formic acid and acetonitrile) and air drying. The loaded plate was then placed in the instrument according to the manufacturer's instructions. Mass spectrum obtained was compared with reference database: a pattern recognition algorithm uses peak positions, peak intensity distributions and peak frequencies, providing identification if the score values meet the criteria established by Bruker Daltonics (Rocca et al., 2019). Based on this comparison, classification of the strain P26 was assigned with the score values ≥ 2.30 and ≤ 3, so the allocation was at highly probable species identification level.

Antimicrobial sensitivity was evaluated using the Kirby–Bauer method, recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (M100, 31st edition). Antimicrobial agents recommended by the Red Nacional de Vigilancia de la Resistencia a los Antimicrobianos WHONET - Argentina (ANLIS Dr. C. G. Malbrán, 2021) were tested. The antimicrobials used for *Pseudomonas* sp. P26 susceptibility testing belong to the following classes: third and fourth-generation cephalosporins (ceftazidime and cefepime, respectively), β-lactam/β-lactamase inhibitor combinations (ceftazidime-clavulanic acid, piperacillin-tazobactam, ceftazidime-avibactam), fluoroquinolones (ciprofloxacin), aminoglycosides (gentamicin, amikacin), monobactams (aztreonam), penicillin (piperacillin), carbapenems (imipenem,

meropenem), and folate pathway inhibitors (trimethoprim-sulfamethoxazole). Minimum Inhibitory Concentrations (MICs) of P26 were determined using the automatized VITEK 2 (BioMerieux®) system for piperacillin, tazobactam, cefotaxime, ceftazidime, cefepime, meropenem, ciprofloxacin, amikacin, gentamicin and trimethoprim-sulfamethoxazole. The results were classified into three categories: susceptible, intermediate resistance, and resistance, according to CLSI breakpoints.

Assessment of clinically and epidemiologically significant quinolone and β-lactam resistance genes

Pseudomonas sp. P26 DNA extracts were prepared by boiling the bacterial suspensions, following the instructions of Clark et al. (1993). Table 1 shows the description of genes investigated. Fluoroquinolone resistant genes (*qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *aac*(6') and *oqxAB*) were determined by multiplex polymerase chain reaction (PCR), according to Adachi et al. (2013). The reaction parameters were as follows: 95°C for 2 min followed by 25 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 15 s; finally, 72°C for 10 min. The presence of genes coding for extended-spectrum β-lactamases and carbapenemases was also investigated by multiplex PCR using specific primers depending on the method available (Dallenne et al., 2010). In pursuit of identifying genes coding for extended-spectrum β-lactamases (*bla*$_{PER}$, *bla*$_{CTX-M}$, and *bla*$_{CMY}$), the reaction parameters were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 2 min; finally, 72°C for 5 min. Additionally, to conduct research on genes responsible for the production of serine- and metallo-carbapenemases (*bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{OXA-48-like}$, *bla*$_{KPC}$), the parameters were: 94°C for 5 min followed by 30-35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min; finally, 72°C for 10 min.

Proteolytic and hemolytic activity

In order to prepare inoculum suspensions, each stored bacterial strain (*Pseudomonas* sp. P26, *P. putida* KT2440 and *P. aeruginosa* PA14) was propagated by streak cultures on LBm agar (LBm added with 1.5% w v^{-1} agar). The plates were incubated for 24 h at 30°C. Cell suspensions in saline (0.85% w v^{-1} NaCl) were prepared from isolated colonies and their concentrations were adjusted to an optical density at 600 nm (OD_{600nm}) of 1 ± 0.1, corresponding to approximately 10^8 CFU mL^{-1}.

For the proteolytic activity assay, the strains of *Pseudomonas* were streaked (10 µL of each inoculum suspension) on skim milk-LBm supplemented agar plates (10% w v^{-1} skimmed milk) and incubated at 30°C for 24 h. The presence of a clear zone surrounding the colonies indicated a positive test result.

To perform the hemolytic activity assay, the basal medium for blood agar preparation was comprised of % w v^{-1}: NaCl, 0.5; meat extract, 1; meat peptone, 1; agar, 1.5; with a pH adjusted to 7.5. Once sterilized (at 121°C for 15 minutes) and cooled to 50°C, 10% v v^{-1} of blood, pre-warmed to 45°C, was gently mixed into the basal medium. The mixture was then poured into plates to solidify. Ten microliters of inoculum suspensions from *Pseudomonas* sp. P26, *P. putida* KT2440, and *P. aeruginosa* PA14 were spotted onto the agar and incubated at 30°C for 24 h. A clear zone around colonies indicated positive hemolytic activity, while its absence signified a negative result."

Galleria mellonella infection and survival monitoring

Cell suspensions of P26, PA14 and KT2440 were prepared from colonies in saline as described above. Subsequently, the infective doses used (10^8, 10^7, 10^6, 10^5 and 10^4 CFU mL^{-1}) were confirmed for each assay by successive dilutions and colony counting.

Galleria mellonella larvae were kindly provided by the Centro Multiplicador de Biocontroladores Nativos (CEMUBIO) - Instituto Nacional de Tecnología Agropecuaria,

(INTA, Río Negro, Argentina), where they were fed with a sterile diet containing essential nutrients and proteins. Those larvae with a healthy appearance (light color) at the end of their instar stage, weighing between 0.2 g and 0.3 g, were placed in groups of 10 in sterile 9-mm Petri dishes (n = 10 per treatment). Each larva was injected with 10 µL of inoculum into the lower left proleg using an insulin syringe and a 0.3 x 13 mm needle. The same volume of sterile saline and dimethyl sulfoxide (DMSO) was used in the positive and negative control groups, respectively. Each larvae group was incubated for 5 days without a food source at 30°C. After infection, the wax moth larvae survival was monitored daily. Treatments were performed in duplicate, on two different days.

Statistical analysis

The analyses of viability percentages data of *Galleria mellonella* were conducted using the Principal Component Analysis (PCA) multivariate statistical analysis method. *Galleria mellonella* survival curves were plotted using the Kaplan–Meier method and statistical analysis was performed using the log-rank test (Mantel–Cox). PCA was performed using the Minitab 17 Statistical Software. Kaplan-Meier was carried out using the GraphPad Prism 8.0.1 statistical software and the significance level was set at $\alpha < 0.05$.

Results and discussion

Bacterial identification and antimicrobial susceptibility testing

To elucidate the phylogenetic group of *Pseudomonas* sp. P26, MALDI-TOF analysis was carried out. This analysis allowed the determination of the relationship between P26 and the *Pseudomonas monteilii* species (score of 2.334), extending genotypic studies previously done (Isaac et al., 2013). The species *P. monteilii* belongs

to the *Pseudomonas putida* cluster (Peña et al., 2019). It is important to note that, although the database primarily comprises organisms of clinical origin, the results from MALDI-TOF MS were comparable to the phylogenetic analysis of P26 obtained previously through 16S rRNA sequencing (Isaac et al., 2013). MALDI-TOF MS has proven to be as effective as traditional methods in accurately identifying various categories of *Pseudomonas*, including species, subspecies, genomovars, and strains, depending on the specific species of *Pseudomonas.* This has contributed to the increasing popularity of MALDI-TOF MS for characterizing and identifying strains of *Pseudomonas*, thereby facilitating the detection of *Pseudomonas* in environmental samples (Silva-Jiménez et al., 2018). In this study, MALDI-TOF MS was a valuable tool for confirming the placement of P26 within the phylogenetic group of *Pseudomonas putida*. However, due to the intricate nature of this group, further molecular studies are planned to confirm the species of P26 (Morimoto et al., 2020).

Table 2 presents the combined results of the Kirby-Bauer Method and VITEK® 2 testing for *Pseudomonas* sp. P26. According to Rodulfo et al. (2019), P26 can be classified as a low-level resistant (LDR) strain, being non-susceptible to fewer than three antimicrobial categories, because it showed sensitivity to most evaluated antimicrobials except for trimethoprim/sulfamethoxazole (Table 2). This resistance is inherent to species within the *Pseudomonas putida* group, such as *P. monteilii*, as noted by Tan et al. (2019).

Studies on *Pseudomonas putida* strains have shown that the determinants of multiple resistance are on both chromosomes and plasmids, enabling the horizontal transfer of antimicrobial resistance determinants (Delgado-Blas et al., 2022; Molina et al., 2014). In this context, Ballaben et al. (2021) isolated a strain of *P. monteilii* that exhibited an extensively drug-resistant phenotype, which only remained susceptible to amikacin, tetracycline, and colistin. Despite this, Kaszab et al. (2021) observed that the

majority of *Pseudomonas* isolates from compost and hydrocarbon-contaminated groundwater or soil exhibited sensitive phenotypes. Moreover, Tohya et al. (2022) studied 42 *Pseudomonas* isolates and evidenced that most of them were sensitive to several antimicrobial agents, except for aztreonam.

Assessment of clinically and epidemiologically significant quinolone and β-lactam resistance genes

Multiplex PCR is a reliable and rapid method for the simultaneous detection of clinically relevant genes, such as those encoding antimicrobial resistance or conferring increased virulence to the organisms carrying them (Faccone et al., 2023). Genes associated with quinolone and β-lactam resistance of epidemiological interest were investigated due to their ability to disseminate and persist (Poirel et al., 2012).

No epidemiologically relevant genes associated with quinolone resistance, extended-spectrum β-lactamases (ESBLs), and carbapenemases were identified (Fig. 1 and 2). The main cause of resistance to β-lactams is the production of hydrolytic enzymes called β-lactamases. These enzymes, mainly plasmid-mediated, are grouped into multiple categories based on their hydrolytic profile (Nagshetty et al., 2021). However, ESBLs and carbapenemases are of the greatest clinical and epidemiological interest due to their potential to spread and persist in the environment (Faccone et al., 2023). The absence of the evaluated genes (Fig. 1 and 2) correlated with P26 high sensitivity to various antimicrobials recommended by the CLSI for the phylogenetic group under consideration (Table 2). These results can contribute to the development of effective bioremediation strategies using *Pseudomonas* sp. P26 within joint action plans aimed at mitigating the spread of antimicrobial resistance in environmental microbial communities by monitoring

and comprehending the dynamics of antimicrobial resistance in these communities (Jofré Bartholin et al., 2023).

Proteolytic and hemolytic activity

Protease production experiments showed that only *P. aeruginosa* PA14 evidenced proteolytic activity in skimmed milk agar. These results were indicated by lysis zones around the colonies ranging from 22 to 34 mm after 24 and 48 h of incubation, respectively (Fig. 3a). Protease is recognized as a significant virulence factor that contributes to tissue protein lysis facilitating bacterial invasion of infected tissues, especially in individuals with burns. Additionally, it serves to shield bacteria from the body's immune defenses (Kareem and Shawkat, 2022). According to the classification of De Longhi et al. (2020), PA14 exhibited a high-potential proteolytic activity. In fact, their results showed that all *P. putida* and *Pseudomonas fluorescens* isolates expressed proteolytic potential, with 71% of *P. putida* strains and 62% of *P. fluorescens* strains showing halos greater than 20 mm after 72 h of incubation. Consistent with the findings of this study, Kareem and Shawkat (2022) reported that 92.2% of *P. aeruginosa* isolates demonstrated the ability to produce protease in skimmed milk agar after 24 h of incubation.

β-hemolysin production is known to be an important virulence factor of *P. aeruginosa* because it plays a significant role in infection dissemination and wound extension due to the complete destruction of red blood cells (El-Mahdy and El-Kannishy, 2019). Hemolysins, which create pores in cell membranes, facilitate the invasion of virulent Pseudomonas into eukaryotic cells. This protective mechanism shields the bacterium from the host's defense mechanisms and antimicrobial treatments, thus contributing to the infection persistence (Rodulfo et al., 2019). Among the *Pseudomonas*

strains grown on blood agar plates, prominent β-hemolysis was observed as a clear zone (halo diameters: 9.6 mm) around the *P. aeruginosa* PA14 colonies after 24 h of incubation (Figure 3b). However, neither zone of hemolysis was found for *Pseudomonas putida* KT2440 nor *Pseudomonas* sp. P26 on blood agar plates. After 48 h of incubation, an increase in the diameter of the halo formed by PA14 was observed (19 mm), with no halos appearing for the two remaining strains (data not shown). While this study was solely conducted with human blood, Nadella et al. (2020) demonstrated that, in general, fish, sheep, and human blood exhibited similar β-hemolytic activity for all bacteria tested with a host specificity depending on strain. Rodulfo et al. (2019) performed tests using different *P. aeruginosa* strains and evidenced that the hemolysins are associated with multidrug resistant and extensively drug-resistant (MDR and XDR, respectively) bacteria. In contrast, studies on *P. monteilii* JK-1 in order to exclude its potential pathogenicity did not detect α- or β-hemolytic activity for this strain cultured on sheep blood agar plates (Qi et al., 2020).

In vivo **experiments with** ***Galleria mellonella*** **larvae**

Conducting experiments within living organisms is essential to identify bacteria safety concerns and the potential loss of activity caused by host-related factors (Ferreira et al., 2019). *Galleria mellonella* larva, commonly known as wax moth, is an innovative model to study microbial virulence and it is inexpensive, simple, and does not require ethical approval. Additionally, it survives at 37°C and possesses an innate immune system that bears significant similarities to that of vertebrates (El Haddad et al., 2022).

The production of melanin by *Galleria mellonella* occurs as a result of an immune response against infection, as melanin assists in trapping and killing microorganisms (Loh et al., 2013). In this work, this phenomenon was partially or completely observed (in cases

of instant or progressive deaths, respectively) and, along with the complete loss of mobility, it was associated with the death of the wax moths. Sterile saline (SS: positive control) injected larvae all remained alive over the 5-day time course. In contrast, DMSO (negative control) injected wax worms were rapidly killed resulting in 100% of instant deaths. Figure 4 shows the PCA loading plot and score plot, providing an indication of the variables contribution to the survival of *Galleria mellonella* inoculated with different inoculum concentrations of *Pseudomonas* strains. The first two principal components, PC1 and PC2, accounted for 82.5% and 16.4% of the variance, respectively, resulting in a total variance of 98.9% altogether. As depicted in the PCA loading plot (Fig. 4a), all the viability percentages (VP1, VP2, VP3, VP4, and VP5) on the first, second, third, fourth, and fifth day overlapped with each other, indicating that the viability percentages obtained on different days of the assay had similar information.

Likewise, in the PCA score plot, it can be observed that the different strains of *Pseudomonas* used to inoculate *Galleria mellonella* larvae at different concentrations were well discriminated in the two-dimensional space represented by the first two principal components, PC1 and PC2.

Combining the PCA loading plot (Fig. 4a) with the PCA score plot (Fig. 4b), it can be noted that the inoculum concentration (IC, in CFU mL^{-1}) is a variable that exhibited a high contrast with the viability percentages variables. In other words, higher inoculum concentrations led to a decreased viability of *Galleria mellonella* larvae, while viability percentages remained similar across the different incubation days.

Furthermore, strains P26 and KT2440 showed similar behaviors throughout the assay period, with higher viability values generally observed towards the upper right part of the plot, in contrast to PA14, which positioned itself on the contrary left side of the

plot. The PCA loading plot and the score plot indicate that the use of higher inoculum concentrations among the three strains (10^8 CFU mL^{-1}) resulted in lower *Galleria mellonella* viability values. Despite this, even in this latter condition, a clear contrast was observed between KT2440 and P26 compared to PA14.

PCA plots revealed significant overlap between the samples from KT2440 and P26. To further assess the distinctions among various inoculum concentrations of each strain, positive and negative controls, a Kaplan-Meier survival analysis was conducted. In general, survival curves correlated with the inoculum concentrations, with greater number of deaths observed at higher concentrations (Fig. 5). Inoculums below 10^8 CFU mL^{-1} of *Pseudomonas* sp. P26 and *P. putida* KT2440 exhibited no significant differences from the positive control (sterile saline) and were not lethal. These behaviors can be seen in Fig. 5a and 5b as shallow slope curves, indicating a lower rate of events (higher survival). Conversely, larvae injected even with the lowest inoculum concentration of the pathogenic strain *P. aeruginosa* PA14 showed no significant differences from the negative control (DMSO) and experienced instant mortality. This phenomenon could be observed as steep slope curves (Fig. 5c), indicating a high rate of events (lower survival). Similarly, Ballaben et al. (2021), demonstrated that, consistent with the relatively low virulence of *P. monteilii* in the clinic, when using the *Galleria mellonella* infection model, *P. monteilii* Pm597/14 did not kill any larvae during the 7 days post-infection with an inoculum of 10^8 CFU mL^{-1}. However, different virulence determinants of *P. monteilii* Pm597/14 were found in that study.

In this *in vivo* technique, an important detail to consider relates to the physiological state of the microbial cultures used for injecting *Galleria mellonella* larvae. In this study, fresh bacterial suspensions were prepared from streaked Petri dishes. Loh et al. (2013) evidenced that fresh cultures in stationary phase are approximately 3 times

more virulent than those in exponential phase. Similarly, cultures that were frozen in exponential phase and thawed at the time of inoculation exhibited lower virulence.

Both *P. putida* and *P. aeruginosa* are categorized as risk level 2 strains due to their known pathogenic properties in humans and vertebrates (Ferreira et al., 2019). Nevertheless, there are non-pathogenic strains within these species that can be safely employed for various applications. In particular, *P. putida* KT2440 is not certified as GRAS (generally regarded as safe) but it is classified in the safety level 1 in the host-vector system (HV): 'HV1'. This HV1 certification indicates that it is considered safe to work with *P. putida* KT2440, and no additional safety measures are required when handling this strain (Kampers et al., 2019). However, the genetic proximity to animal (*P. aeruginosa*) and plant (*Pseudomonas syringae*) pathogens underscores the need to assess the biosafety of related *P. putida* species, such as *Pseudomonas* sp. P26 (Passarelli-Araujo et al., 2021). In fact, Kaszab et al. (2021) isolated various environmental isolates of *P. aeruginosa*, where the majority (65.9%) were virulent with a mortality rate of 75-100% in the *G. mellonella* model. *P. aeruginosa* PA14 falls within a category of bacterial pathogens known as ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, P. aeruginosa*, and *Enterobacter* species). These pathogens have raised concerns due to the increasing prevalence of multidrug-resistant and pan drug-resistant strains. Both in the military and civilian population, infections caused by *P. aeruginosa* result in numerous clinical complications and hundreds of fatalities (Medina-Rojas et al., 2020).

The consistency of results with the *G. mellonella* model is influenced by several factors, including larval weight and injected volume, pre-treatments applied to the larvae, the preparation of microbial inocula (with a well-defined concentration and growth phase), the method of inoculation (such as intrahemocoelic injection, the most commonly

used, or oral gavage for intestinal pathogens), the criteria used to define larval death (some researchers consider melanization, while others also observe larval movement and/or responsiveness to touch), and the number of larvae used in the experiment (Andrea et al., 2021; Banville et al., 2012; Pereira et al., 2020).

A commonly used pretreatment involves incubating the larvae at low temperatures and starving them from one to several days. This practice reduces the immune response of *G. mellonella*, and the virulence of a tested microorganism can be overestimated (Banville et al., 2012; Pereira et al., 2020). In this study, *Galleria mellonella* larvae aged no more than 10 days incubated between 16°C and 20°C were used. Browne et al. (2015) demonstrated that larvae incubated at 15°C for 3, 6, or 10 weeks were the most susceptible to infection with *Candida albicans*, with survival rates at 24 h post-infection being 43.3 ± 13.3% ($p < 0.01$), 46.7 ± 6.6%, and 30.0 ± 10.0% ($p < 0.05$), respectively, while larvae infected after 1 week of pre-incubation showed 73.3 ± 3.3% survival at the same time point.

Conclusion

The results of this study suggest that *Pseudomonas* sp. P26 is a safe strain for its application in environmental bioremediation processes. This bacterium displayed low level of phenotypic antimicrobial resistance and lacked quinolone and β-lactam resistance genes. In the *in vitro* tests, no virulence factors (protease and hemolysin production) were evidenced. The *Galleria mellonella in vivo* model proved to be a valuable tool for evaluating bacterial virulence, providing confirmation of the *in vitro* safety profile of the strain P26. To the best of our knowledge, this is the first study exploring safety attributes, including antimicrobial resistance and virulence characteristics, in a *Pseudomonas* strain

specialized in removing polycyclic aromatic compounds. Additional studies will be conducted to ensure the safety of this bacterium against other organisms.

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Figure captions

Fig. 1. Multiplex PCR for amplification of quinolone resistance genes. Lanes 1 and 9: negative controls; lanes 2, 3, 7, 10 and 11: *Pseudomonas* sp. P26; lane 4: *qnrA* positive sample (*Escherichia coli* 7932) showing a typical band size of 579 pb; lane 5: *qnrB* positive sample (*Klebsiella pneumoniae* 48-3) showing a typical band size of 263 pb; lane 6: *qnrS* positive sample (*K. pneumoniae* 1153) showing a typical band size of 427 pb; lane 12: *qnrD* and *aac(6')* positive samples (*Proteus mirabilis* 54-2) showing typical band sizes of 645 pb and 264 pb, respectively; lane 13: *oqxAB* positive sample (*K. pneumoniae* 1153) showing a typical band size of 448 pb; lane 14: *qnrC* positive sample (*P. mirabilis* 11452) showing a typical band size of 308 pb; lane 15: *aac(6')* and *oqxAB* positive samples (*K. pneumoniae* 55-4) showing typical band sizes of 264 pb and 448 pb, respectively.

Fig. 2. Multiplex PCR for amplification of genes coding for extended-spectrum β-lactamases (ESBLs), and carbapenemases of clinic and epidemiologic interest. **a**. Lane 1: negative control; lanes 2 and 3: *Pseudomonas* sp. P26; lane 4: bla_{PER}, $bla_{\text{CTX-M}}$ and bla_{CMY} positive samples (*Klebsiella pneumoniae* 1803) showing typical band sizes of 739 pb, 593 pb and 462 pb, respectively. **b**. Lane 1: bla_{NDM} positive sample (*K. pneumoniae* 54-2) showing a typical band size of 512 pb; lane 2: bla_{IMP} positive sample (*K. pneumoniae* 52-3) showing a typical band size of 404 pb; lane 3: bla_{VIM} positive sample (*Escherichia coli* 49-1) showing a typical band size of 261 pb; lane 4: bla_{OXA} positive sample (*Providencia stuartii* 51-1) showing a typical band size of 775 pb; lane 5: bla_{KPC} positive sample (*K. pneumoniae* 51-2) showing a typical band size of 916 pb; lane 6: negative control and lane 7: *Pseudomonas* sp. P26.

669 **Fig. 3.** Skimmed milk agar plates spot (**a**)and human blood agar plates spot (**b**) inoculated
670 with different *Pseudomonas* strains. **1:** *Pseudomonas* sp. P26; **2:** *Pseudomonas putida*
671 KT2440; **3:** *Pseudomonas aeruginosa* PA14.

672 **Fig. 4.** Principal Component Analysis (PCA) plot of *Galleria mellonella* viability
673 percentages (VP) inoculated with different *Pseudomonas* strains at different inoculum
674 concentrations (IC). **a.** PCA loading plot representing the following variables: IC (CFU
675 mL^{-1}); VP at the first, second, third, fourth and fifth day of the assay (VP1, VP2, VP3,
676 VP4, and VP5, respectively). **b.** PCA score plot representing the samples (*Pseudomonas*
677 strains).

678 **Fig. 5.** Kaplan-Meier survival curves of *Galleria mellonella* injected with different
679 *Pseudomonas* strains (**a**: *Pseudomonas* sp. P26; **b**: *Pseudomonas putida* KT2440; **c**:
680 *Pseudomonas aeruginosa* PA14) at different inoculum concentrations (CFU mL^{-1}), sterile
681 saline (SS) as positive control and dimethyl sulfoxide (DMSO) as negative control. *$p <$
682 0.05 (log-rank (Mantel-Cox) test).

683

684 **Table 1.** Quinolone and β-lactam resistance genes studied in *Pseudomonas* sp. P26.

Gene group	Resistance gene	Gene description	Amplicon size (bp)	Reference strain used as positive control
Fluoroquinolone resistant gene	***qnrA***	Quinolone resistance pentapeptide repeat protein	579	*Escherichia coli* 7932
	qnrB	Quinolone resistance pentapeptide repeat protein	263	*Klebsiella pneumoniae* 48-3
	qnrS	Quinolone resistance pentapeptide repeat protein	427	*K. pneumoniae* 1153
	oqxAB	Multidrug efflux RND transporter periplasmic adaptor/permease subunits	448	*K. pneumoniae* 1153 and 55-4
	qnrC	Quinolone resistance pentapeptide repeat protein	308	*Proteus mirabilis* 11452
	qnrD	Quinolone resistance pentapeptide repeat protein	645	*P. mirabilis* 54-2
	***aac*(6')**	Aminoglycoside 6'-N-acetyltransferase	264	*P. mirabilis* 54-2 and *K. pneumoniae* 55-4
Production of ESBLs	bla_{PER}	Extended-spectrum class A β-lactamase family	739	*K. pneumoniae* 1803
	bla_{CTX-M}	Extended-spectrum class A β-lactamase family	593	*K. pneumoniae* 1803
	bla_{CMY}	Class C β-lactamase family	462	*K. pneumoniae* 1803
Production of carbapenemases	bla_{NDM}	Subclass B1 metallo- β-lactamase family	512	*K. pneumoniae* 54-2
	bla_{IMP}	Subclass B1 metallo- β-lactamase family	404	*K. pneumoniae* 52-3
	bla_{VIM}	Subclass B1 metallo- β-lactamase family	261	*E. coli* 49-1
	$bla_{OXA-48-like}$	Class D β-lactamase family	775	*Providencia stuartii* 51-1
	bla_{KPC}	Carbapenem-hydrolyzing class A β-lactamase family	916	*K. pneumoniae* 51-2

685 ESBLs: Extended-spectrum β-lactamases.

686 Gene descriptions database: National Center for Biotechnology Information (NCBI)

687 (https://www.ncbi.nlm.nih.gov/).

688

689 **Table 2.** Results of phenotypic antimicrobial susceptibility testing of *Pseudomonas* sp.

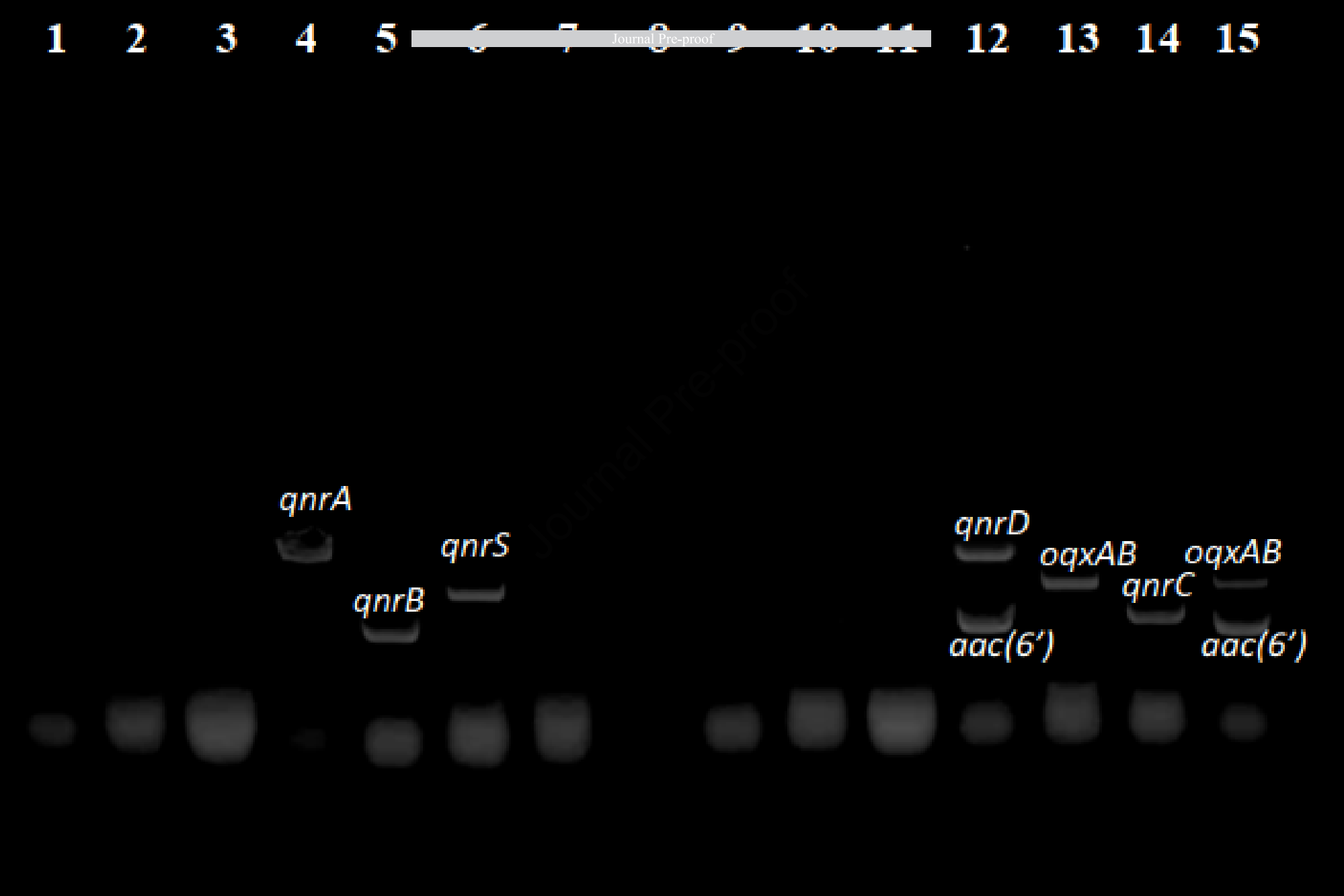
690 P26.

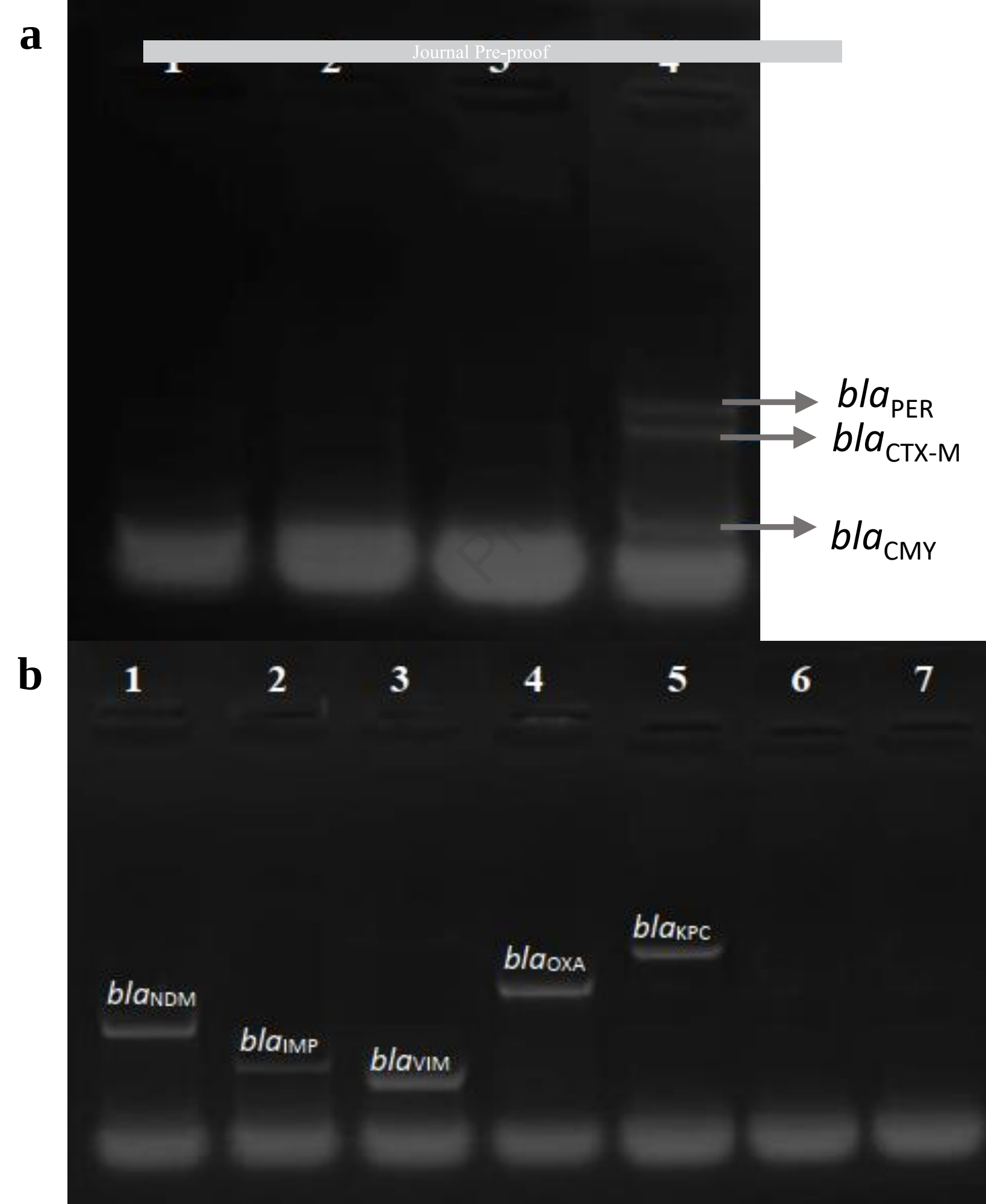
Antimicrobial agent	Results of Kirby-Bauer method	Results of automated system VITEK 2 (BioMérieux®)	
		MIC (µg mL^{-1})	Interpretation
Ceftazidime	S	≤1	S
Ceftazidime-Clavulanic acid	S	ND	ND
Ciprofloxacin	S	≤0.25	S
Gentamicin	S	≤1	S
Amikacin	S	≤2	S
Aztreonam	S	ND	ND
Cefepime	S	≤1	S
Piperacillin-Tazobactam	S	8	S
Meropenem	S	≤0.25	S
Imipenem	S	≤0.25	S
Ceftazidime-Avibactam	S	ND	ND
Cefotaxime	ND	8	S
Trimethoprim-Sulfamethoxazole	R	≥320	R

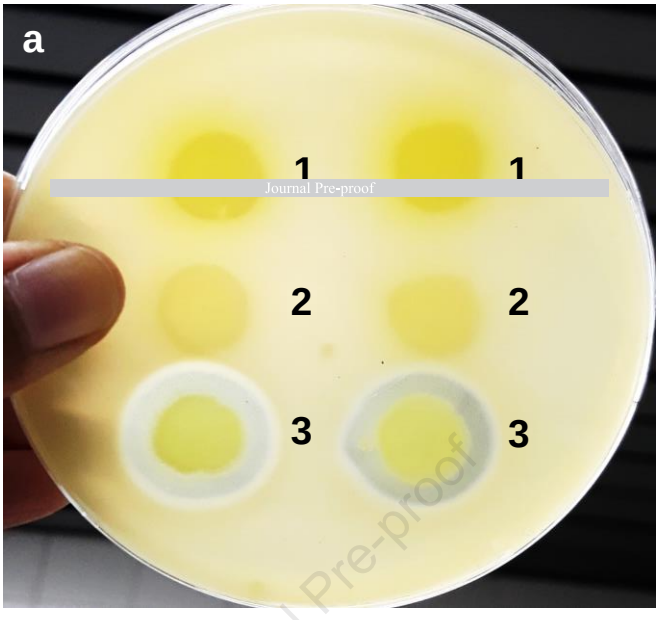
691 MIC: Minimal Inhibitory Concentration; S: Susceptible; R: Resistance; ND: Not

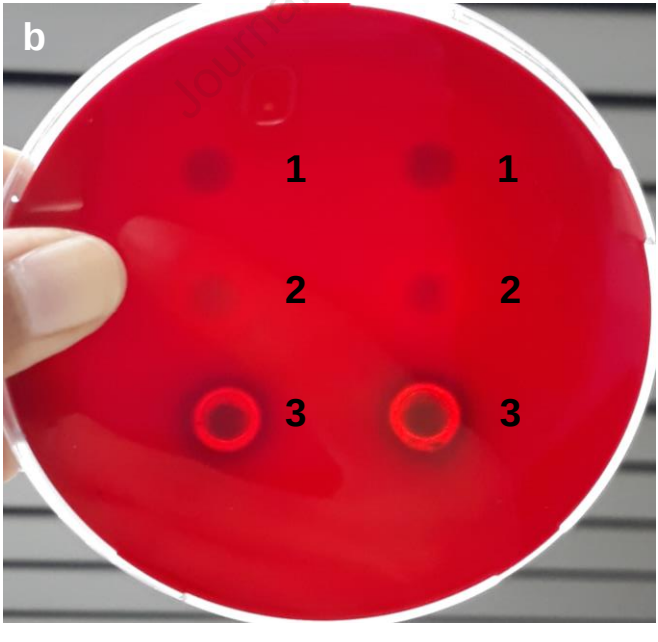
692 determined.

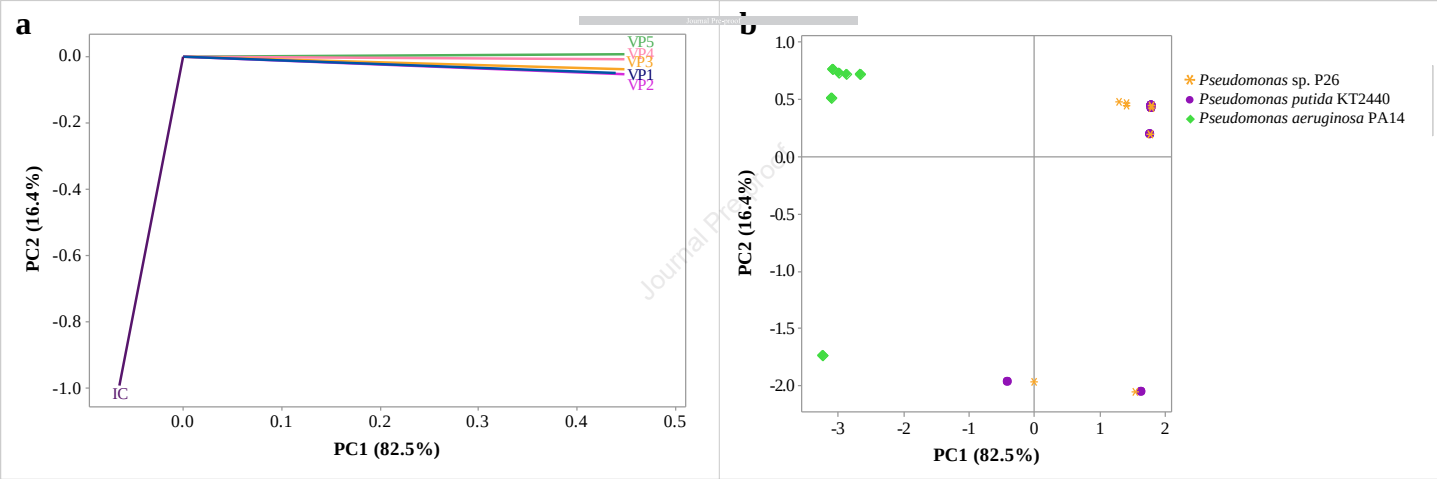
693



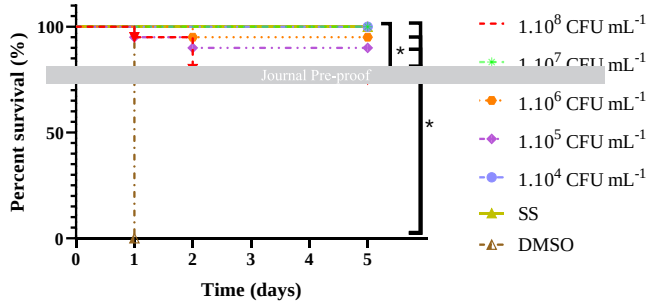




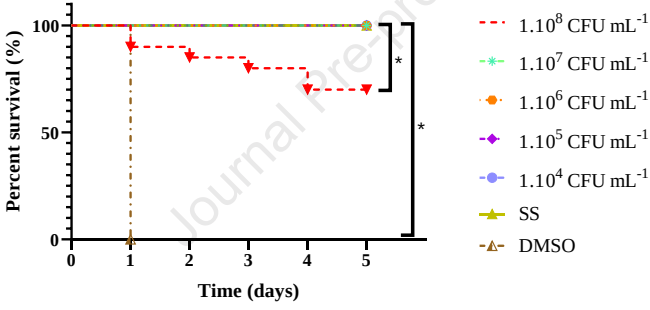



a
VP5
VP4
VP3
VP1
VP2
IC
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
0.0
0.1
0.2
0.3
0.4
0.5
PC2 (16.4%)
PC1 (82.5%)
b
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-3
-2
-1
0
1
2
PC2 (16.4%)
PC1 (82.5%)
Pseudomonas sp. P26
Pseudomonas putida KT2440
Pseudomonas aeruginosa PA14

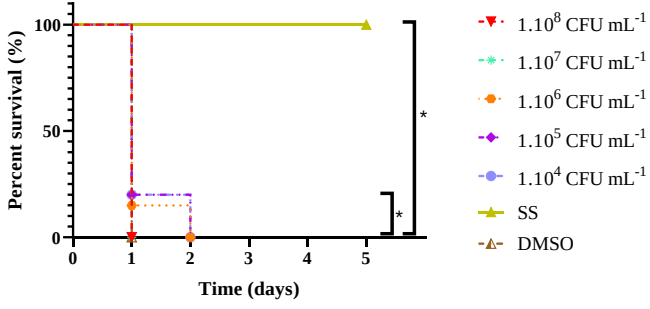
a ***Pseudomonas* sp. P26**



b ***Pseudomonas putida* KT2440**



c ***Pseudomonas aeruginosa* PA14**



Highlights

- The study of safety features of a strain for use in bioremediation is essential.
- *Pseudomonas* sp. P26 displayed low level of phenotypic antimicrobial resistance.
- No quinolone and β-lactam resistance genes were identified.
- The *Galleria mellonella* model was a useful tool for studying bacterial virulence.
- *Pseudomonas* sp. P26 lacked virulence factors and did not affect larval survival.

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: