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Research Article

Biofilm Formation and Inhibitory Effect of Essential Oils in Multidrug-Resistant *Pseudomonas aeruginosa*

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Abstract

Biofilm formation is a major concern in medicine, as well as in the food industry. Some infections related to bacterial biofilms such as *Pseudomonas aeruginosa* are a real public health challenge. This study aimed to show the activity of essential oils on *P. aeruginosa* biofilm formation. *Pseudomonas aeruginosa* isolates consisting of animal (100), environmental (20) and clinical strains (42) were identified by PCR and sequenced. Biofilm formation was assessed by the microplate method. Antibiotic susceptibility testing was performed by using Kirby–Bauer disc-diffusion method. The average biofilm formation percentages vary from 1.2 to 2.1 in 24h and from 2.3 to 3.2 in 48h. The median biofilm formation value was higher in environmental strains (1.4 ± 0.2) than in clinical (1.2 ± 0.4) and animal (1.1 ± 0.4). In decreasing order of importance, the essential oils of Mentha piperita (90 ± 5.12% at 100%), Eucalyptus globulus (34 ± 0.08% at 100%) and Lavandula angustifolia (12 ± 0.71% to 100%) showed distinct inhibitory effects on biofilm formation ($p < 0.05$). The rate of resistance of *P. aeruginosa* to the antibiotics imipenem, ceftazidime, cefepime, fosfomycin and colistin varied from 12.7% to 48.4% in the biofilm status while that of plankton cells ranged from 2.3% to 15.0%. Moreover, resistance to ticarcillin, ticarcillin clavulanic acid, piperacillin and ciprofloxacin ranged from 56.4% to 83.1% in biofilm and from 29.4% to 51.4% in planktonic cells. In general, biofilm is more resistant to different antibiotics than free cells. The tested essential oils could be an effective natural control against microbial biofilm formation.

Keywords: *P. aeruginosa*; Biofilm; Essential oils; Antibiotics resistance

Introduction

Biofilms are an aggregate of microorganisms frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) [1]. These microorganisms adhere to each other and/or to a surface, protecting them from environmental stresses. Biofilms are the source of persistent infections caused by many pathogenic microbes [2]. Moreover, bacterial biofilms can survive antibiotics due to impaired antibiotic diffusion, antibiotic efflux, expression of biofilm-specific genetic mechanisms, selection of resistant mutants or nutrient and oxygen limitation [3-5]. Over the last few decades, the study of microbial biofilms has been gaining interest among the scientific community.

Various studies indicate that the lifestyle of biofilms, their structure and their composition lead to an increase in resistance to antimicrobial agents [6]. Thus, biofilm bacteria resist the host's immune response and may be 10 to 1000 times more resistant to antimicrobial agents than planktonic bacterial cells [7]. The biofilm formation can have a negative impact in different sectors within society; namely in agriculture, food industries, veterinary and human health, as it could lead to substantial economic losses [2].

In the food industry, biofilms are the source of many problems, in terms of hygiene and deterioration of the organoleptic qualities of food products [8,9]. In dairy industries, for example, bacterial species can remain on certain parts of the equipment, which promotes their development in the form of biofilms and thus contaminate the finished product [10,11]. Likewise, the presence of biofilm on surfaces found on the farm, at the slaughterhouse in water pipes or at the processing plant will affect the effectiveness of the disinfection protocol [9].

In medicine, biofilms are of particular importance because they are implicated in a wide range of infections in humans. In addition, nearly 80% of human bacterial infections are biofilm-associated. Infections resulting from biofilms pose real public health problems [8,9].

Among the germs involved in the formation of biofilms, *Pseudomonas aeruginosa* is increasingly mentioned for each health insecurity factor, whether in terms of food poisoning or nosocomial infections [6,12]. *Pseudomonas aeruginosa* is an opportunistic pathogen frequently implicated in biofilm-related infections [1].

This microorganism has several virulence mechanisms that promote its pathogenesis, in particular the production of biofilm [13]. Although *P. aeruginosa* has been widely used as a study model in biofilm, to date there are no guidelines for the treatment of biofilm infections caused by *P. aeruginosa* [14].

Therefore, *P. aeruginosa* biofilm infections present a pharmacological and medical challenge. Anti-infective agents that selectively interrupt virulence pathways to prevent or cure infection are less likely to promote the emergence of resistance [13]. Among these anti-infective agents, certain natural essential oils show

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promising therapeutic properties to fight against emerging resistance [15].

This study aimed to determine the inhibition of biofilm formation by essential oils in *P. aeruginosa* isolates obtained from various origins.

Materials and Methods

Isolation and identification of *P. aeruginosa*

A total of 162 strains of *P. aeruginosa* isolated from animal infections (100), clinical (42) and environmental (20) settings were studied. The isolates were identified by classical microbiology and biochemical characters using API 20NE (bioMérieux, Marcy l'Etoile, France). The molecular identification of *P. aeruginosa* strains using Polymerase Chain Reaction (PCR) was conducted using the 16S gene. The reference strain *P. aeruginosa* PA14 was used as quality control.

Extraction and purification of the genomic DNA of *P. aeruginosa*

Pseudomonas aeruginosa strains were harvested from an overnight broth culture. Genomic DNA was extracted and purified according to the method described by Amutha and Kokila [16]. After extraction, DNA was diluted and stored at -20°C to serve as a DNA template for polymerase chain reactions (PCR).

Amplification of the 16S rDNA Gene for *P. aeruginosa* detection

The 50µL reaction mixture consisted of 38µL of sterile Milli-Q water (milli-Q™, Millipore Corporation, Foster City, CA, USA) ; 5µL of 10 X concentration loading buffer; 1µL of $Mgcl_2$, 25mM (Promega Corporation, Madison, WI, USA) ; 1µL of d'NTPs, 10mM; 1µL of each primer 27F and 1492R, 10mM (TranS, AP111 5U, Macau City, China); 0.5µL of BSA, 20mg/mL and 0.5µL of Easy Tag® DNA polymerase with a final concentration of 1.5U (TranS, AP111 5U, Macau City, China) and 2µL of the DNA matrix.

Sterile Milli-Q water and the reference strain *P. aeruginosa* PA14 were used as negative control and positive control, respectively, for each PCR reaction run.

Amplification of the 16S rDNA gene was performed according to the method described by Amutha and Kokila [16] using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (3′-TACGGYTACCTTGTTACGACTT-5′). The amplification program included an initial denaturation of 5min at 94°C followed by 35 cycles of denaturation (94°C for 30s), annealing (55°C for 40s) and extension (72°C for 30s), with a single final extension of 10min at 72°C. The samples were stored at 4°C until the Thermocycler was stopped.

Sequencing of *P. aeruginosa* strains

After purification of the PCR products using a commercial kit (EZ-10Spin Column PCR Products Purification Kit, Foster City, CA, USA), these products were sequenced with primers 27F and 1492R in an automated DNA sequencer 310 (Applied Biosystem, Foster City, CA, USA). The sequences obtained were analyzed in the NCBI database using BLAST for strains confirmation, as described in our previous publications [17].

Biofilm formation by the microplate method

Biofilm formation: Biofilm-forming ability of *P. aeruginosa* was measured in 96-well polystyrene microplates by using the method described by [18,19] with some adaptations. Different suspensions with a final volume of 1.2mL consisting of LB medium diluted 1:10 and Casamino acid with a final concentration of 0.5% are produced in different Eppendorf tubes for each strain of *P. aeruginosa*. The overnight cultures were adjusted to a 0.5 McFarland (10^8CFU/mL). Each strain was tested in 5 replicates after inoculation of a standardized culture in Tryptic Soy Broth (TSB) added with 0.2% of glucose. Negative control wells contained noninoculated Tryptic Soy Broth (TSB) with glucose. Samples (200µL) were dispensed into wells, and the microplates were covered and incubated aerobically without agitation at 37°C for 24 and 48 h.

Biofilm assay: In detail, after 24h and 48h, the bacterial suspension was aspirated, and each well was washed three times with phosphate-buffered saline (PBS) (Sigma). The plates were dried at room temperature and stained with 200µL 1% (v/v) of crystal violet solution used for Gram staining (Merck Millipore) for 30min. The excess crystal violet is removed by 3 to 10 successive manual washings of the plates with milliQ water and dried at room temperature. After that, the biofilm was fixed with 300µL of ethanol (95%) for 15min, and was later removed. The dye bound to the adherent cells was resolubilized with 160µL of 33% (v/v) glacial acetic acid (Sigma) per well. The dye concentration or absorbance (OD) is measured with a spectrophotometer at 595nm wavelength with the Cytation™ 5 Cell Imaging Multi-Mode Reader (BioTek Instruments, Innovation, CA, USA), linked to the analysis software Data Gen 5 v. 2.04 ™ (BioTek Instruments, Innovation, Foster City, CA, USA).

Finally, the strains were grouped into: OD595 <0.1, nonproducers (NP) ; OD595 = 0.1-1.0, weak producers (WP); OD595 = 1.1-3.0, moderate producers (MP); and OD595 >3.0, strong producers (SP).

Determination of the antibiofilm effect of essential oils

The antibiofilm activity of three essential oils (*Lavandula angustifolia* (true lavender), *Mentha piperita* (peppermint) and *Eucalyptus globulus* (eucalyptus)) was evaluated. This antibiofilm activity was compared with that of furanone (reference antibiofilm molecule) according to different doses of essential oils and the biofilm formation time. Thus, furanone and essential oils were introduced at a dose of 100%, 50% and 25% after formation of the biofilm in 24h.

The percentage inhibition of biofilm formation (PI) is determined according to the formula below [20]. On the one hand, it was a question of comparing the optical densities (OD) of the white wells without bacteria (B) with the OD of the control wells without antibiofilm molecule (C). On the other hand, to compare the optical densities (OD) of the same white wells without bacteria (B) with the ODs of the wells containing the antibiofilm molecules (S), we used the following equation.

$$PI = \frac{(C-B)-(S-B)}{(C-B)} * 100 \qquad (1)$$

Antimicrobial susceptibility of planktonic cell and biofilm

Antibiotic susceptibility testing was performed by using the Kirby–Bauer disc-diffusion method according to the CA-SFM/EUCAST recommendations [21]. The antibiotics tested and their sensi-disk concentrations are mentioned in Table 1. The standard

reference strain of *P. aeruginosa* ATCC 27,853 was used as a quality control. The method remains the same for the determination of biofilms resistance, but the inoculum of approximately 10^6CFU/mL is obtained from the 24h biofilm taken from a well of microplates.

Statistical analysis

Data were entered into the SPSS Statistics 20.0 data processing software (IBM Corporation, SPSS Inc, Chicago, IL, USA) and transferred to Excel. Data were entered with Data Gen 5 v. 2.04™. The data were analyzed by t-test at a statistical level of $\alpha < 0.05$ (Excel, MS office, Chicago, IL, USA).

Results

Strains of *P. aeruginosa*

Laboratory diagnosis of infections caused by *P. aeruginosa* is most often performed by conventional methods such as growth on specific culture media. In this study, molecular identification using the 16S gene (Figure 1) and sequencing of *P. aeruginosa* strains confirmed the identity of the isolates from the samples analyzed.

Biofilm formation over time

The study demonstrated the ability of Pseudomonas strains of various origins to form biofilms. *P. aeruginosa* strains of animal, environmental and clinical origin form biofilms in 48h as well as in 24h. The average percentages of adhered cells vary, respectively from 1.2 to 2.1 in 24h and from 2.3 to 3.2 in 48h, in the three groups of above-mentioned strains (Figure 2). The results also show that only strains of environmental origin adhere significantly within 12h (Figure 2). All strains were biofilm producers, classified as strong, moderate and weak producers (Table 2).

Median value of biofilm formed in *P. aeruginosa*

The study revealed that the median value of biofilm formed is higher in environmental strains (1.4 ± 0.2) than in clinical strains (1.2 ± 0.4) and animal strains (1.1 ± 0.4) (Table 3). The different median values obtained (1.1; 1.2 or 1.4) indicate that at least 50% of the values of the statistical series are below the median and 50% of these statistical values are above the median (Figure 3).

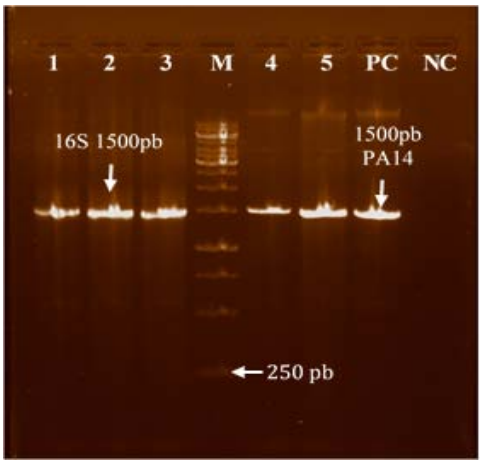

Figure 1: 16S profiles of *P. aeruginosa* isolates. Lanes 1-5: Presence of *P. aeruginosa*; (PC: Positive Control (*P. aeruginosa* PA 14); NC: Negative Control; M: Marker Gene Ruler 250 bp (Bench Top, 1kb DNA Ladder, Promega Corporation, Foster City, CA, USA).

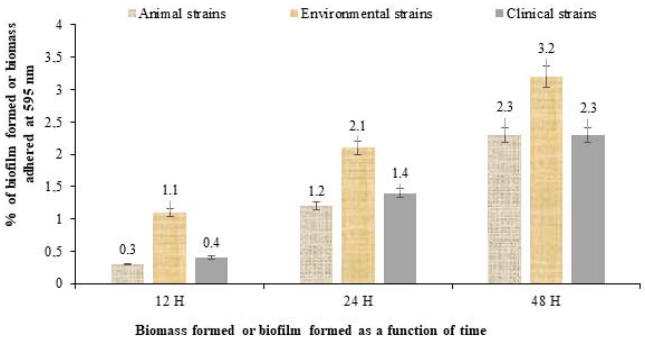

Figure 2: Evaluation of the adhered biomass or biofilms formed as a function of time in *P. aeruginosa*.

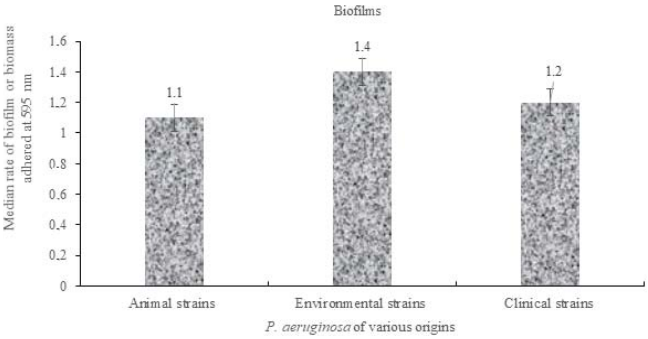

Figure 3: Adhered biomass or biofilms formed after 48 in LB in 96-well microplates, with *P. aeruginosa* strains of various. Origins (Median ± standard deviation).

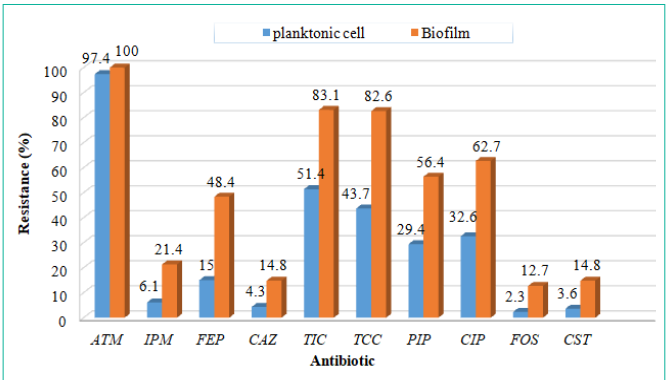

Figure 4: Antibiotic resistance rate of biofilm and planktonic cells in *P. aeruginosa*. TIC: Ticarcillin; TCC: Ticarcillin/Clavulanic Acid; FEP: Cefepime; CAZ: Ceftazidime; IPM: Imipenem; ATM: Aztreonam; CIP: Ciprofloxacin; PIP: Piperacillin; FOS: Fosfomycine; CST: Colistin.

Antibiofilm effect of essential oils

The results showed that at 100% concentration, true lavender, peppermint and eucalyptus oils inhibit and disperse biofilm formation in *P. aeruginosa* (Table 4). At this concentration, the essential oils tested have the same efficacy as furanone, a molecule known for its inhibitory action on biofilm formation. Among the essential oils tested, that of peppermint showed an efficacy much closer to that of furanone on the formation and dispersion of *P. aeruginosa* biofilms with percentages of inhibition ranging from 90 ± 5.12 to 100% (Table 4).

Table 1: The antibiotics tested.

Class of Antibiotic	Antibiotic	Abbreviation	Concentration
Beta-lactams	Ticarcillin	TIC	75µg
	Ticarcillin clavulanic acid	TCC	75-10 µg
	Aztreonam	ATM	30µg
	Imipenem	IPM	10µg
	Piperacillin	PIP	100µg
	Cefepime	FEP	30µg
	Ceftazidime	CAZ	10µg
Fosfomycin	Fosfomycin	FOS	200µg
Aminosides	Kanamycin	K	30µg
Polymyxins	Colistin	CST	10µg
Fluoroquinolones	Ciprofloxacin	CIP	100µg

TIC: Ticarcillin; TCC: Ticarcillin/Clavulanic Acid; FEP: Cefepime; CAZ: Ceftazidime; IPM: Imipenem; ATM: Aztreonam; CIP: Ciprofloxacin; PIP: Piperacillin; FOS: Fosfomycine; CST: Colistin.

Antibiotic resistance of planktonic cells and *P. aeruginosa* biofilms

The results revealed that the resistance of *P. aeruginosa* biofilms to the antibiotics IMP, CAZ, FEP, FOS and CST is three times that of planktonic cells. The resistance rates for the antibiotics vary, respectively from 12.7% to 48.4% at the level of the biofilms and from 2.3% to 15.0% for the planktonic cells. The rate of resistance to TIC, TCC, PIP and CIP is twice as high in *P. aeruginosa* with biofilms (56.4% to 83.1%) than in planktonic cells (29.4% to 51.4%) (Figure 4). Planktonic cells and biofilms exhibited almost the same rates of natural resistance to aztreonam (ATM).

Discussion

The biofilm problem remains typical for *P. aeruginosa* [22]. Indeed, one of the characteristics of biofilms, especially *P. aeruginosa*, will be a loss of sensitivity to treatments available, including antibiotics commonly used as antipyocyanics [9]. This study therefore demonstrated the ability of *P. aeruginosa* strains of various origins to form biofilms. This study shows that *P. aeruginosa* strains (animal, environmental and clinical) form biofilms in both 24h and 48h. All strains were biofilm producers, classified as strong, moderate and weak producers [11]. Only strains of environmental origin adhere significantly within 12h.

In addition, we observed that biofilm formation in *P. aeruginosa* strains was promoted within 48h. Mainly, the strains unable to produce a biofilm within 48h were nonproducers or particularly weak producers. It is believed that biofilm formation is promoted by cell motility, especially when mediated by flagella, and under certain environmental conditions flagella are required for biofilm formation in *P. aeruginosa*. Thus, we can hypothesize that the biofilm-forming ability of the strains here studied could be due to bacterial surface appendages that can be time dependent.

The study revealed that the median value of biofilm formed is higher in environmental strains (1.4 ± 0.2) than in clinical (1.2 ± 0.4) and animal strains (1.1 ± 0.4). These results indicate that environmental strains may be more resistant to antimicrobial treatments than animal and clinical strains [23].

This observation has been made by several authors who have shown that the composition and availability of nutrients in the environment could promote the formation of biofilms [9,11].

Indeed, during their growth, bacteria produce diffusible signaling molecules or homoserine lactone which are involved in quorum sensing [24]. These molecules were accumulated in the environment and promote the formation of biofilm. Therefore, bacterial growth could influence biofilm formation. The OD or adhesion rate obtained in clinical strains (1.2 ± 0.4) could be explained by intrinsic changes and by resistance due to inappropriate use of antibiotics [25]. The ability of animal strains to form biofilms could be explained by poor hygiene and poor cleaning systems in indus-tries processing these animal products [11]. Indeed, these inappropriate cleaning

Table 2: Percentage of *P. aeruginosa* strains capable of producing biofilm after 24h or 48h of incubation.

Origins	Total Strains	Percentage after 24h				Percentage after 48h			
	(*n*)	NP	WP	MP	SP	NP	WP	MP	SP
Animal strains	100	3%	34%	37%	26%	0	13%	47%	40%
Environmental strains	20	0	10%	70%	20%	5%	15%	30%	50%
Clinical strains	42	4%	12%	60%	24%	2%	7%	48%	43%

NP: Non producers; WP: Weak Producers; MP: Moderate Producers; SP: Strong Producers.

Table 3: Median values of adhered biomass or biofilm formed at 595nm.

Adhered biomass (biofilm)	Minimum OD	OD Median	Maximum OD
Animal strains n = 100	0.2	1.1 ± 0.4	2.3
Environmental strains n = 20	0.3	1.4 ± 0.2	3.4
Clinical strains n = 42	0.3	1.2 ± 0.4	2.3

Table 4: Antibiofilm effect of essential oils in *P. aeruginosa*.

Concentration (µL/mL)	% Inhibition of *P. aeruginosa* Biofilm in 48h			
	Furanone	*Lavandula angustifolia* (*True Lavender*)	*Eucalyptus globulus* (*Eucalyptus*)	*Mentha piperita* (*Peppermint*)
100%	100	100	100	100
50%	100	25 ± 0.50	74 ± 7.78	100
25%	98 ± 0.71	12 ± 0.71	34 ± 0.08	90 ± 5.12

and disinfection systems promote an accumulation of organic matter, allowing bacteria to adhere to surfaces and the formation of microcolonies involved in the formation of biofilms [9]. This study also showed that the essential oils studied, (*Lavandula angustifolia, Mentha piperita* and *Eucalyptus globulus*) exhibited an ability to disrupt biofilm formation in *P. aeruginosa*. The introduction of these oils at different doses in the biofilm formation cycle or in 24 or 48 h biofilms demonstrated the ability of these three essences to disrupt biofilm formation in *P. aeruginosa*. Peppermint (*Mentha piperita*) oil, with characteristics very close to those of furanone, exhibited an antibiofilm effect, capable of modifying the kinetics of biofilm formation and significantly reducing the number of adhered cells. Peppermint oil exhibited antibiofilm activity with a percentage inhibition of adherent cells ranging from 90 ± 5.12% to 100%.

This result suggests that a cytotoxicity test combined with a cell adhesion inhibition test could define the potential interest of this selected essence in the preventive or curative treatment of *P. aeruginosa* infections [8,9,15]. The efficiency of this oil in eliminating and dispersing the biofilms of *P. aeruginosa* shows that this essential oil could act on the different LasR/LasI, RhlR/RhlI and Pqs (Pseudomonas quinolone signals) systems involved in quorum sensing in *P. aeruginosa* [8,9,26,27]. These results suggest that these essential oils could constitute new therapeutic molecules acting on new targets in *P. aeruginosa* infections [15]. These results indicate that these natural substances may reduce the virulence of *P. aeruginosa* and may have implications in the development of alternative approaches to control bacterial infections [11,28].

Several studies confirm the potential of essential oils to inhibit biofilms in *P. aeruginosa* and even in *E. coli* [29,30]. These studies have shown on the one hand that the antibacterial activities of certain essential oils are attributed to considerable alterations in the structure of cell envelopes [30]. On the other hand, these studies have shown that these natural substances only affect biofilm susceptibility of bacterial strains [30,31].

The results finally revealed that the resistance of *P. aeruginosa* biofilms to certain cephalosporins (CAZ, FEP) and carbapenem (IMP) as well as to fosfomycin (FOS) and colistin (CST) is three times that of planktonic cells [32]. The rate of resistance to penicillins (TIC, TCC, PIP) and fluoroquinolones (CIP) is twice as high in biofilm strains of *P. aeruginosa* than in planktonic cells [33]. This same observation was made by [33] who reported that bacteria in biofilm were more resistant to antibiotics than planktonic bacteria. The greater resistance of *P. aeruginosa* biofilms to antibiotics could be attributed to efflux pumps in its biofilm, actively expelling antimicrobial components [6,27].

In fact, in *P. aeruginosa*, four efflux systems are described: MexAB-OprM; MexCD-OprJ; MexEF-OprN and MexXY-OprM. Among these systems, the MexAB-OprM and MexXY-OprM efflux pumps can, when overproduced, help to increase the resistance of the bacteria to several families of antibiotics [34]. This phenomenon is linked to the occurrence of spontaneous mutations in the regulatory genes of these systems. MexAB-OprM, MexCD-OprJ and MexXY-OprM confer resistance to β-lactams in clinical isolates of *P. aeruginosa* [25].

MexAB-OprM pumps have also been implicated in ticarcillin resistance and its expression is statistically linked to aztreonam resistance [34].

The presence of the exopolysaccharide matrix also slows down the penetration of antibiotics and biocides. This polymeric matrix acts as a barrier reducing or preventing the diffusion of antimicrobial agents. In [35], it was indicated that the metabolism of bacteria in a biofilm also plays a very important role in the resistance of the biofilm to antimi-crobials.

Indeed, the low concentration of certain nutrients and the oxygen gradient cause certain cells of the biofilm to be in their dormant form and not very metabolically active [34,36]. These dormant bacterial cells are probably responsible for a large part of the tolerance associated with biofilms [34]. Finally, the spatial proximity of bacteria within a mature biofilm probably promotes horizontal gene transfer and increased resistance to antibiotics.

Conclusions

This study revealed the ability of *P. aeruginosa* strains to form biofilms. The impact of biofilms on animal health and public health is very evident. The essential oils of true lavender (*Lavandula angustifolia*), peppermint (*Mentha piperita*) and eucalyptus (*Eucalyptus globulus*) showed distinct inhibitory effects on biofilm formation. This study indicates that these natural essential oils may reduce the virulence of *P. aeruginosa* and have implications in the development of alternative approaches to control bacterial biofilm infections.

Declaration

Author contributions: CKDB and AD: Conceived and designed the study. CKDB and AD: Performed the experiments. CKDB, KA, YCTB and YPA: Analyzed the data. CKDB, AD and YPA: Contributed reagents, materials and analysis tools. CKDB, YPA and YCTB: Wrote the paper. All the authors read, reviewed, and approved the final manuscript.

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