Archives of Microbiology (2022) 204:709
https://doi.org/10.1007/s00203-022-03308-8

ORIGINAL PAPER

In vitro virulence activity of *Pseudomonas aeruginosa*, enhanced by either *Acinetobacter baumannii* or *Enterococcus faecium* through the polymicrobial interactions

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Received: 10 October 2022 / Revised: 10 October 2022 / Accepted: 26 October 2022 / Published online: 16 November 2022

Abstract
Microbes within an infection impact neighbors' pathogenicity. This study aimed to address in vitro virulence activity of *Pseudomonas aeruginosa* under the binary interaction with *Acinetobacter baumannii* or *Enterococcus faecium*, co-isolated from two chronic wound infections. The biofilm formation of *Pseudomonas* was enhanced 1.5- and 1.4-fold when it was simultaneously cultured with *Acinetobacter* and *Enterococcus*, respectively. *Pseudomonas* motility was increased by 1.9- and 1.5-fold (swimming), 3.6- and 1.9-fold (swarming), and 1.5- and 1.5-fold (twitching) in the dual cultures with *Acinetobacter* and *Enterococcus*, respectively. The synergistic hemolysis activity of *Pseudomonas* was observed with the heat-killed *Acinetobacter* and *Enterococcus* cells. The minimum inhibitory concentration of ciprofloxacin against *Pseudomonas* was increased from (μg mL^{-1}) 25 to 400 in the individual and mixed cultures, respectively. The pyocyanin production by *Pseudomonas* in the single and mixed cultures with *Acinetobacter* and *Enterococcus* was (μg/mL) 1.8, 2.3, and 2.9, respectively. The expression of *lasI*, *rhlI*, and *pqsR* genes was up-regulated by 1.0-, 1.9-, and 16.3-fold, and 4.9-, 1.0-, and 9.3-fold when *Pseudomonas* was incubated with *Acinetobacter* and *Enterococcus*, respectively. Considering the entire community instead of a single pathogen may lead to a more effective therapeutic design for persistent infections caused by *Pseudomonas*.

Keywords Biofilm · Poly-microbial infection · *Pseudomonas* · *Acinetobacter* · *Enterococcus* · Quorum sensing

Introduction

Progress in culture-dependent studies, along with the development of culture-independent methods, demonstrates the community composition of many infections and raised the possible impact of species interaction on the progression of diseases (Peters et al 2012; Cummings et al 2020). The term poly-microbial infection is defined as pathological manifestations caused by more than one strain/species. Co-existing microorganisms can affect their neighbors through the mechanisms like direct cell–cell contact, cell–cell communication via diffusible molecules, alteration of the host environment, and the utilization of others' metabolic by-products (Peleg et al 2010).

Chronic wounds are characterized by the impairment of the normal wound healing process in an orderly and timely manner (Frykberg and Banks 2015). They impose an enormous burden on the healthcare systems, and it is estimated that 2–3% of the healthcare budgets are related to chronic wounds. *P. aeruginosa* has been identified as a significant pathogen in chronic wound infections. This Gram-negative opportunistic pathogen can cause mild to life-threatening infections. *P. aeruginosa* often causes chronic infection due to its ability to form biofilms. The presence of microbes within the biofilms protects them from the host immune system, antibiotics, and other stressful conditions (Donlan 2002). The most studied biofilms were known to have a diverse population which means more than one species formed a cosmopolitan community. Therefore, pathogens (Like *Pseudomonas*) within biofilm may behave differently from single-cell planktonic life (or single sessile) due to the microbial interaction within these three-dimensional

Communicated by Muhammad Bilal.

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structures. Members of a poly-microbial infection can coordinate various social behaviors of neighboring populations, such as pathogenesis, by affecting their quorum sensing systems like L*as* and Rhl (depend on *N*-acyl-homo-serine lactone) and PQS (an alkylquinolone (AQ)-dependent quinolone signal system) that regulate much virulence-associated genes of *Pseudomonas* (Warrier et al 2021; Kostylev et al 2019).

The interactions of *Pseudomonas* with *Staphylococcus aureus* showed that poly-microbial infections are more virulent than mono-culture infections with either species (Limoli and Hoffman 2018). However, poly-microbial interactions of *Pseudomonas* with other bacteria have been less considered. In this study, we assessed the effect of *Pseudomonas aeroginosa* interaction with the two co-isolated strains from chronic wounds, i.e., *Acinetobacter baumannii* and *E. faecium*, on its in vitro virulence activities. We showed that the dual interactions could promote some virulence activity of *Pseudomonas*, including biofilm formation, hemolysis, motility, pigmentation, and antibiotic resistance. The up-regulations of quorum-sensing genes upon the microbial interaction were also observed.

Materials and methods

Bacterial strains

Pseudomonas aeroginosa/Acinetobacter baumannii and *Pseudomonas aeroginosa/Enterococcus faecium* were co-isolated from two skin chronic wounds. Ethics approval or waiver has been obtained with their letter number IR.UM.REC.1401.075 dated 2021. Bacterial identifications were confirmed by the conventional biochemical tests and molecular method based on the amplification and sequencing of their 16S rRNA genes.

Single and dual biofilm formation

The ability of *Pseudomonas* to form biofilm in the presence of *Acinetobacter* and *Enterococcus* was evaluated by the competition, displacement, and exclusion methods. All three strains were cultured overnight in Tryptic soy broth (TSB) medium at 37 °C and the standard cell concentrations (10^6 cells mL^{-1}) were prepared. Regarding the competition assay, 1000 µL of each strain suspension's (*Pseudomonas* and *Acinetobacter*/*Enterococcus*) was simultaneously added into the wells of 12-well polystyrene plates containing polystyrene disks with the area of 25 mm^2 placed in the bottom and incubated for 24 h at 37 °C. For the displacement test, *Pseudomonas* cells were transferred on 12-well plates for 24 h at 37 °C, the growth medium was replaced by fresh media containing *Acinetobacter*/*Enterococcus* suspension (10^6 CFU/mL). Exclusion assay was achieved when *Acinetobacter*/*Enterococcus* suspensions were pre-inoculated, and after 24 h incubation at 37 °C, the medium was aspirated, and fresh medium containing *Pseudomonas* cells (10^6 CFU/mL) was added to the wells and kept at 37 °C for further 24 h. Single species biofilms were formed as control by adding *Pseudomonas* suspension and TSB 1:1 to wells, followed by incubation for 24 h (or 48 h for displacement) at 37 °C. The polystyrene disks were removed from wells and washed three times with PBS. The attached *Pseudomonas* cells were scraped, and 100 µL aliquots of the desired dilutions were seeded on plates containing TSA medium and incubated for 24 h at 37 °C. The *Pseudomonas* colony forming units per milliliter (CFU/mL) were determined for the single and dual biofilms.

Motility investigation

Swimming, swarming, and twitching motilities of *Pseudomonas* were determined in the single and dual cultures. For the swimming and swarming motilities, 5 µL of each fresh bacterial suspension (as mentioned above) was mixed and inoculated directly into the center of semisolid (0.3% W/V agar) and solid (1.0% W/V agar) TSA medium, respectively. The twitching motility was assessed by 10 µL deep inoculation of mixed bacterial suspension into the agar–Petri dish interface. The diameters of the swimming and swarming motility zones were measured after 24 h incubation at 37 °C. To visualize the twitching motility, the agar medium was carefully removed with tweezers, and the plates were stained with 0.1% (V/W) crystal violet (5 min). In the single motility experiments as the control, *Pseudomonas* and TSB were co-inoculated 1:1. The presence of *Pseudomonas* in the colonies' edge was confirmed by the conventional Gram staining method (Cai et al 2020).

Antibiotic susceptibility test

Resistance of *Pseudomonas* to ciprofloxacin (Sigma-Aldrich, Germany) in the single and dual cultures was compared by the micro-dilution method. For that, TSB media contained single and dual bacterial cells (1:1 of standard fresh bacterial suspensions) were supplemented with different antibiotic concentrations of 0 to 100 ppm. The cultures were incubated at 37 °C for 24 h, and *Pseudomonas* growth was measured by counting colony-forming units (CFUs).

Hemolysin assay

Hemolysin activity of *Pseudomonas* singly and in the presence of *Acinetobacter*/*Enterococcus* live, dead, and cell-free supernatants (CFS) was investigated as described elsewhere (Sperandio et al 2010). The released hemoglobin in

the supernatant was measured at 540 nm using an ELISA reader, and the percentage (%) of total cell lysis was calculated as follows:

$$\text{Hemolsis}\,(\%):\frac{\text{OD treatment}-\text{OD negative control}}{\text{OD positive control}-\text{OD negative control}} \quad (1)$$

The negative and positive controls were prepared by mixing RBC suspension with RPMI and SDS (1% W/V), respectively. The fractional inhibitory concentration (FIC) index defined additive, antagonist, synergism, and indifferent hemolysis activity. The sum of the FICs (ΣFIC) was calculated for all experiments with the below equation:

$$\sum \text{FIC} = \text{FIC}_A + \text{FIC}_B$$

where FIC_A was the hemolysis percent of *Pseudomonas* in combination/ hemolysis percent of *Pseudomonas* alone, and FIC_B was the *Acinetobacter*/*Enterococcus* live, dead, and CFS hemolysis in combination/ *Acinetobacter*/*Enterococcus* live, dead and CFS hemolysis activity alone. FICI was interpreted as synergism, indifferent, additive and antagonism when $\leq 0.5, > 0.5$ and $\leq 1, > 1.0$ and ≤ 4.0, and > 4.0, respectively (Fadwa et al 2021).

Pigmentation

P. aeruginosa strain singly and mixed with *Acinetobacter*/*Enterococcus* was cultured in nutrient broth medium supplemented by glycerol and statically incubated at 37 °C for four days. The pigment was extracted from filtrated supernatant by chloroform following the methods described by DeBritto et al. (2020). The UV–visible spectrum of the pigment was recorded between 200 and 800 nm, and the intensity of pyocyanin concentration (μg ml^{-1}) was calculated according to the following equation:

$$\text{Concentration of pyocyanin}\,(\mu g/\text{mL}): \text{OD}_{520} \times 17.072$$

Electron microscope image

The cell pellets were fixed with 2.5% glutaraldehyde for two hours, serially dehydrated with ethanol, and then coated with gold. The samples were observed under LEO 1450 VP-Zeiss scanning electron microscope (SEM) (Jena, Germany) at the resolution of 2.5 nm and an accelerating voltage of 20 kV.

Gene expression analysis

Quantitative reverse transcriptase PCR (qRT-PCR) was performed against Rhl, LasI, and PQS genes using The ABI 7500 Real-Time PCR system (Applied Biosystems Co, California, USA). The transcripts were normalized to GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) levels (Shi et al 2019).

Statistical analysis

The standard error mean was calculated and shown as error bars on the figures. One-way analysis of variance and *t* test were performed by SPSS ver. 16 software (IBM Co) to determine the significance of results (*p* value less than 0.05).

Results

Bacterial strains were identified as *P. aeruginosa* (GenBank/EMBL/DDBJ accession number ON514172), *A. baumannii* (ON514174), and *E. faecium* (ON514173) and confirmed by biochemical tests (data not shown).

Single and dual biofilms of *Pseudomonas* with either *Acinetobacter* or *Enterococcus*

Pseudomonas biofilm was enhanced 1.5- and 1.4-fold when simultaneously cultured with *Acinetobacter* and *Enterococcus*, respectively (competition). *Pseudomonas* biofilm was decreased to 0.51- and 0.37-fold when the pre-formed biofilms were treated by *Acinetobacter* and *Enterococcus* strains, respectively (displacement). When *Pseudomonas* was added to the substratum with the attached *Acinetobacter* and *Enterococcus* cells in the exclusion methods, its biofilm formation was not changed (*Acinetobacter*) ($p > 0.05$) or reduced by half in comparison to the single biofilm (*Enterococcus*) (Fig. 1). However, *Pseudomonas* growth was not affected in the planktonic co-cultures with *Acinetobacter* or *Enterococcus* (Fig. 1. inset).

Pseudomonas motility in the single and dual cultures

The swimming motility of *Pseudomonas* was enhanced 1.9- and 1.5-fold in the presence of *Acinetobacter* and *Enterococcus*, respectively. The swarming motility was much affected in the co-culture with *Acinetobacter* and *Enterococcus* and was enhanced 3.7- and 1.9-fold compared to the axenic *Pseudomonas* culture, respectively. Regarding the twitching motility, no significant difference was observed between *Acinetobacter* and *Enterococcus*, and both strains increased 1.5-fold *Pseudomonas* motility in the dual cultures (Fig. 2).

The ciprofloxacin susceptibility and pigment production

The concentration of ciprofloxacin inhibited the single cell *Pseudomonas* biofilm was equal to 25 μg mL^{-1}. However,

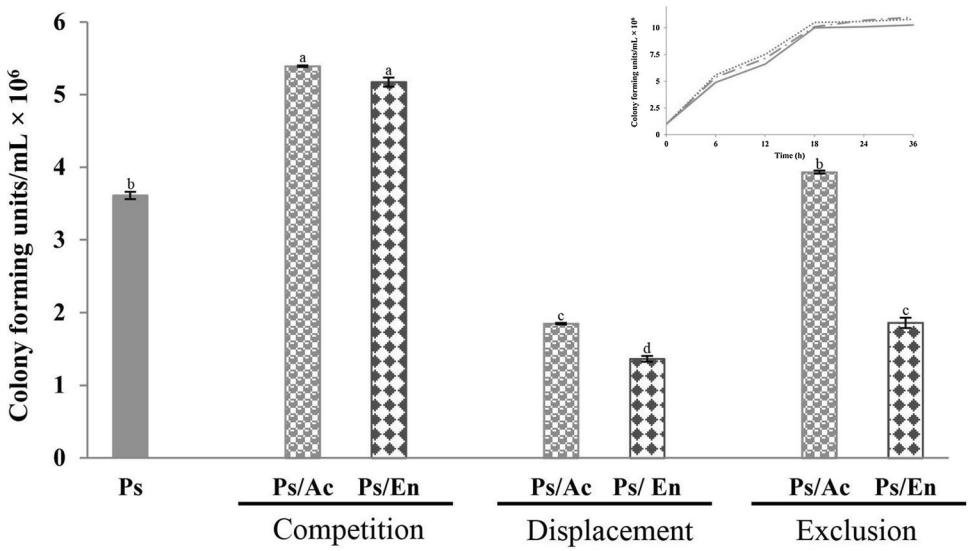

Fig. 1 Biofilm formation of *Pseudomonas aeruginosa* in the mono-culture (solid) and co-cultures with *Acinetobacter* (sphere) and *Enterococcus* (diamond) by competition, displacement, and exclusion. Error bars indicate standard deviation for three replicates, and different letters indicate significant differences between strains ($p < 0.05$). Inlet: *Pseudomonas* growth curve; alone (solid line), and in the presence of *Acinetobacter* (dash-dot line) and *Enterococcus* (dot line)

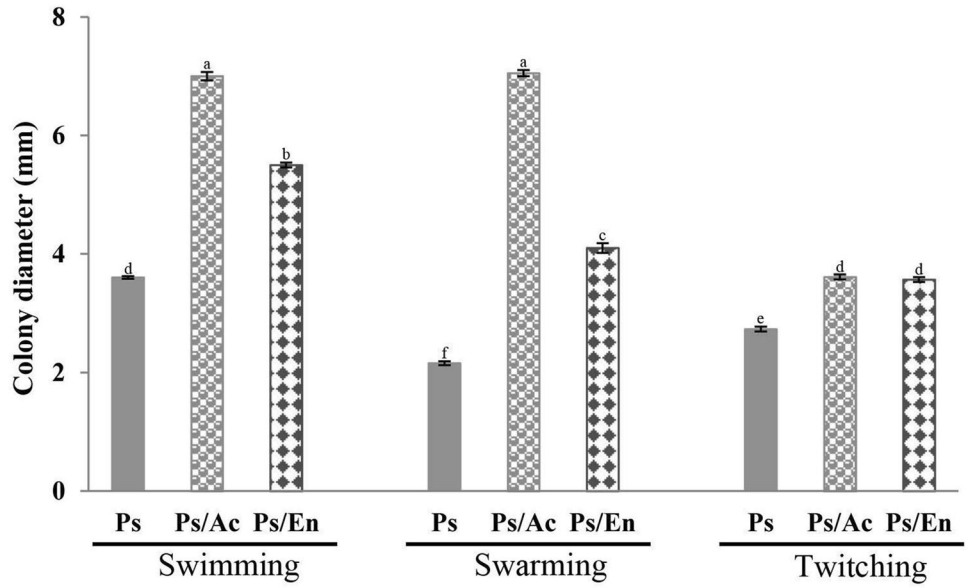

Fig. 2 *Pseudomonas* swimming, swarming, and twitching motility in the mono-culture (solid) and co-cultures with *Acinetobacter* (sphere) and *Enterococcus* (diamond). Error bars indicate the standard deviation for three replicates, and different letters indicate significant differences between strains ($p < 0.05$)

the *Pseudomonas* cells could tolerate up to 400 µg ciprofloxacin in the mixed biofilms with *Acinetobacter*/*Enterococcus* (Fig. 3). The UV–Vis spectrum of pigment showed a similar pattern in the single and dual *Pseudomonas* cultures except for the absorbance peaks seen in the *Pseudomonas*–*Acinetobacter* at 290 nm (Fig. 1. inlet). The crude pyocyanin production titer in the single *Pseudomonas* culture was 1.8 µg/ml, whereas it increased up to 2.3, and 2.9 µg/ml in the co-culture with *Acinetobacter* and *Enterococcus*, respectively.

Hemolysis activity

The synergistic hemolysis activity of *Pseudomonas* was observed with dead *Acinetobacter* and *Enterococcus* cells. Simultaneous culture of *Pseudomonas* and live *Acinetobacter*/*Enterococcus* reduced its hemolysis activity (antagonist). *Acinetobacter* CFS showed an additive effect on *Pseudomonas* hemolysis, while the *Enterococcus* cell-free supernatant did not increase nor decrease the *Pseudomonas* hemolysis (indifferent) (Table 1).

Las, Rhl, and PQS genes expression in single and dual *Pseudomonas* cultures

Rhl was the most affected *Pseudomonas* quorum-sensing system where its expression increased 16.3- and 9.3-fold in the mixed culture with *Acinetobacter* and *Enterococcus*, respectively. The expression of PQS and LasI was not significantly varied in *Pseudomonas* co-cultured with *Acinetobacter*/*Enterococcus*, respectively ($P > 0.05$), but

increased 4.9- and 1.9-fold when *Pseudomonas* were co-cultured with *Enterococcus* and *Acinetobacter*, respectively (Fig. 4).

Scanning electron microscope image

SEM analysis was conducted to represent the morphology of the *Pseudomonas* cells in the presence of *Acinetobacter* and *Enterococcus*. The micrographs represent that *Pseudomonas* morphology was not affected by the co-cultivation, and the intact rod-shaped cells could be observed in the mixed culture by *Acinetobacter* and *Enterococcus* (Fig. 4 inlet).

Discussion

It is estimated that more than 60% of the chronic wounds are caused by microbial biofilms (Høiby et al 2015). Dual biofilm formation of *Pseudomonas* with *S. aureus*, *E. coli*, and *A. baumannii* was previously reported (Solis-Velazquez et al 2021). We showed here that

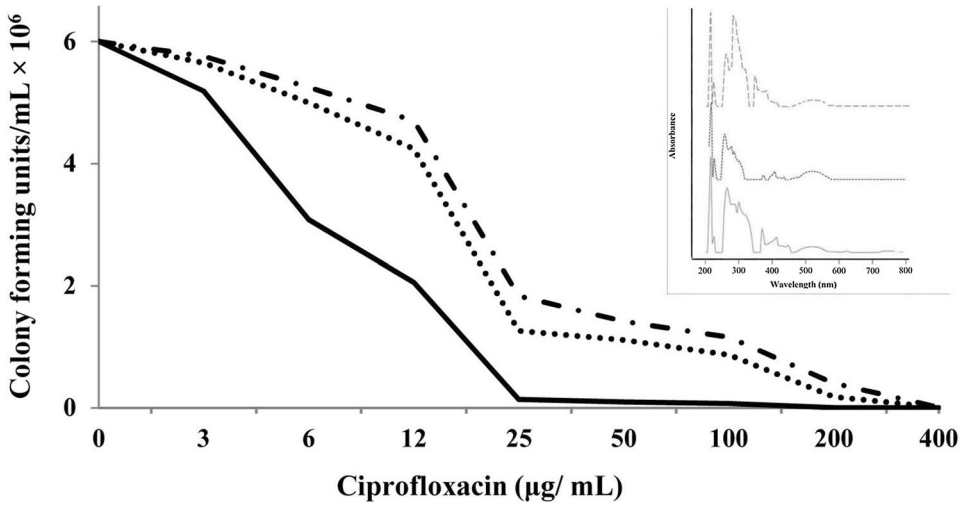

Fig. 3 *Pseudomonas* growth in the presence of various ciprofloxacin concentrations. Solid line: mono-culture, dash-dot line: *Pseudomonas* in dual culture with *Acinetobacter*, dot line: *Pseudomonas* in dual culture with *Enterococcus*. Inlet: UV/vis spectrum of crude pigment by *Pseudomonas*

Table1 Hemolysin activity of *Pseudomonas* strain in the presence of live, dead and cell-free supernatant (CFS) of *Acinetobacter* and *Enterococcus*

	Acinetobacter			*Enterococcus*		
	Live	Dead	CFS	Live	Dead	CFS
FIC	6.3	0.33	1.2	6.3	0.2	0.83
Effect	Ant	Syn	Add	Ant	Syn	Ind

Ant antagonist, *Syn* synergism, *Add* additive, *Ind* indifferent

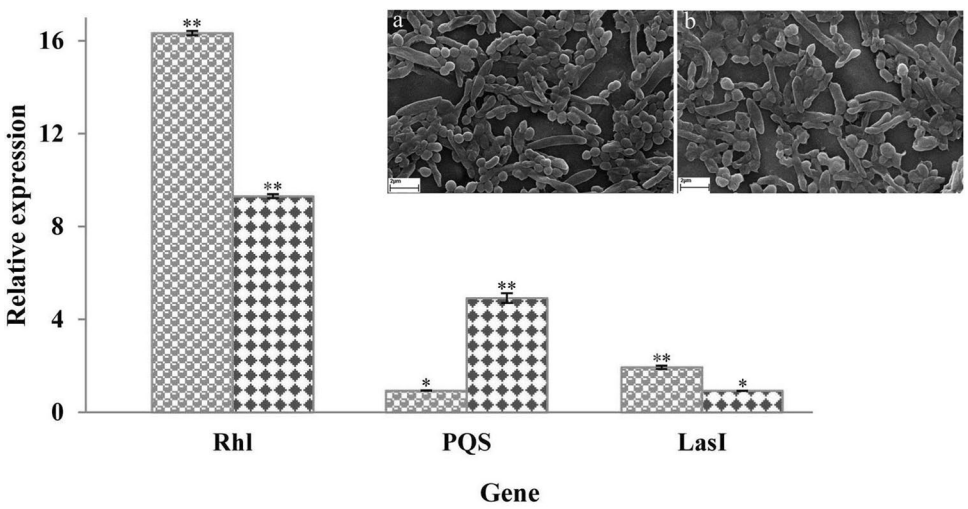

Fig. 4 Relative expression of *lasI*, *rhlI*, and *pqsR* genes in dual *Pseudomonas- Acinetobacter/Enterococcus* cultures. Sphere: *Acinetobacter*, and diamond: *Enterococcus*. Error bars indicate the standard deviation for three replicates. *Not significant difference with control ($P > 0.05$), **Significant differences with control ($p < 0.05$). Inlet: scanning electron micrographs of *Pseudomonas* in co-culture with *Acinetobacter* (**a**) and *Enterococcus* (**b**)

Acinetobacter/*Enterococcus* enhanced biofilm formation of *Pseudomonas* in dual biofilms. The correlation between cell auto-aggregation and biofilm formation and the dependence of *Pseudomonas* auto-aggregation on the fimbrial adhesins were previously determined (D'Argenio et al 2002; Sorroche et al 2012). The ability of *P. aeruginosa* cells to produce adhesins and attachment to epithelial cells was reported to be dependent on the Rhl quorum-sensing system (Glessner 1999). Accordingly, because the *Pseudomonas* Rhl system was over-expressed in the dual cultures with *Acinetobacter*/*Enterococcus*, we hypothesize that *Acinetobacter*/*Enterococcus* enhanced *Pseudomonas* biofilm formation by enhancing its auto-aggregation due to the overproduction of some aggregative adhesins. Interestingly, adding *Acinetobacter*/*Enterococcus* to the pre-formed *Pseudomonas* biofilm reduced its attachment. While the surfactant production was reported for both *Acinetobacter* and *Enterococcus* strains, it is not surprising that they could disrupt the pre-formed *Pseudomonas* biofilms (Gupta et al 2020; Chaurasia et al 2022). Cells could also leave the biofilm by dispersion, the active mechanisms that cause cells to separate from the biofilm. The involvement of PQS and Rhl quorum sensing systems in *Pseudomonas* dispersal seems evident from our data that these genes were much expressed in the presence of *Acinetobacter* and *Enterococcus* (Kim and Lee 2016).

Motility plays a significant role in the bacterial pathogenesis (at least in the initial phases of the infection), where non-motile bacteria are severely reduced in their virulence (Josenhans and Suerbaum 2002). *Acinetobacter* and *Enterococcus* induced all motility types of *P. aeruginosa* in the interspecies interactions. Overhage et al. present the association between swarming and up-regulation of *rhlR* (Overhage et al 2008). While both *Acinetobacter* and *Enterococcus* strains induced *rhlI* expression, it is not surprising that they enhanced *Pseudomonas* flagella-mediated motility. A proteomic study of *P. aeruginosa* and *Staphylococcus aureus* dual-species biofilm also represents the abundance of *Pseudomonas* flagellar and pilus proteins which result in the increase of the swimming/swarming and twitching motilities (Reigada et al 2021). Pigments like pyocyanin and pyoverdine play an essential role in the pathogenesis of *P. aeruginosa* by suppressing the host immune response and their role in iron acquisition. Pigmentation was significantly reduced in the *Pseudomonas–Staphylococcus* co-culture (Hall et al 2016). The sufficient iron concentration in this mixed biofilm can decrease the need for production of the iron acquisition system (pyocyanin) and pigmentation (Reigada et al 2021). However, we observed that *Pseudomonas* produces more pyocyanin in the presence of *Acinetobacter* and *Enterococcus*. While the iron acquisition systems were well determined in *Acinetobacter* and *Enterococcus*, competition between these strains and *Pseudomonas* to uptake iron may cause more pigmentation in co-cultures in comparison to monocultures.

In many poly-microbial biofilms, interspecies interactions significantly decrease bacterial susceptibility to antimicrobials compared to single species biofilm (Bottery et al 2022). *Staphylococcus* increased *Pseudomonas* susceptibility to ciprofloxacin, gentamicin, and amikacin but decreased to polymyxin B (Trizna et al 2020; Reigada et al 2021). The role of Rhl quorum sensing system in *Pseudomonas* antibiotic resistance was determined through the induction of the resistance genes like drug efflux pumps and is consistent with the results of the gene expression study in our research (Rezaie et al 2018).

The LasR is the central regulator of *Pseudomonas* QS and triggers the expression of most virulence factor-encoding genes. The third *Pseudomonas* QS signal is a quinolone-based QS system (PQS), where its null mutation may result in reduced biofilm formation and decreased production of some virulence factors (Lee and Zhang 2015). In this study, we observed that the Rhl is the central QS system and is affected in the dual cultures with *Acinetobacter*/ *Enterococcus*, with the more effects for *Acinetobacter*. This QS system plays a crucial role in the regulation of *Pseudomonas* virulence and biofilm formation, which is promoted by interspecies interactions. The result may relate to unknown metabolites produced by *Acinetobacter*/*Enterococcus* to induce the *Pseudomonas* QS system; however, the exact active molecule(s) remains to be identified.

In conclusion, dual-species cultures of *P. aeruginosa* with two co-isolated species, viz. *Acinetobacter* and *Enterococcus* from chronic wounds were characterized. Multiple *Pseudomonas* virulence factors, including biofilm formation, motility, hemolysis, antibiotic resistance, and pigmentation, were promoted in the dual cultures. The Rhl quorum sensing of *Pseudomonas* was sharply over-expressed in the dual cultures and may activate various virulence factors. Since the synergistic effects of *Acinetobacter*/*Enterococcus* on *Pseudomonas* pathogenesis are probably due to its QS system, the detection of quorum quenching molecules can be used to overcome the *Pseudomonas* poly-microbial chronic infection.

Acknowledgements This work was supported by a grant from Ferdowsi University of Mashhad (50614/3).

Author contributions A.M and S.A contributed to the study conception and design. G.H and A.M contributed to Material preparation, data collection and analysis. The first draft of the manuscript was written by A.M and G.H. All authors read and approved the final manuscript

Funding The authors have not disclosed any funding.

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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