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Interference of AHL signal production in the phytophatogen *Pantoea agglomerans* as a sustainable biological strategy to reduce its virulence

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ABSTRACT

Pantoea agglomerans is considered one of the most ubiquitous and versatile organisms that include strains that induce diseases in various crops and occasionally cause opportunistic infections in humans. To develop effective strategies to mitigate its impact on plant health and agricultural productivity, a comprehensive investigation is crucial for better understanding its pathogenicity. One proposed eco-friendly approach involves the enzymatic degradation of quorum sensing (QS) signal molecules like *N*-acylhomoserine lactones (AHLs), known as quorum quenching (QQ), offering potential treatment for such bacterial diseases. In this study the production of C4 and 3-oxo-C6HSL was identified in the plant pathogenic *P. agglomerans* CFBP 11141 and correlated to enzymatic activities such as amylase and acid phosphatase. Moreover, the heterologous expression of a QQ enzyme in the pathogen resulted in lack of AHLs production and the attenuation of the virulence by mean of drastically reduction of soft rot disease in carrots and cherry tomatoes. Additionally, the interference with the QS systems of *P. agglomerans* CFBP 11141 by two the plant growth-promoting and AHL-degrading bacteria (PGP-QQ) *Pseudomonas segetis* P6 and *Bacillus toyonensis* AA1EC1 was evaluated as a potential biocontrol approach for the first time. *P. segetis* P6 and *B. toyonensis* AA1EC1 demonstrated effectiveness in diminishing soft rot symptoms induced by *P. agglomerans* CFBP 11141 in both carrots and cherry tomatoes. Furthermore, the virulence of pathogen notably decreased when co-cultured with strain AA1EC1 on tomato plants.

1. Introduction

The continual expansion of the global human population demands food production that operates within sustainable and eco-friendly frameworks. By 2030, it is crucial to significantly reduce the use of chemical plant protection within European Agriculture, recognizing the significance of adopting sustainable crop production methods to ensure food security and food safety across Europe (Finger and Möhring, 2024; Schneider et al., 2023). Considering the significant threat posed by phytopathogenic bacteria to both food production and ecosystem stability on a global scale, innovative alternatives to traditional chemical-based agricultural approaches are essential for effectively addressing this challenge (FAO, 2023).

Pantoea agglomerans is a plant-associated Gram-negative enterobacterium of the family *Erwiniaceae* that includes strains belonging to the "*Erwinia herbicola-Enterobacter agglomerans* complex" (Gavini et al., 1989). This species is considered one of the most ubiquitous organisms in nature, but its role is ambiguous (Dutkiewicz et al., 2015). *Pantoea* spp. cause disease in a wide range of plants, resulting important economic losses in many countries (Gutiérrez-Barranquero et al., 2019; Dutkiewicz et al., 2016a). Typically, these bacteria infiltrate plants through wounds or natural openings, manifesting symptoms like leaf spots, blights, wilts, and fruit rot. The type III secretion system (T3SS) seems to be a crucial factor in the pathogenicity of numerous *Pantoea* species (Coburn et al., 2007; Dutkiewicz et al., 2016a). It facilitates the colonization and initiation of disease by injecting bacterial effector proteins, which interfere with defense signaling in host cells (Alfano, Collmer, 2004). Specific strains of *P. agglomerans* possess tumorigenic traits due to the natural acquisition of pathogenicity plasmid from the pPATH family, which harbors genes encoding *hrp/hrc* type III secretion system genes (T3SS), type III effectors and phytohormones (Nissan et al., 2018; Weinthal et al., 2007). Notably, the best studied gall-forming plant pathogens are *P. agglomerans* pv. *gypsophilae* which triggers galls formation on the ornamental plant gypsophila (*Gypsophila paniculate*),

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and *P. agglomerans* pv. *betae*, which induces galls on both table beet (*Beta vulgaris*) and gypsophila (Dutkiewicz et al., 2016a). Moreover, this versatile bacterium also occurs in animals, water, soil, dust, and air, and occasionally in humans where it could be a cause of the opportunistic infections (Cruz et al., 2007; Dutkiewicz et al., 2016a). On the contrary, this species can be found in the rhizospheres of plants where it suppresses the development of fungal and bacterial plant pathogens (Dutkiewicz et al., 2016b; Matilla et al., 2023; Walterson and Stavrinides, 2015), promotes plant growth and appears as a potentially efficient bio-fertilizer (Walterson and Stavrinides, 2015; Lorenzi et al., 2022) and could be used for the prevention and/or treatment of human and animal diseases (Dutkiewicz et al., 2016b).

In many agriculture pathogens, the production of hydrolytic enzymes as well as other phenotypes related to virulence factors are controlled by a cell density-dependent gene expression mechanism called quorum sensing (QS) (Baltenneck et al., 2021; Von Bodman et al., 2003; Williams et al., 2000). This sophisticated bacterial communication system promotes coordinate behavior within a population and implies the production, secretion and recognition of small signal molecules known as autoinducers (Fuqua et al., 1994; de Kievit and Iglewski, 2000). The most studied autoinducers in Gram negative bacteria are *N*-acylhomoserine lactones (AHLs) that are diffused or exported to the surrounding medium where they are accumulating as the cell concentration increases. Once the concentration of AHLs reaches a threshold level, a coordinate expression of different genes is activated, including antibiotic, pigments, exopolysaccharides and exoenzymes production and biofilm formation (Abisado et al., 2018; Papenfort and Bassler, 2016; Whiteley et al., 2017).

To date, the expression of many important bacterial virulence factors is regulated by AHL signal molecules in important phytopathogenic bacteria such as *Agrobacterium fabrum* (Haudecoeur and Faure, 2010; Lang and Faure, 2014), *Erwinia amylovora* (Piqué et al., 2015; Venturi et al., 2004), *Dickeya solani* (Crépin et al., 2012; Potrykus et al., 2018), *Pectobacterium carotovorum* (Moleleki et al., 2017; Pollumaa et al., 2012), *P. atrosepticum* (Smadja et al., 2004) and *Pseudomonas syringae* (Cheng et al., 2016; Quinones et al., 2005). Regarding *Pantoea* species, limited information exists concerning QS systems. A phylogenetic analysis of *P. agglomerans* strains's genomes, including endophytic, plant pathogenic, and clinical strains, highlights the presence of conserved homologs of the synthase *luxI* and transcriptional regulator *luxR,* named *pagI* and *pagR,* respectively. This suggests an important role of AHL signaling in the adaptive survival of the bacterium across various ecological niches (Jiang et al., 2014).

AHL-based QS systems has been identified in the endophytic *P. agglomerans* YS19, which promotes host plant growth. This strain produces 3-oxo-C8-HSL to control the formation of symplasmata, a multicellular aggregate used in the colonization of host rice plant and the endophytic life of the strain (Jiang et al., 2014).

In relation to the role of QS in the virulence of host plants, in *P. stewartia* subsp. *stewartia*, a pathogen that primarily infects field and sweet corn, causing wilt and leaf blight, QS regulates the production of EPS, leading to vascular occlusion. In this species, the main AHL produced is 3-oxo-C6-HSL (von Bodman and Farrand, 1995; von Bodman et al., 1998). In *P. agglomerans* pv. *gypsohilae*, the production of C4-HSL and C6-HSL were identified as major and minor signal compounds and the disruption of the QS system impacts gall tumor formation, diminishing its size due to the influence on *hrp* regulatory genes (Chalupowicz et al., 2008). Regulatory connections among QS, phytohormones such as auxin and cytokinin, and the Hrp regulon are associated with gall formation of *P. agglomerans* pv. *gypsohilae* (Chalupowicz et al., 2009).

The interference of QS systems in plant pathogens has become an interesting and promising alternative to fight with bacterial diseases instead of the use of antibiotics and chemical pesticides in agriculture (Defoirdt, 2018; Sharma et al., 2022; Verma et al., 2021). Indeed, eco-friendly alternative strategies are necessary to replace the current methods and to avoid the increase of emergence antibiotic-resistant bacteria (FAO, 2023). In case of QS interruption, this mechanism attenuates pathogen virulence without killing or affecting its growth as occurs with antibiotics and consequently, reducing the risk of inducing resistances (Munguia and Nizet, 2017; Muras et al., 2018). Two of the most studied mechanisms to interfere QS systems are the production of AHL antagonist (quorum sensing inhibitor compounds, QSI) and the enzymatic degradation or modification of AHLs (quorum quenching, QQ) through three main types of enzymes: lactonases, acylases and oxidorreductases (Fetzner, 2015; Grandclément et al., 2016; Uroz et al., 2009). To date, AHL-degrading bacteria is commonly found among diverse bacterial taxa, including gram-positive and gram-negative bacteria, being more abundant in saline environments (Torres et al., 2019).

A few of the AHL-degrading or QQ bacteria has also been recognized as plant growth-promoting bacteria (PGPB), a well-known and effective tool used in agriculture for many decades as biofertilizer and to combat bacterial pathogens (Liu et al., 2023; Yu et al., 2022; Vejan et al., 2016; Wang et al., 2020; Zhou et al., 2022; Zhu et al., 2023). For instance, PGPB are recognized to produce a high number of hydrolytic enzymes, siderophores, antibiotics, bacteriocins that interfere with the pathogen growth as well as induce plant systemic resistance (Kumari et al., 2019). Recently, PGP-QQ bacteria in co-cultivation with important plant pathogens have demonstrated to promote plant growth as well as attenuate their virulence as the signs of infections reduced (Roca et al., 2024; Rodriguez et al., 2020; Vega et al., 2020).

The aim of this study was to analyze AHL production in the plant pathogenic strain *P. agglomerans* CFBP 11141 and deepen our understanding of its role in the virulence of this species, given the limited information on this aspect. This knowledge was proved crucial to demonstrate the potential biocontrol of this phytopathogen using two recognized PGP-QQ bacteria, *Bacillus toyonensis* AA1EC1 (Roca et al., 2024) and *Pseudomonas segetis* P6 (Rodríguez et al., 2020), as an innovative and environmentally friendly approach.

2. Material and methods

2.1. Bacterial strains, media and growth conditions

The pythopathogen *P. agglomerans* CFBP 11141 (CIP 105196 https://bacdive.dsmz.de/strain/139406) (Gavini et al., 1989) and the PGP-QQ bacteria *Pseudomonas segetis* P6 (Rodriguez et al., 2020) and *Bacillus toyonensis* AA1EC1 (Roca et al., 2024) were used in this study. The biosensor strains used were *Agrobacterium tumefaciens* NTL4 (pZLR4) (Shaw et al., 1997), *Chromobacterium subtsugae* CV026 (Harrison and Soby, 2020), formerly *C. violaceum* CV026 (McClean et al., 1997) and *C. violaceum* VIR07 (Morohoshi et al., 2008). All strains were routinely grown in Luria-Bertani (LB) medium at 28°C and at 120 rpm in a rotary shaker. In the case of biosensor NTL4, it was grown in LB medium supplemented with 2.5 mM $CaCl_2 \cdot 2\ H_2O$ and 2.5 mM $MgSO_4 \cdot 7\ H_2O$ (LB-MC) or AB medium (Chilton et al., 1974). If necessary, kanamycin (Km) and gentamicin (Gm) were used with final concentrations of 50 μg mL^{-1} and tetracyclin (Tc) at 10 μg mL^{-1}.

2.2. Detection of AHL production by well-diffusion agar-plate assay

The production of AHLs in the bacterial wild-type strain was detected by a well-diffusion agar assay as previously reported (Torres et al., 2013). Briefly, *P. agglomerans* CFBP 11141 was grown in 5 mL of LB at 28 °C until the early stationary phase was reached (DO_{600} 2.0). Then, 100 μL-aliquots of this culture were dispensed in wells made on the surface of LB agar plates (for biosensors CV026 or VIR07) and on AB agar plates supplemented with 80 μg mL^{-1} of 5-bromo-4--chloro-3-indolyl-ß-D-galactopyranoside (X-Gal) (biosensor NTL4). An overnight culture of each biosensor was previously spread on the surface of the corresponding medium. After 24 h of incubation at 28°C, the appearance of blue and purple halos around the wells was visually inspected. LB medium was used as negative control. The assay was

repeated three times.

2.3. AHL identification by ultrahigh performance liquid chromatography – triple quadrupole mass spectrometry

The identification of specific AHLs produced by *P. agglomerans* CFBP 11141 was accomplished using ultrahigh performance liquid chromatography – triple quadrupole mass spectrometry (UPLC-MS/MS). To obtain the AHL crude extract, the strain was grown in 25 mL of LB until stationary phase and the entire culture was extracted twice with equal volumes of acidified ethyl acetate, evaporated, and dissolved in 1 mL of the mixture of methanol and deionized water (35:65 v/v) (Marketon et al., 2002).

The UPLC-MS/MS analyses were performed with a Acquity UPLC System I-Class (Waters) equipped with an Acquity UPLC BEH C18™ column (2.1 mm×100 mm, 1.7 μm particle size). Elution of the column was carried out with a mixture of methanol-water (Kušar et al., 2016). MS experiments were conducted on a low-resolution spectrometer Triple Cuadrupole XEVO-TQ-XS (Waters) equipped with a Turbolon source using positive-ion electrospray (ESI). The MS signals were used to generate relative quantification information by comparison a calibration curve constructed for pseudomolecular-ion abundance, using the appropriate AHL synthetic standards.

The following synthetic AHLs were tested: C4-HSL (*N*-butyryl-DL-homoserine lactone), C6-HSL (*N*-hexanoyl-DL-homoserine lactone), 3-oxo-C6-HSL (*N*-3-oxo-hexanoyl-DL-homoserine lactone), C8-HSL (*N*-octanoyl-DL-homoserine lactone), 3-oxo-C8-HSL (*N*-3-oxo-octanoyl-DL-homoserine lactone), 3-OH-C8-HSL (*N*-3-hydroxyoctanoyl-DL-homoserine lactone), C10-HSL (*N*-decanoyl-DL-homoserine lactone) C12-HSL (*N*-dodecanoyl-DL-homoserine lactone) and 3-oxo-C12-HSL (*N*-3-oxo-dodecanoyl-DL-homoserine lactone) (Sigma-Aldrich).

2.4. Expression of AHL-lactonase gene in P. agglomerans CFBP 11141

The strain *P. agglomerans* CFBP 11141 was transformed by electroporation, introducing the recombinant plasmid pME6010::*hqiA*, containing an AHL-lactonase gene (Torres et al., 2017) as well as the empty plasmid pME6010 (Tc^R) (Heeb et al., 2000). Competent cells were obtained from 25 mL of 24 h culture and washed with sucrose 300 mM following the methodology previously described (Torres et al., 2017). Then, the transformants were confirmed by PCR amplification of the *hqiA* gene using the following specific primers: hqiA-T forward 5'-ATGAGTGAAATCACGTTGGC-3' and hqiA-T reverse 5'-CTTTACCCGAAGGATCGTAA-3'.

To investigate the impact of *hqiA* expression on AHL production by the strain *P. agglomerans* CFBP 11141, a well-diffusion agar-plate assay was conducted following the previously described method, employing NTL4 (pZLR4) and CV026 as bioindicator strains.

To assess the impact of AHL degradation on QS-regulated virulence factors, various cellular functions were examined in the confirmed transformants (pME6010::*hqiA* and pME6010) as well as in the wild-type strain. Thus, hydrolysis of starch (Barrow and Feltham, 1993), gelatin (Pickett et al., 1991), Tween 20 and Tween 80 (Sierra, 1957) as well as the production of β-glucosidase (Gong et al., 2012), lecithinase (Larpent and Larpent-Gourgand, 1957), alkaline phosphatase (Baird--Parker, 1963), acid phosphatase (Pikovskaya, 1948), phytase (Hosseinkhani and Hosseinkhani, 2009), indole-3-acetic acid (Naik et al., 2008) and siderophores (Alexander and Zuberer, 1991) were determined by spotting 10 μL of each culture in the corresponding media. Swimming (Ha et al., 2014a) and swarming (Ha et al., 2014b) motility were tested by spotting 2 μL of each culture in LB 0.3 and 0.5% (w/v) agar respectively. Each assay was repeated three times.

2.5. Antagonist assay

The antagonist activity of each PGP-QQ strain (*P. segetis* P6, *B. toyonesis* AA1EC1) against *P. agglomerans* CFBP 11141 was assessed using the well diffusion method (Balouiri et al., 2016). An overlay of the pathogen was spread onto the surface of an LB agar plate, and 100 μL of the supernatants of a 5-day culture of each QQ bacterium were poured into the pre-made wells. Following 48 h of incubation at 28°C, the presence of growth inhibition zones surrounding the wells was visually examined.

2.6. Co-culture assays

Co-culture experiments were conducted involving *P. agglomerans* CFBP 11141 and PGP-QQ strains (*P. segetis* P6 and *B. toyonensis* AA1EC1) employing the previously outlined methodology (Torres et al., 2016; Reina et al., 2019). Briefly, 50 μL of 24 h culture of pathogen (10^9 CFU mL^{-1}) was added to 5 mL of a 24 h culture of each PGP-QQ strain (10^9 CFU mL^{-1}) in LB medium. Monocultures of each bacterium were grown under similar conditions as controls. After 24 h incubation at 28ºC, the remaining AHLs from each co-culture and monoculture was detected using the well-diffusion agar-plate method previously described, using *A. tumefaciens* NTL4 (pZLR4) and *C. subtsugae* CV026 as bioindicator strains. The abundance of the pathogen and each PGP-QQ bacterium in the co-cultures was quantified by serial dilutions and plate counts on LB agar, with colonies differentiated by their different colors and morphologies (*P. agglomerans* CFBP 11141 produces yellow mucoid colonies whereas strains P6 and AA1EC1 produce cream colonies).

To assess how AHL degradation affects the virulence of *P. agglomerans* CFBP 11141, various cellular functions were examined in the co-cultures and monocultures as described in 2.4.

2.7. Virulence assays in cherry tomatoes and carrot

The interference of PGP-QQ bacteria with the virulence of *P. agglomerans* CFBP 11141 was evaluated in cherry tomatoes and carrots according to the methodology previously outlined with modifications (Torres et al., 2017; Rodríguez et al., 2020). Briefly, cherry tomatoes (*Solanum lycopersicum* L. var. *cerasiforme*) and carrots (*Daucus carota* L., cv. Amsterdam 2) were surface sterilized. Then, four tomatoes were placed in Petri dishes while carrots were cut into slices and four of them placed in Petri dishes with a wet filter paper to maintain moisture. Six replicates were carried out for the following treatments: sterilized distillated water, *P. agglomerans* CFBP 11141, strain P6, strain AA1EC1, pathogen-AAE1C co-culture (ratio 1:100) and pathogen-P6 co-culture (ratio 1:100). One milliliter of each culture (10^9 CFU mL^{-1}) was centrifuged at 12,000 rpm for 3 min and the cells were suspended in 1 mL of sterilized distillated water. For each culture condition, 15 μL was injected into the tomatoes and carrot slices. Sterilized distillated water was similarly inoculated on tomatoes and carrots slices as negative control. After 5 days of incubation at 28°C, the maceration areas were visually examined and the spatial extent of the damage was calculated by image analysis using ImageJ software (Schneider et al., 2012).

The potential of *hqiA* to disrupt the patogen's virulence was evaluated in carrot slices and cherry tomato assays following the same procedure.

2.8. Virulence assay in tomato plants

The interference of QS-associated virulence factors of *P. agglomerans* CFBP 11141 was examined in tomato plants (*Solanum lycopersicum* L. var. Roma) according to the technique previously described (Yan et al., 2008; Vega et al., 2020). Briefly, 50 sterilized tomato seeds were sown in individual pots containing sterilized vermiculite: perlite (ratio 3:1). The pots were incubated for three weeks in an indoor green house during a long-day photoperiod (16:8 h light: dark) at 25°C. Three pots were used for each treatment as follows: sterile distilled water, *P. agglomerans* CFBP 11141, strain P6, strain AA1EC1, pathogen-P6 co-culture (ratio 1:100) and pathogen-AA1EC1 co-culture (ratio 1:100). Previously to the

infection, to induce stomatal opening, each pot was exposed to 100% humidity for 24 h and then, they were sprayed with 5 mL of each treatment (10^9 CFU mL^{-1}). Relative humidity was maintained at 100% for a further 24 h to facilitate pathogen infection under similar growth-controlled conditions. After 5 days post-inoculation, leaves were examined for the appearance of infection symptoms. Healthy, necrotic-chlorotic and dead leaves were counted in each treatment.

2.9. Statistical analysis

The results presented in this work were analyzed using the GraphPad Prism 9 program. The normality of the data was assessed using the Shapiro-Wilk test or the D'Angostino and Pearson test. They were analyzed by simple ANOVA using Tukey's test for the comparison of means. In all cases, the confidence interval was set at 95%.

3. Results

3.1. Production of AHLs by P. agglomerans CFBP 11141

The synthesis of AHL signal molecules were firstly examined in cultures of the pathogen by using a well-diffusion agar-plate assay. The biosensor strains *Agrobacterium tumefaciens* NTL4 (pZLR4) and *Chromobacterium subtsugae* CV026 were activated in the presence of the pathogen, while no response was seen for *C. violaceum* VIR07 (Fig. 1).

To investigate the type of AHLs produced by *P. agglomerans* CFBP 11141, culture extracts from 25 mL of an early stationary phase was analyzed by UPLC-MS/MS. A wide range of synthetic AHLs were used as standards for the identification of these molecules and only C4-HSL and 3-oxo-C6-HSL were detected in a concentration of 6.7 mg L^{-1} and 0.153 mg L^{-1}, respectively (Fig. 1).

3.2. Cellular functions regulated by AHL-QS system

The cellular functions controlled by AHLs in *P. agglomerans* CFBP 11141 were evaluated by expressing the HqiA enzyme, an AHL lactonase which hydrolyzes the lactone ring of AHL signal molecules. The plasmid construction pME6010::*hqiA* as well as the empty plasmid pME6010 were transformed into the pathogen. Transformants were confirmed by the amplification of the expected AHL-lactonase coding gene (750 bp) and the lack of the ability to activate the biosensors *C. subtsugae* CV026 and *A. tumefaciens* NTL4 (pZLR4). Transformants containing the empty plasmid were used as negative control.

P. agglomerans CFBP 11141, *P. agglomerans* (pME6010::*hqiA*) and *P. agglomerans* (pME6010) were tested for the production of different hydrolytic enzymes synthesizes by the pathogen such as amylase, gelatinase, lipases (hydrolysis of lecithin, Tween 20 and Tween 80), phosphatases (phytase, acid and alkaline phosphatases), protease and β-glucosidase, the synthesis of indole-3-acetic acid (IAA) and siderophores, swimming and swarming motilities. The phenotypic analyses were carried out in triplicate and the results obtained were consistent. The lack of the production of AHLs in the pathogen in our assay conditions resulted in the reduction of acid phosphatase and amylase activities (Fig. 2).

To confirm the expression of *hqiA* gene interfered in the virulence and maceration activity of the *P. agglomerans* CFBP 11141, virulence assays in carrot slices and cherry tomatoes were performed. The transformants were inoculated on the surface of carrot and the expression of *hqiA* in the phytopathogen reduced the capacity to cause soft rot (9.85 ± 1.65% maceration), while a maceration area of 21.83 ± 2.92% and 23.61 ± 2.71% was produced by *P. agglomerans* (pME6010) and the wild-type strain respectively (Fig. 2). Cherry tomatoes inoculated with QQ-expressing strain showed no signs of infection, while those inoculated with the wild-type and *P. agglomerans* (pME6010) strains exhibited a distinct maceration area.

3.3. Interference of bacterial phytopathogen AHL-QS system and impact on associated phenotypes by PGP-QQ bacteria

Once the AHL-QS system has been demonstrated to be involved in the virulence of *P. agglomerans* CFBP 11141, co-cultures of the phytopathogen with two PGP-QQ bacteria, *P. segetis* P6 and *B. toyonensis* AA1EC1, were conducted to assess the capacity to degrade AHL and reduce the expression of QS associated cellular functions in *P. agglomerans* CFBP 11141. Previously, an antagonist experiment to discard any inhibitory effect of the PGP-QQ strains with the growth of the pathogen was performed (data not shown). The pathogen was grown in a co-culture with each of the PGP-QQ strains in a ratio 1:100 for 24 h. The concentration of each bacterium remained consistent throughout the entire assay (10^7:10^9 CFU mL^{-1}). Subsequently, AHL production in each co-culture condition was assessed through a well-diffusion agar-plate assay employing the biosensor *C. subtsugae* CV026. The findings revealed that both AA1EC1 and P6 strain effectively degraded the AHLs produced by *P. agglomerans* CFBP 11141 in co-culture, as evidenced by the absence of signal activation of the biosensor. Under these assay conditions, the pathogen control activated the biosensor strain, whereas

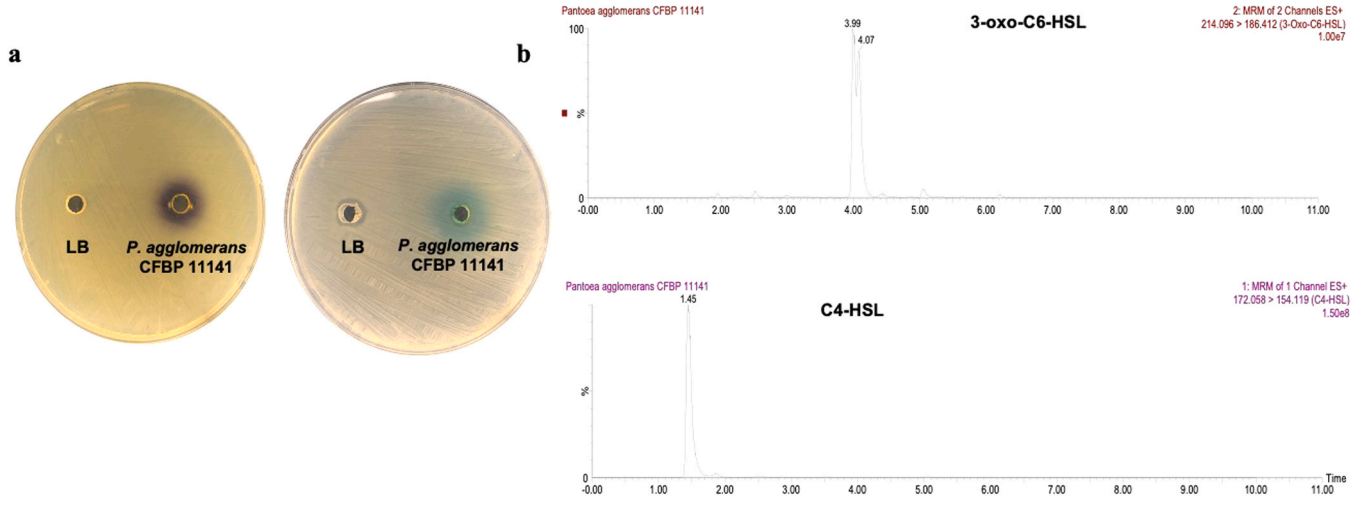

Fig. 1. Well-diffusion agar-plate assay to detect the production of AHLs by *P. agglomerans* CFBP 11141. 100 μL aliquots of culture was placed in each well. LB (negative control). The biosensors strains used were *Agrobacterium tumefaciens* NTL4 (pZLR4) (a) and *Chromobacterium subtsugae* CV026 (b). Extracted chromatogram by UPLC-MS/MS showing the peaks corresponding 3-oxo-C6-HSL and C4-HSL.

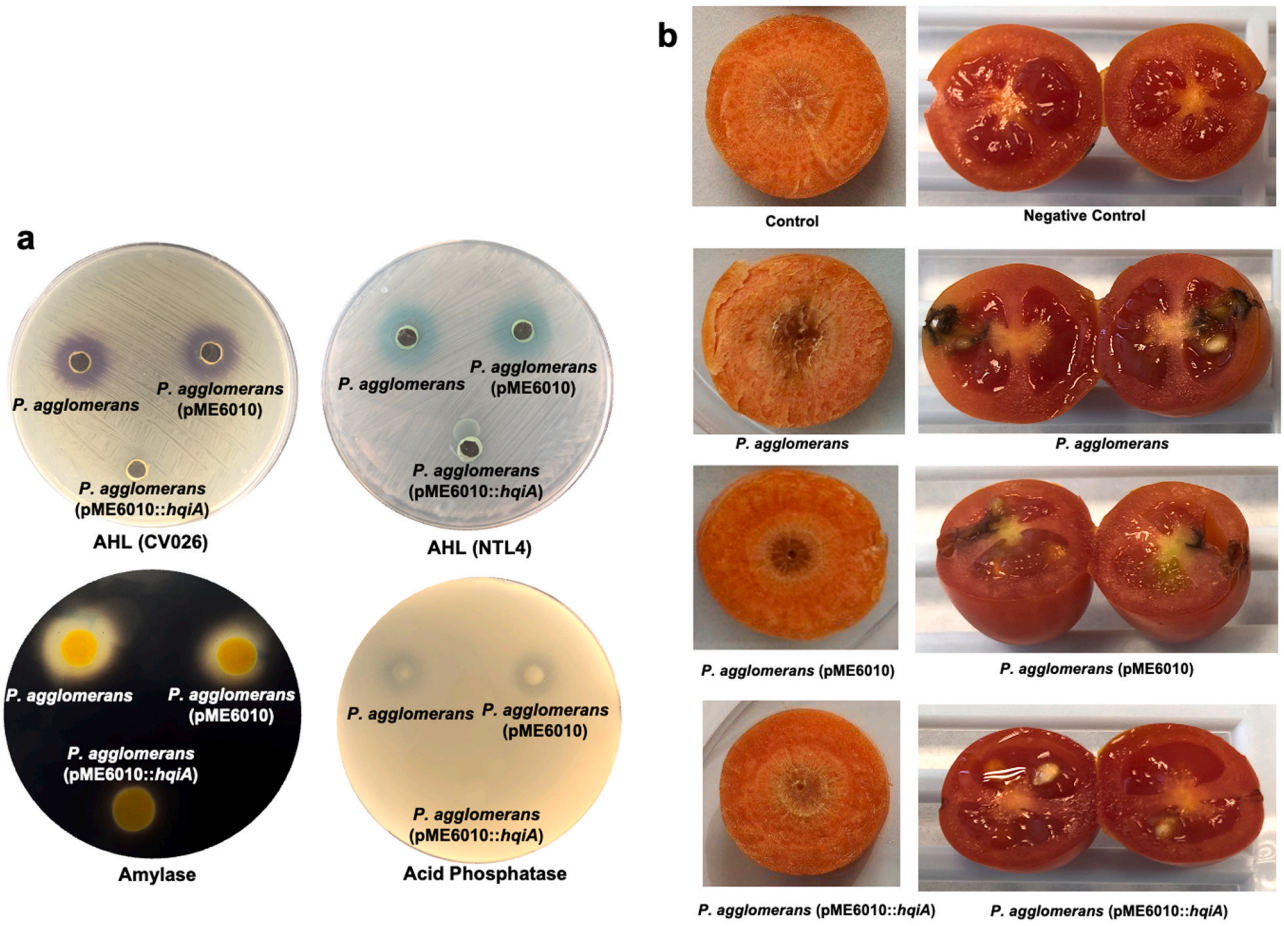

Fig. 2. AHL degradation by the expression of HqiA lactonase in *P. agglomerans* CFBP 11141 and virulence assays in carrot slices and cherry tomatoes. a. Well-diffusion agar-plate assay to detect the production of AHL in the wild-type *P. agglomerans* CFBP 11141 and its transformants expressing *hqiA* gene (pME6010::*hqiA*) and the empty plasmid (pME6010) using the biosensor *Agrobacterium tumefaciens* NTL4 (pZLR4) and *Chromobacterium sutsugae* CV026. **b.** Virulence and maceration produced by the wild-type strain and its transformants on the surface of carrot slices and cherry tomatoes after 5 days of incubation. Sterile water was used as negative control.

no signal was seen in cultures of strains P6 and AA1EC1 (Fig. 3).

The same pathogen and PGP-QQ co-cultures, as well as the monocultures, were also examined to assess the influence of AHL degradation on QS-associated phenotypes of the pathogen. Phenotypes known to be produced by the PGP-QQ strains were not included in the analysis. Some phenotypes of *P. agglomerans* CFBP 11141 showed reductions when co-cultured with PGP-QQ strains (Fig. S1). Specifically, phytase and acid phosphatase production of the pathogen were diminished in the presence of AA1EC1 (Fig. S2). In the case of co-cultures with P6, amylase production and swimming motility of the pathogen were decreased (Fig. S3).

3.4. Interference of bacterial phytopathogen virulence by PGP-QQ strains

The impact of AHL degradation activity exhibited by the two PGP-QQ strains on the virulence of *P. agglomerans* CFBP 11141 was assessed through virulence assays conducted on carrot slices and cherry tomatoes. Co-cultures of *P. agglomerans* CFBP 11141 with *P. segetis* P6 and *B. toyonensis* AA1EC1 were prepared in conditions similar to those mentioned previously and inoculated onto both type of vegetables. Additionally, *P. agglomerans* CFBP 11141 and the PGP-QQ strains, along with sterile water were individually inoculated in each experiment as controls (Fig. 3). As a result, strains AAE1C1 and P6 significantly diminished the capacity of the pathogen to induce soft rot in carrots, with maceration rates of 0 and 10.37 ± 0.66% respectively, as compared to the 23.61 ± 2.71% observed by the pathogen in monoculture. Regarding cherry tomatoes, strain P6 decrease the virulence of *P. agglomerans* CFBP 11141, exhibiting mild infection symptoms, while no tissue damage was observed in the presence of AA1EC1. In all of cases, the inoculation of PGP-QQ strains did not induce any infection symptoms (Fig. 3).

To assess the possible use of PGP-QQ strains as biocontrol agents against *P. agglomerans* CFBP 11141, *in vivo* assays of tomato plants were carried out. Co-cultures and monocultures were inoculated and the number of affected (necrotic, chlorotic and dead leaves) and healthy leaves were counted after 5 days post-inoculation (Fig. 4). The results indicated that strain AA1EC1 reduced the virulence of the pathogen in co-culture showing plants with less damaged than those infected with the pathogen alone. There was an increase of 17.74% in healthy leaves treated with the co-culture with respect to the plants infected with the pathogen. Furthermore, there was a notable reduction in the number of dead and necrotic-chlorotic with values of 2.62 and 19.56% respectively, compared to plants infected with the pathogen (18.79 and 21.14% respectively). In the case of the co-cultures with *P. segetis* P6, the effect on the reduction of virulence of *P. agglomerans* CFBP 11141 was not remarkable. Plants treated with sterile water (negative control) and strains AA1EC1 and P6 exhibited a few leaves attributed with natural senescence (Fig. 4). As evidenced by the photographs of plants in Fig. 4, leaves treated with the pathogen alone showed severe symptoms, characterized by chlorotic leaves and necrotic lesions. However, the reduction of these symptoms was significant in leaves treated with the pathogen in the presence of AA1EC1.

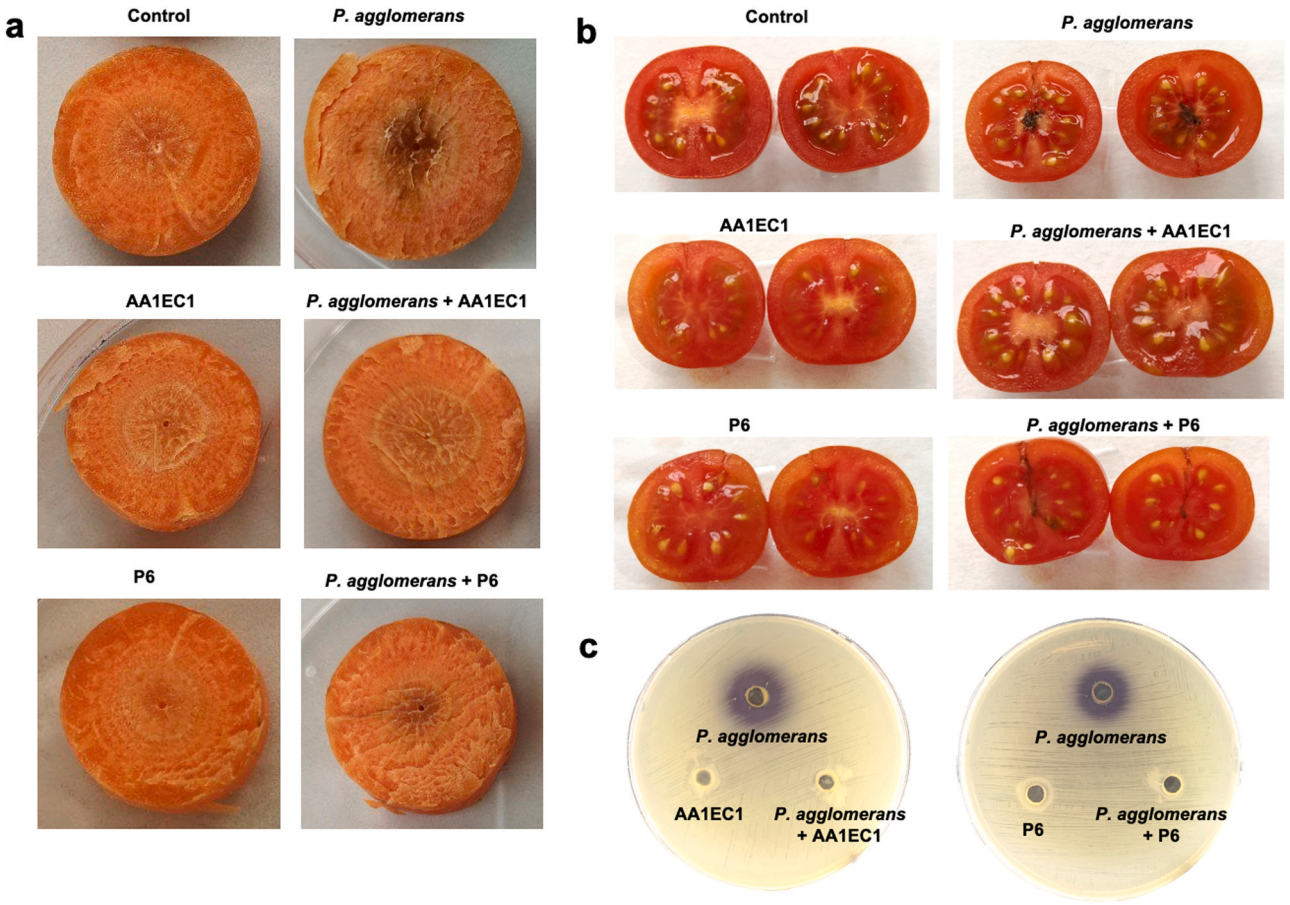

Fig. 3. Impact of AHL-degrading activity of AA1EC1 and P6 against *P. agglomerans* CFBP 11141. Assessment of virulence and maceration of monocultures and co-cultures of AA1EC1 and P6 with the pathogen inoculated on carrot slices (**a**) and cherry tomatoes (**b**) after 5 days of incubation. Sterile water was used as negative control. **c**. Detection of AHLs in the monocultures and co-cultures of AA1EC1 and P6 with the pathogen using *C. sutsugae* CV026 as biosensor.

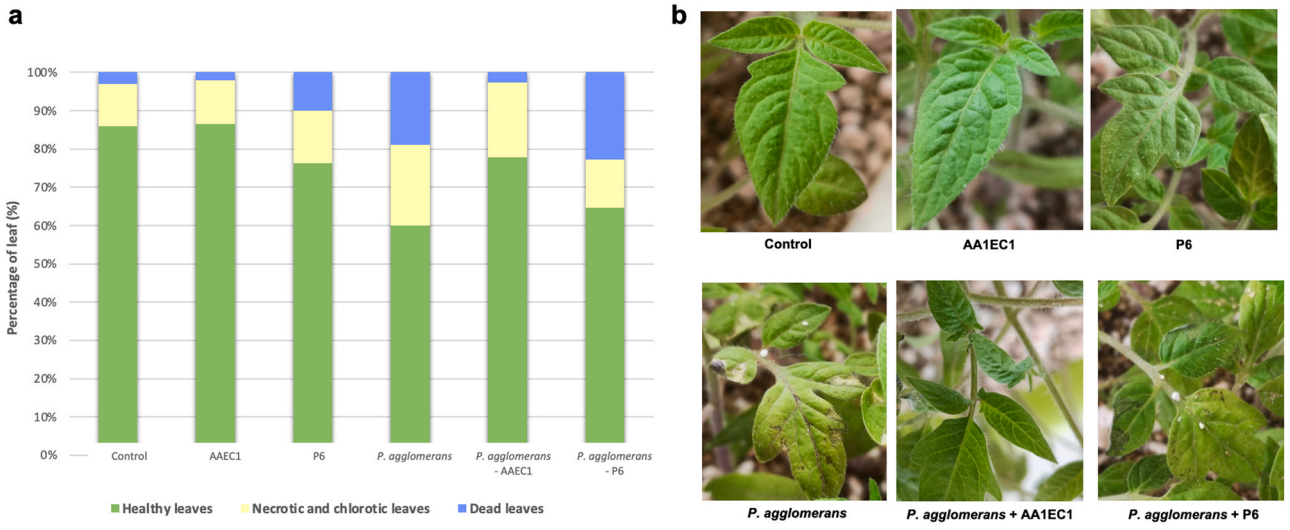

Fig. 4. Infection assay in tomato plants with monocultures and co-cultures of *P. agglomerans* CFBP 11141 and PGP-QQ strains AA1EC1 and P6. a. Total percentage of healthy, dead, necrotic and chlorotic leaves. **b.** Photographs of the leaves of the plants inoculated with the different treatments.

4. Discussion

Crop losses caused by pest and pathogens is continue increasing and reach up to 30% of the total agricultural production worldwide (Delgado-Baquerizo et al., 2020; Savary et al., 2019). The overuse of chemical compounds like fertilizers and pesticides to manage bacterial plant diseases in recent decades has an impact in ecosystems and human health. These consequences include the emergence of microbial resistance strains, damage to soil health and loss of biodiversity (Tilman et al., 2002). Addressing this urgent situation necessitates the

exploration of sustainable methods for crop protection that reduce reliance on pesticides (FAO, 2023). Among these methods, numerous PGPB have proven effective as biocontrol agents against plant diseases due to their ability to produce beneficial compounds (i.e. hormones, antibiotics, exopolysaccharides, enzymes and siderophores) and activate induced indirect systemic resistance in plants (Lyu et al., 2019; Santoyo et al., 2012). Likewise, an emerging and promising alternative strategy involves the enzymatic degradation of AHLs signal molecules in Gram-negative phytopathogens, given their significant role in virulence (Torres et al., 2019; Verma et al., 2021).

P. agglomerans stands out as one of the nature's most ubiquitous and adaptable organisms. While certain strains are known to induce diseases in various crops and occasionally cause opportunistic infections in humans, others exhibit beneficial properties, serving as efficient biofertilizers (Dutkiewicz et al., 2015; 2016a,; Lorenzi et al., 2022). Given the limited understanding of AHL signaling in *P. agglomerans*, conducting a comprehensive investigation is crucial for better understanding its pathogenicity and develop effective strategies to mitigate its impact on plant health and agricultural productivity. Furthermore, research in this field will be significant due to its potential implications for human health.

In this study, we utilized a well-diffusion agar-plate assay along with three biosensor strains that respond to short- medium- and long-chain AHLs. This approach facilitated the straightforward detection of signal molecules production in the plant pathogen *P. agglomerans* CFBP 11141. Our results indicated that this strain induces the biosensors *A. tumefaciens* NTL4 (pZLR4) and *C. subtsugae* CV026 but no activation was shown in *C. violaceum* VIR07. This methodology has proven successfully not only in our laboratory but also among other researchers for identifying AHL-QS systems in a wide array of Gram-negative bacterial species (Kato et al., 2015; Torres et al., 2018; 2019). To characterize the signal molecules, organic crude extracts from a 24-h pathogen culture were examined by UPLC-MS/MS to detect AHLs, with various commercial AHLs serving as controls. The analysis identified the presence of C4-HSL and 3-oxo-C6-HSL in this strain, with the former being more predominant. The production of AHL in plant pathogenic strains of this species has only been reported in *P. agglomerans* pv. *gypsophilae* where C4-HSL and C6-HSL were identified as major and minor signal compounds through mass spectral analysis (Chalupowicz et al., 2008). In the case of *Pantoea stewartia*, a species related to *P. agglomerans* that causes a disease known as Stewart's wilt or bacterial leaf blight in sweet corns (*Zea mays*), the main molecule produced is 3-oxo-C6-HSL (von Bodman et al., 1998).

As the expression of virulence factors and colonization of the plant is regulated by AHLs in numerous phytopathogens (Baltenneck et al., 2021; Torres et al., 2019), and the understanding of AHLs' role in plant pathogenic strains of *P. agglomerans* is still limited, an AHL-lactonase was expressed into strain CFBP 11141. Our results demonstrated a marked reduction in AHL production upon *hqiA* expression, as indicated by the lack of activation in the biosensor strains. In addition, certain enzymatic activities such amylase and acid phosphatase synthesis were also diminished. According to Lin et al. (2021), amylase production stands out as a recognized virulence factor in *Xanthomonas campestris*. However, the significance of acid phosphatase in this context remains unexplored. Acid phosphatase plays a role in plant colonization by phytopatogenic bacteria such *Xanthomonas campestris*, an enzyme involved in the hydrolysis of phosphate ester bonds under acidic conditions, releasing phosphate ions from various organic phosphates in the plant environment. In this bacterium, as well as in other plant pathogenic Gram-negative bacteria, a T2SS secretion system is used to export hydrolytic enzymes related to the degradation of different plant substrates such as amylases (Benali et al., 2014).

Furthermore, the AHL-defective strain *P. agglomerans* (pME6010::*hqiA*) did not induce maceration in carrots and cherry tomatoes, suggesting a potential role of AHLs in attenuating its virulence. Similar findings have been reported in other plant pathogens such as *Pectobacterium carotovorum, P. atrosepticum* and *Dickeya solani,* where AHL regulate the production of extracellular enzymes necessary for cell wall degradation (Cui et al., 1995; Monson et al., 2013; Potrykus et al., 2018). To our knowledge, little information has been disclosed regarding to the role of AHL-based QS in the plant pathogenic *Pantoea* species. In *P. agglomerans* pv. *gypsophilae*, AHLs signals control gall formation (Chalupowicz et al., 2008) whereas in *P. stewartia* subsp. *stewartii*, AHLs regulate the production of the exopolysaccharide, motility, and carotenoids pigments, which are virulence factors of this species (von Bodman et al., 1998).

Based on our results showing a correlation between AHLs and the virulence of *P. agglomerans* CFBP 11141, we carried out *in vitro* and *in vivo* tests to investigate a strategy involving the co-cultivation of the pathogen and AHL-degrading bacteria, aiming to develop a potential treatment for the disease in the future. This approach has been demonstrated to be efficient against other phytopathogens such as *D. solani*, *P. carotovorum* subsp. *carotovorum*, *P. atrosepticum* and *P. syringae* pv. tomato DC3000, where the attenuation of virulence was achieved (Fan et al., 2020; Garge and Nerurkar, 2017; Rodríguez et al., 2020; Vega et al., 2020; Zhang et al., 2020).

Thus, in this study, *P. agglomerans* CFBP 11141 was co-cultured with the PGP-QQ strains *B. toyonensis* AA1EC1 and *P. segetis* P6. Both bacteria were chosen for this study due to their previously demonstrated high AHL-degradation activity against a broad spectrum of AHLs (Roca et al., 2024; Rodriguez et al., 2020). The *in vitro* virulence tests demonstrated that both PGP-QQ strains were able to the severity of soft rot disease induced by the pathogenic strain CFBP 11141 in carrots and cherry tomatoes. Particularly remarkable was the effect observed for strain AA1EC1 against the virulence of the pathogen as no tissue damage was observed. Furthermore, strain AA1EC1 significantly attenuated bacterial virulence on tomato plants by notably reducing the number of dead and necrotic-chlorotic leaves with respect to the severe symptoms induced by the pathogen.

5. Conclusions

This study enhances our understanding of AHL signaling and its role in the plant pathogenic *P. agglomerans*. Our findings establish a correlation between AHL and the virulence of the plant pathogenic strain CFBP 11141. Moreover, this research enforces the effectiveness of an innovative and environmentally friendly strategy involving the utilization of PGP bacteria that produce enzymes capable of degrading QS signal molecules to control the phytopathogen *P. agglomerans*.

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CRediT authorship contribution statement

Alba Amaro-da Cruz: Methodology, Investigation. **Mónica Cabeo:** Methodology, Investigation. **Ana Durán-Viseras:** Methodology, Investigation, Data curation. **Inmaculada Sampedro:** Investigation, Funding acquisition, Conceptualization. **Inmaculada Llamas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare no competing interests.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2024.127781.

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