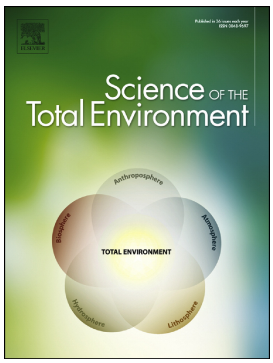

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Drinking-water isolated *Delftia acidovorans* selectively coaggregates with partner bacteria and facilitates multispecies biofilm development

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Abstract

Coaggregation plays an important role in the development of multispecies biofilms in different environments, often serving as an active bridge between biofilm members and other organisms that, in their absence, would not integrate the sessile structure. The ability of bacteria to coaggregate has been reported for a limited number of species and strains. In this study, 38 bacterial strains isolated from drinking water (DW) were investigated for their ability to coaggregate, in a total of 115 pairs of combinations. Among these isolates, only *Delftia acidovorans* (strain 005P) showed coaggregating ability. Coaggregation inhibition studies have shown that the interactions mediating *D. acidovorans* 005P coaggregation were both polysaccharide-protein and protein-protein, depending on the interacting partner bacteria. Dual-species biofilms of *D. acidovorans* 005P and other DW bacteria were developed to understand the role of coaggregation on biofilm formation. Biofilm formation

by *Citrobacter freundii* and *Pseudomonas putida* strains highly benefited from the presence of *D. acidovorans* 005P, apparently due to the production of extracellular molecules/public goods favouring microbial cooperation. This was the first time that the coaggregation capacity of *D. acidovorans* was demonstrated, highlighting its role in providing a metabolic opportunity for partner bacteria.

Keywords: Bacterial fitness; Cell-cell interaction; *Delftia acidovorans;* Multispecies biofilm; Public goods

1. Introduction

In natural aquatic environments, bacterial cells are commonly found in close association with wet surfaces and water-air interfaces in the form of biofilms (Romaní et al., 2016). Interspecies interactions are promoted inside the biofilm and can shape the development, structure, and function of these communities (Burmølle et al., 2014; Elias and Banin, 2012; Rendueles and Ghigo, 2012; Yang et al., 2011). Interspecies spatial and metabolic interactions contribute to the organization of multispecies biofilms, being able to alter the physiology of individual biofilm species, as well as the functions of the entire community (Wimpenny et al., 2000). In addition, cell-cell interactions in multispecies biofilms appear to play a key role in cell adhesion, dispersal and biofilm resistance to antimicrobials (Giaouris et al., 2015; Kaplan, 2010; Simões et al., 2010).

Some studies have focused on the importance of coaggregation, a form of cell–cell interaction, defined as a highly specific mechanism of recognition and adhesion of different bacterial species to each other (Buswell et al., 1997; Min and Rickard, 2009; Rickard et al., 2000, 2003b; Simões et al., 2008), mediated by lectin–saccharide interactions between cell surface molecules (Rickard et al., 2003a). Coaggregation is a key mechanism in biofilm

formation, which facilitates the interaction and integration of bacterial species in biofilms (Rickard et al., 2003b). It is believed that coaggregation contributes to the development of biofilms by two routes (Rickard et al., 2003a): the first route occurs when single cells in suspension specifically adhere to genetically distinct cells in the developing biofilm; the second route occurs by a prior coaggregation of secondary colonizers in suspension followed by subsequent adhesion of this coaggregate to the developing biofilm (Rickard et al., 2003a). Coaggregation was first recognized among human dental plaque bacteria (Gibbons and Nygaard, 1970). The number of studies on the coaggregation between aquatic bacteria is reduced, an aspect related to the inexistence of bacteria with such functional activity (Afonso et al., 2021). These studies focus on the identification of coaggregating species (Rickard et al., 2002, 1999; Simões et al., 2008; Stevens et al., 2015; Vornhagen et al., 2013), characterization of the type of molecules involved in this mechanism (Rickard et al., 2000, 1999; Simões et al., 2008), and the study of factors that influence coaggregation (Min et al., 2010; Rickard et al., 2004, 2000). For bacteria from freshwater environments, coaggregation has been observed between members of the same species (intraspecies coaggregation), members of the same genus (intrageneric coaggregation) and different genera (intergeneric coaggregation) (Rickard et al., 2002, 2003b). So far, intraspecies coaggregation has only been described in freshwater, possibly related to the constantly changing environmental conditions and the possibility of contact between resident bacteria in the biofilm with bacteria from other niches (Rickard et al., 2003a). Coaggregation between freshwater bacteria depends on different biotic and abiotic factors. Biotic factors include the microbial growth phase, expression of adhesins and receptors or the production of extracellular polymeric substances (EPS), while examples of abiotic factors are the presence of solutes, the hydrodynamic conditions and the environmental physicochemical properties (Afonso et al., 2021). Data on the role of coaggregation in multispecies biofilm formation is scarce (Afonso et al., 2021).

The discovery of a coaggregating strain has the potential to advance our understanding of the development and recalcitrance of biofilms in a specific ecosystem (Afonso et al., 2021; Rickard et al., 2003a). Considering what is known so far about coaggregation in other environments (Afonso et al., 2021; Daep et al., 2008), we hypothesize that similar events may occur with bacteria from aquatic systems, mainly regarding coaggregation contribution to multispecies biofilm formation. Furthermore, we also believe that coaggregation facilitates interactions between species within the biofilm. However, coaggregation is not a generic functional ability of bacteria (Afonso et al., 2021; Rickard et al., 2003a), making the identification of a bacterium with this ability an advance to understand this phenomenon. For that, 38 bacterial strains isolated from drinking water (DW) in the north of Portugal were studied regarding intergeneric coaggregation. Coaggregation was detected by a visual assay and epifluorescence microscopy. The surface-associated molecules involved in the coaggregation process were also investigated by sugar reversal tests and heat and protease treatment. The role of the coaggregating strain in biofilm formation was assessed in dual-species biofilms formation and characterized in terms of biomass content and the number of culturable cells.

2. Material and Methods

2.1.Bacteria isolation and culture conditions

A total of 38 strains (Table S1) isolated from DW in the north of Portugal were used. These strains belong to the personal collection of Maria José Saavedra (Saavedra, 2000; Saavedra et al., 2003) and were cryopreserved at -80 °C, in aliquots of BHI (Brain Heart Infusion) medium (Oxoid, UK) with 15% (v v^{-1}) of glycerol. Bacterial cells were grown under batch using R2A broth [peptone 05 g L^{-1} (Oxoid, UK), glucose 0.5 g L^{-1} (Chem-Lab, Belgium), magnesium sulfate heptahydrate 0.1 g L^{-1} (Merck, Germany), sodium pyruvate 0.3 g L^{-1}

(Merck, Germany), yeast extract 0.5 g L^{-1} (Merck, Germany), casein hydrolysate 0.5 g L^{-1} (Oxoid, UK), starch soluble 0.5 g L^{-1} (Sigma-Aldrich, Portugal) and di-potassium phosphate trihydrate 0.4 g L^{-1} (Aplichem Panreac, USA)], at room temperature (23 ± 2 ºC), under agitation (150 rpm), until reaching the stationary growth phase. The stationary phase of growth was selected because coaggregation is growth-phase-dependent, being maximum in the stationary phase (Rickard et al., 2000). The medium R2A was used to ensure optimal growth and successfully recover heterotrophic bacteria from DW since R2A has been validated previously as an adequate medium for freshwater bacteria (Reasoner and Geldreich, 1985).

2.2.Screening for auto and coaggregation ability

2.2.1. Auto and coaggregation visual assay

A visual aggregation assay, with some modifications from the method of Cisar et al. (1979), was used to assess the ability of bacteria to auto and coaggregate. After incubation until the stationary phase of growth, cells were harvested by centrifugation (20 min, 3,100 ×g) (Eppendorf centrifuge 5810R, Eppendorf, Germany), washed three times in coaggregation testing medium and resuspended in a certain volume of the same medium to reach an OD_{600} nm of 1. To assess the best media to study aggregation, the assays were performed with sterile distilled water, sterile synthetic tap water (STW) [$NaHCO_3$ 100 mg L^{-1} (ThermoFisher Scientific, USA), $MgSO_4.7H_2O$ 13 mg L^{-1} (Merck, Germany), K_2HPO_4 0.7 mg L^{-1} (Aplichem Panreac, USA), KH_2PO_4 0.3 mg L^{-1} (Chem-Lab, Belgium), $(NH_4)_2SO_4$ 0.01 mg L^{-1} (Labkem, Spain), NaCl 0.01 mg L^{-1} (Merck, Germany), $FeSO_4.7H_2O$ 0.001 mg L^{-1} (VWR, Portugal), $NaNO_3$ 1 mg L^{-1} (Labkem, Spain), $CaSO_4$ 27 mg L^{-1} (Labkem, Spain), humic acids 1 mg L^{-1} (Sigma-Aldrich, Portugal)] and phosphate-buffered saline [PBS: 8 g L^{-1} of NaCl (Labkem, Spain), 0.2 g L^{-1} of KCl (VWR, Portugal), 1.44 g L^{-1} of Na_2HPO_4 and 0.24 g L^{-1} of KH2PO

(Chem-Lab, Belgium), pH 7.4]. To determine autoaggregation and coaggregation, a volume of 2 mL (for each bacterial isolate) was placed in a glass test tube. The mixtures were then vortexed for 10 s, and the tubes were rolled gently for 30 s. The visual differences were observed over time (2, 24 and 48 h). The aggregation score classification was based on the original scoring scheme of Cisar et al. (1979): 0, no visible coaggregates in the cell suspension; 1, very small uniform coaggregates in a turbid suspension; 2, easily visible small coaggregates in a turbid suspension; 3, clearly visible coaggregates which settle, leaving a clear supernatant; 4, very large flocs of coaggregates that settle almost instantaneously, leaving a clear supernatant. Coaggregation occurred when the score of the bacterial mixtures was equal to or higher than the autoaggregation score of each strain.

2.2.2. Epifluorescence microscopy visualizations

Bacterial coaggregates were observed, after 24 h, by epifluorescence microscopy using a DNA binding stain, 4,6-diamino-2-phenylindole (DAPI; Sigma-Aldrich, Portugal), according to Simões et al. (2008). Briefly, aliquots (15 µL) of bacterial aggregates were filtrated through a 25 mm black Nuclepore® polycarbonate membrane with a pore size of 0.2 µm (Whatman, UK). After filtration, bacterial aggregates were stained with 100 µg mL^{-1} DAPI for 5 min and preparations were stored at 4 °C in the dark until their visualization. Autoaggregates and coaggregates were observed under a LEICA DMLB2 epifluorescence microscope (LEICA Microsystems, Germany) equipped with a filter sensitive to DAPI fluorescence (359 nm excitation filter in combination with a 461 nm emission filter). Several microphotographs of the stained samples were obtained using a LEICA DFC300 FX camera and LEICA IM50 Image Manager - Image processing and archiving software.

2.2.3. Inhibition of autoaggregation and coaggregation with simple sugars

The reversal or inhibition of aggregation was determined by the addition of simple sugars: D-(+)-galactose, N-acetyl-D-glucosamine, D-(+)-fucose and D-(+)-lactose (Sigma-Aldrich, Portugal) to the coaggregating pairs. Briefly, filter-sterilized solutions of each simple sugar (500 mM in sterile deionised water) were immediately added to the coaggregating pairs (t = 0 h), to a final concentration of 50 mM. Mixtures were then vortexed and analyzed by the visual coaggregation assay (after 2, 24 and 48 h) (Simões et al., 2008) and by epifluorescence microscopy.

2.2.4. Inhibition of autoaggregation and coaggregation by heat treatment

After adjusting the optical density (OD_{600} = 1), the bacterial suspensions were heated for 30 min at 80 ºC. Heat-treated and untreated bacterial cells were then combined in reciprocal pairs, and the capacity for the bacterial cells to coaggregate was assessed by the visual coaggregation assay (Simões et al., 2008) and by epifluorescence microscopy.

2.2.5. Inhibition of autoaggregation and coaggregation by protease treatment

Protease type XIV from *Streptomyces griseus* (P5147, Sigma-Aldrich, Portugal) was added to bacterial suspensions with adjusted optical density, ensuring a final concentration of 2 mg mL^{-1}. Protease pre-treatment of bacteria was carried out at 37 ºC, and cells were harvested after 2 h by centrifugation (20 min, 3,100 ×g) (Eppendorf centrifuge 5810R, Eppendorf, Germany) and washing three times with coaggregation testing medium. The bacterial suspensions were then readjusted to an optical density of 1, at 600 nm (Simões et al., 2008). Protease treated and untreated cells were mixed and their ability to coaggregate was determined using the visual assay and epifluorescence microscopy. For all tests, inhibition or reversal of coaggregation was detected by the decrease in coaggregation score.

2.3.Single and dual-species biofilm formation

Biofilms were developed according to the modified microtiter plate test proposed by Stepanovic et al. (2000). Briefly, for each condition, at least sixteen wells of a sterile 96-well microtiter plate (Tissue Culture Plate, VWR, Portugal) were filled with 200 µL of overnight cultures in R2A broth (with a cell density 10^8 cells mL^{-1}). Note that, for the dual combinations, 100 µl of each inoculum was added. Negative control wells contained R2A broth without bacterial cells. Plates were incubated aerobically on a shaker (IKA KS 130 shaker, Sigma-Aldrich, Portugal) at 150 rpm, at room temperature, for 24 and 48 h. For the 48 h plates, the growth medium was carefully discarded and replaced by a fresh one after 24 h of incubation. After the incubation period, the content of the well was removed and washed with 0.85% (v v^{-1}) sterile saline solution to remove the reversibly adherent bacteria. After that, plates were air-dried, and the remaining attached biofilms were analysed regarding their total biomass and bacterial culturability.

2.3.1. Biofilm mass quantification

Biomass of single and dual-species biofilms was quantified by the crystal violet (CV) staining method, according to Gomes et al. (2018). Briefly, the previously air-dried biofilms were fixed with 250 µl of 99% (v v^{-1}) ethanol for 15 min. After that, ethanol was removed and the plates were allowed to evaporate for a few minutes. Then, fixed biofilms were stained with 250 µl of 1% (v v^{-1}) CV (Merck, Germany) for 5 min. CV was removed, and the dye bound to the adherent cells was resuspended by adding 200 µl of 33% (v v^{-1}) glacial acetic acid (Merck, Germany). The absorbance was measured at 570 nm using a microplate reader (SPECTROstar® Nano, BMG LABTECH).

2.3.2. Cell culturability

The number of culturable biofilm cells was determined in terms of colony forming units (CFU). For that, after each biofilm formation period, the biofilm was washed with 200 µl of 0.85% (v v^{-1}) sterile saline. Then, biofilms were scraped three times (during periods of 1 minute each) after the addition of 200 µL of sterile saline solution to resuspend the attached sessile cells. The content of each well was transferred to independent sterile labelled microcentrifuge tubes (VWR, Portugal). Then, each tube contained 200 µL of each scrapping step (a total of 600 µL after the three scrapping steps) and an additional 400 µL of the sterile saline solution was added into each tube to achieve a total volume of 1 mL. Subsequently, ten-fold serial dilutions in sterile saline solution were performed and plated in triplicate on R2A agar. Plates were incubated at 25 ºC for 24 h. For dual-species biofilms, different agar media were used for better distinction of colonies: Luria Bertani (LB) agar-Chloramphenicol 25 mg mL^{-1} for the pair *D. acidovorans* 005P *D. acidovorans* 545P and LB agar-Gentamicin 12.5 mg mL^{-1} for all the other pairs (LB: Sigma-Aldrich, Portugal; Chloramphenicol and Gentamicin: Merck, Germany). The antibiotics and concentrations used were selected considering the susceptibility profiles of the strains (data not shown). The number of cultivable bacterial cells in biofilms was determined and expressed per area of the well (log CFU cm^{-2}).

2.3.3. Bacterial growth evaluation

A high throughput 96-well microtiter plate method was used to evaluate bacterial growth, taking multiple optical density measurements at various time intervals, using a microplate reader (SPECTROstar® Nano, BMG LABTECH) (Malheiro et al., 2019). Briefly, overnight bacterial cultures in R2A broth (25 ± 2 ºC, 150 rpm) were adjusted for their OD_{600} to 0.2. Then, 200 µL of the bacterial suspensions were added to the plate wells (at least in duplicate). Empty wells were filled with sterile fresh medium. The bacterial growth was measured at 600

nm using the microplate reader for 24 h at room temperature without continuous agitation. Optical density was measured in intervals of 1 h, with prior shaking.

The bacterial growth rate, μ (h^{-1}), was calculated through equation 3 for all pairs of consecutive values of OD_{600} according to the following equation:

$$\mu = ln\left(\frac{C_{i+1}}{C_i}\right) / (t_{i+1} - t_i) \quad (1)$$

where, C_i and C_{i+1}represent the OD_{600} values of any two consecutive time points ($t_i$and t_{i+1}, respectively).

Calculating bacterial growth rate, it was possible to determine the doubling time (dt; h) through the following equation:

$$dt = \frac{\ln(2)}{\mu} \quad (2)$$

2.4. Statistical analysis

Data analysis was performed using the statistical program SPSS version 27.0 (Statistical Package for the Social Sciences). Descriptive statistics were used to calculate the mean and standard deviation (SD). The data were analysed using a paired samples t-Test, since the variables were normally distributed, and were based on a confidence level ≥ 95 % ($P < 0.05$ was considered statistically significant). Three independent experiments with three replicates were performed for each assay.

3. Results and Discussion

3.1. Autoaggregation and coaggregation ability of the drinking water isolated bacteria

Aggregation was assessed visually since it is a rapid and simple method, which provides reproducible results with enough sensitivity to detect significant interactions (Buswell et al.,

1997). Distilled water was used to assess autoaggregation and coaggregation based on Min et al. (2010), who reported that standard laboratory saline buffers such as PBS can inhibit coaggregation due to the high concentration of ions in these solutions. In that study, the presence of 60 mM NaCl decreased coaggregation by 1 score (the concentration of NaCl in PBS is 137 mM). The interference of $MgSO_4$ (present in STW) in coaggregation was also studied and it was observed that, from low concentrations onwards, there was a decrease in coaggregation, with a complete absence of the formation of aggregates from a concentration of 40 mM (Min et al., 2010). Thus, the presence of NaCl, $MgSO_4$ and other salts in a media seems to be the reason for the absence of this event. Charged groups can associate or dissociate with cell surface polymers through changes in the ionic strength and/or pH (Poortinga et al., 2002), inducing changes in the conformation of structures and appendages of the cell surface (*e.g.*, surface polysaccharides, fibrils, fimbriae, and flagella) (Donlan, 2002). Furthermore, the ionic strength (*I*) could also be involved in the absence of aggregation with PBS (I = 68 mM). In the case of STW, since the ionic strength has very low values, I = 0.06 mM, the most plausible reason for coaggregation inhibition is the presence of salts. Despite the inhibition of macroscopic aggregation in ionic solutions, the formation of small (microscopic) aggregates may occur.

In this study, all the 38 DW-isolated bacterial strains were visually assessed for their ability to autoaggregate and, out of a total of 741 pairs of possible combinations, 115 pairs were studied for their ability to coaggregate. Coaggregation was evaluated between bacterial strains with autoaggregation ability, combined with different bacterial genera. The pairs tested for coaggregation and their respective scores are presented in Table S2. It was observed that, among the 38 strains evaluated, only nine were able to autoaggregate. Table 1 presents the positive autoaggregation results over time in distilled water. Among the 115 pairs tested, five of them (*D. acidovorans* 005P - *C. freundii* 002L; *D. acidovorans* 005P - *C.*

freundii 003L; *D. acidovorans* 005P - *P. fluorescens* 008P; *D. acidovorans* 005P -*P. putida* 011P; and *D. acidovorans* 005P - *E. cloacae* 023L) formed aggregates, suggesting the occurrence of coaggregation. Interestingly, all the positive results of coaggregation took place in the presence of *D. acidovorans* 005P (Table 2). *D. acidovorans* was formerly named *Comamonas acidovorans* in 1999, having undergone this rename after sequencing the 16s rRNA (Wen et al., 1999). Strains of *Comamonas* had already been shown to coaggregate (Bossier and Verstraete, 1996; Cheng et al., 2014; Jiang et al., 2006). Bossier and Verstraete (1996) demonstrated coaggregation between *Comamonas testosteroni* isolated from activated sludge and yeast cells. Cheng et al. (2014) reported the coaggregation of *C. testosteroni* from wastewater with bacteria from other water environments. In Jiang et al. (2006), the strain *Comamonas* sp. PG-08 coaggregated with *Propionibacterax*-like PG-02, both isolated from phenol-degrading aerobic granules. This is the first time that coaggregation has been demonstrated for *D. acidovorans* and the genus *Delftia* in drinking water. *D. acidovorans* is an environmental bacteria found in water and soil, generally non-pathogenic (Højgaard et al., 2022). However, it has been reported in immunocompetent and immunocompromised individuals, as well as those with underlying diseases (Højgaard et al., 2022). In addition, cases of nosocomial infections associated with this species have already been described (Ta et al., 2020; Yassin et al., 2020).

From the analysis of Table 1, after 2 h of bacterial interaction coaggregation was higher for *D. acidovorans* 005P - *C. freundii* 003L (coaggregation score of 1 / 2). This score increased at 24 h (score of 3) and remained with an invariable score until 48 h of the experiment. Overall, comparing coaggregation scores over time, it is shown that the maximum scores were achieved at 24 h and maintained until a period of 48 h. The same was observed in other works for aquatic bacteria, such as *Acinetobacter calcoaceticus* (Simões et al., 2008), *Blastomonas natatoria* and *Micrococcus luteus* (Rickard et al., 2000). In Rickard et al.

(2000), the overall maximum coaggregation capacity was maintained until 50 h and then declined to zero (Rickard et al., 2000). The coaggregation results were confirmed by epifluorescence microscopy using DAPI stain. Microscopy has also been successfully applied to assess bacterial coaggregation (Douterelo et al., 2014; Simões et al., 2008). Figure 1 shows several representative microphotographs of various interactions between distinct DW bacteria with and without visual coaggregation (Figure S1 presents the micrographs of other coaggregating pairs). Epifluorescence microscopy analysis revealed a greater degree of interaction than the visual coaggregation assay. This feature was evident for all interactions, even for autoaggregation.

Inhibition assays were performed to determine the surface-associated molecules involved in coaggregation. The addition of simple sugars and heat/protease treatment assays were selected considering that previous studies have demonstrated the involvement of lectin-saccharide-type interactions in coaggregation (Buswell et al., 1997; Min and Rickard, 2009; Rickard et al., 2000, 2003b; Simões et al., 2008). Inhibition or reversal of coaggregation was determined as a reduction in the coaggregation score. Bacterial aggregation was partially inhibited by the addition of simple sugars (D-(+)-galactose, N-acetyl-D-glucosamine, D-(+)-fucose and D-(+)-lactose). No sugar was able to completely reverse autoaggregation and coaggregation (Table 3). The coaggregating pair *D. acidovorans* 005P - *C. freundii* 003L was disaggregated by all sugars, except by D (+)-lactose. On the other hand, D (+)-lactose was the only sugar capable of partial disaggregation of the pair *D. acidovorans* 005P - *E. cloacae* 023L. For the coaggregating pairs *D. acidovorans* 005P - *P. putida* 011P and *D. acidovorans* 005P - *P. fluorescens* 008P, no disaggregation was observed with any sugar. In this case of coaggregation between *D. acidovorans* 005P and both *Pseudomonas* strains the interactions seem to be unrelated to saccharides since no disaggregation was observed, regardless of the sugar used. Autoaggregation of *D. acidovorans* 005P was partially inhibited by all sugars,

suggesting an interaction not as specific as coaggregation, and that protein-saccharide interaction may not be the only one involved in the autoaggregation process. It was expected that the addition of simple sugars would reverse the lectin-saccharide (protein-carbohydrate)-like interactions, as already observed for oral coaggregating bacteria (Buswell et al., 1997). However, for bacteria from aquatic systems it has been observed that such interactions are very specific (Rickard et al., 2000, 2003b) and even dependent on the type of sugar and the concentration used (Simões et al., 2008). The effect of heat and protease treatment on aggregation scores was also evaluated (Table 4). Regarding *D. acidovorans* 005P autoaggregation, when one partner was treated (heat or protease) the score was maintained, suggesting that this interaction is not dealing exclusively with protein-protein interaction. The complete inhibition of aggregation when both partners were treated indicates that proteins are essential in this type of interaction, corroborating the previous hypotheses that this interaction depends not only on protein-saccharide bonds but also on these protein-protein. For coaggregating pairs, heat and protease treatment led to complete coaggregation inhibition of all the partnerships studied, when the treatments were applied to both partners. When only *D. acidovorans* 005P was treated with heat either complete inhibition (*D. acidovorans* 005P - *P. fluorescens* 008P and *D. acidovorans* 005P - *E. cloacae* 023L) or a reduction in the score (*D. acidovorans* 005P - *C. freundii* 002L, *D. acidovorans* 005P - *C. freundii* 003L and *D. acidovorans* 005P - *P. putida* 011P) was observed. In the case of *D. acidovorans* 005P being the only partner treated with protease, complete inhibition of coaggregation was observed for the pairs studied, except for *D. acidovorans* 005P - *C. freundii* 002L and *D. acidovorans* 005P - *C. freundii* 003L. For the combinations between *D. acidovorans* 005P and *C. freundii* 002L, *C. freundii* 003L or *E. cloacae* 023L, the inhibition assays suggested that the interactions are particularly dependent on proteins. This is because, more evident changes in the coaggregation score were observed when bacteria were treated with heat and protease;

although protein-saccharide interactions also exist, as suggested by the sugar addition tests. For combinations of *D. acidovorans* 005P with both *Pseudomonas* strains (*P. fluorescens* 008P and *P. putida* 011P), the results suggest that the coaggregation may be mediated by protein-protein interactions. Here, inhibition of coaggregation only occurred when one of the bacterial strains was heat or protease-treated. This hypothesis was also corroborated by the sugar addition test, as coaggregation was not reversed in the presence of any of the sugar molecules tested. So far, protein-protein interactions had never been identified for strains from aquatic environments, having only been observed in studies involving oral strains (Daep et al., 2008).

3.2. Impact of coaggregation in biofilm formation

To understand the role of coaggregation in the establishment of sessile communities, dual-species biofilms were allowed to develop for 24 and 48 h. These biofilms were formed by the five bacterial combinations positive for coaggregation (*D. acidovorans* 005P - *C. freundii* 002L; *D. acidovorans* 005P - *C. freundii* 003L; *D. acidovorans* 005P - *P. fluorescens* 008P; *D. acidovorans* 005P - *P. putida* 011P; and, *D. acidovorans* 005P - *E. cloacae* 023L) and characterized in terms of mass and culturability. Single-species biofilms were formed for each bacterial strain, for comparison. Figure 2 shows an over time increase of single-species biofilms mass ($P < 0.05$), except for *C. freundii* 002L and *P. putida* 011P ($P > 0.05$). For dual-species biofilms, an increase in biomass over time ($P < 0.05$) was observed for the combinations of *D. acidovorans* 005P with *C. freundii* 002L, *C. freundii* 003L and *E. cloacae* 023L. In all the other cases, the biomass was not different between the 24 and 48 h-old biofilms ($P > 0.05$). For the combinations between *D. acidovorans* 005P - *C. freundii* 003L and *D. acidovorans* 005P - *P. fluorescens* 008P, a significant increase in biomass was observed in comparison to *C. freundii* 003L and *P. fluorescens* 008P single-species biofilms

($P < 0.05$) at 48 h and 24 h, respectively (Figure 2). The 24 h-old *D. acidovorans* 005P - *C. freundii* 002L dual-species biofilms and the 48 h-old *D. acidovorans* 005P - *P. putida* 011P dual-species biofilms had lower mass than the single species counterparts ($P < 0.05$). In the remaining cases, significant alterations in dual-species biomass were not observed in comparison to the values obtained for single-species biofilms, proposing a neutral effect of coaggregation and interspecies interactions in biofilm production. These findings are in line with the work by Simões et al. (2008). These authors evaluated biomass formation of multispecies biofilms in the presence/absence of a coaggregating strain of *A. calcoaceticus* and generally observed an increase over time and when this strain was part of the biofilm. However, using a strain exclusion process, they observed different behaviors in biomass production, suggesting that certain strains benefited more from the presence of *A. calcoaceticus* than others.

In terms of cell culturability (Figure 3), both single and dual-species biofilms had comparable CFU at 24 h and 48 h, for almost all cases ($P > 0.05$). The exceptions were *D. acidovorans* 005P and *E. cloacae* single-species biofilms, where there was an increase from 24 h to 48 h ($P < 0.05$). When comparing dual-species biofilms with these single-species (of other bacteria than *D. acidovorans* 005P), significant alterations in the number of culturable cells were also not observed ($P > 0.05$). The exception was the comparison between *E. cloacae* 023L biofilms and *D. acidovorans* 005P - *E. cloacae* 023L biofilms at 48 h, in which the number of CFU was significantly higher for these from single-species ($P < 0.05$). The increase in biomass but not in culturability observed for *D. acidovorans* 005 - *C. freundii* 003L and *D. acidovorans* 005 - *P. fluorescens* 008P, can be explained by the higher productivity of extracellular polymeric substances (EPS) and cell replication. The CV method not only quantifies cells but also EPS (Stiefel et al., 2016). Therefore, some differences between the results obtained by the CV method and by CFU enumeration may be related to the EPS

content (Dertli et al., 2015). Interestingly, for 24 and 48 h-old biofilms formed by *D. acidovorans* 005P combined with *C. freundii* 002L or *P. putida* 011P, the CFU values were much lower than that observed for the single-species biofilms ($P < 0.05$). To understand this result, bacterial growth dynamics were characterized in terms of growth rate and doubling time (Table 5). *D. acidovorans* 005P, *P. fluorescens* 008P and *E. cloacae* 023L were those with the highest growth rates and, consequently, with the lowest doubling time, respectively ($P > 0.05$). Both *C. freundii* strains and *P. putida* 011P were those with the lowest specific growth rates. These results do help to understand the dual-species biofilm results. However, they suggest that even if the planktonic cells were able to coaggregate they antagonize when forming the dual-species biofilms. When comparing the specific growth rates and doubling times of these two strains with *D. acidovorans* 005P, the differences were significant ($P < 0.05$). It is accepted that the spatial arrangement of different species within biofilms strongly influences the relative fitness benefits of cooperative and competitive phenotypes (Nadell et al., 2016). Besides that, social phenotypes include not only growth rate regulation, but also the secretion of compounds (Mitri et al., 2011). Some secondary metabolites may even interfere with the growth rate of other bacteria when in co-culture. Alkaloids produced by *Citrobacter freundii* grown associated with the marine sponge *Cliona* sp. showed antimicrobial activity against multidrug-resistant pathogens (Skariyachan et al., 2016). A decrease in the growth rate of *Candida albicans* was observed when in co-culture with *P. aeruginosa*, due to the production of the phenazine 5-MPCA (Doing et al., 2020). On the other hand, some compounds secreted can behave as public goods. In a cooperative interaction, these kinds of compounds would be exploited by non-producing cells (Drescher et al., 2014), which means that they can also be easily exploited by cheaters (Smith and Schuster, 2019). Cheaters are non-cooperative individuals in the population who benefit from compounds (public goods) produced by cooperators but do not share their production costs

(Smith and Schuster, 2019). A simple way to confirm the cheating of microorganisms is to assess the relative growth rate individually and in co-culture. When co-cultured, non-producers grow faster than the producers. Conversely, when these strains are grown individually, producers grow at a faster rate than non-producers (Smith and Schuster, 2019). The same was observed in this study through the analysis of log CFU cm^{-2} and growth dynamics of individual bacteria. Moreover, density dependence (Ross-Gillespie et al., 2009) and frequency dependence (Ross-Gillespie et al., 2007) studies revealed to be basic properties of public goods cheating.

4. Conclusion

There is a lack of understanding of coaggregation-based interactions and how they influence multispecies biofilm formation in aquatic environments. In this study, a *D. acidovorans* strain was described for the first time as capable of coaggregating with other DW strains. Inhibition assays characterized *D. acidovorans* 005P coaggregation as mediated by polysaccharide-like receptors and protein adhesins, depending on the bacterial species involved. When the coaggregation partner was a *Pseudomonas* strain (*P. fluorescens* 008P or *P. putida* 011P), the predominant interactions were found to be mediated by protein-protein bonds. In addition, and since biofilm communities found in nature consist of a variety of microorganisms, dual-species biofilms were formed between *D. acidovorans* 005P and other bacteria isolated from DW to understand the role of this coaggregating bacterium in DW biofilms. Biomass quantification assay suggested that the biofilms of *C. freundii* 003L and *P. fluorescens* 008P benefited from the presence of the coaggregating strain. On the other hand, the culturability assay did not show differences between single and dual-species biofilms. However, a decrease in the number of cells of *D. acidovorans* 005 biofilms when cultivated with *C. freundii* 002L and *P. putida* 011P suggests a superimposition of these strains. Furthermore,

the findings regarding growth rate indicate a case of producers (*D. acidovorans* 005P) and non-producers (the other DW species, especially *C. freundii* 002L and *P. putida* 011P) of public goods. Thus, this study shows that *D. acidovorans* may provide a metabolic opportunity for other species, creating a functional cooperating microbial community.

Declaration of Competing Interest

Authors declare no conflict of interests.

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Figure Captions

Figure 1. Microscopy visualizations by epifluorescence microscopy (DAPI stained) of aggregation between DW bacteria. Visually assigned scores for each sample at 24 h: **a)** *D. acidovorans* 005P autoaggregation (score of 1 / 2); **b)** *C. freundii* 002L (score of 1) autoaggregation; **c)** *D. acidovorans* 005P - *C. freundii* 003L (score of 3) coaggregation; **d)** *D. acidovorans* 005P - *P. fluorescens* 008P (score of 2) coaggregation; **e)** *C. freundii* 002L - *P. putida* 011P (score of 0) coaggregation; **f)** *C. freundii* 003L autoaggregation (score of 0). bar = 5 μm (epifluorescence photomicrographs).

Figure 2. Values of $OD_{570\ nm}$ correspond to the biomass of single and dual-species biofilms for 24 h (black) and 48 h (white). **a)** *D. acidovorans* 005P single species biofilms; **b)** *C. freundii* 002L single and dual-species biofilms; **c)** *C. freundii* 003L single and dual-species biofilms; **d)** *P. fluorescens* 008P single and dual-species biofilms; **e)** *P. putida* 011P single and dual-species biofilms; **f)** *E. cloacae* single and dual-species biofilms. The experiment was performed in triplicate and repeated three times. The error bars indicate SD.*$P < 0.05$; **$P < 0.01$.

Figure 3. Cellular culturability of single (filled) and dual-species (stripes) biofilm represented by log (CFU cm^{-2}) values at 24 h (black) and 48 h (white). **a)** *C. freundii* 002L single and dual-species biofilms; **b)** *C. freundii* 003L single and dual-species biofilms; **c)** *P. fluorescens* 008P single and dual-species biofilms; **d)** *P. putida* 011P single and dual-species biofilms; **e)** *E. cloacae* single and dual-species biofilms. The experiment was performed in triplicate and repeated three times. The error bars indicate SD. *$P < 0.05$.

Figure 1

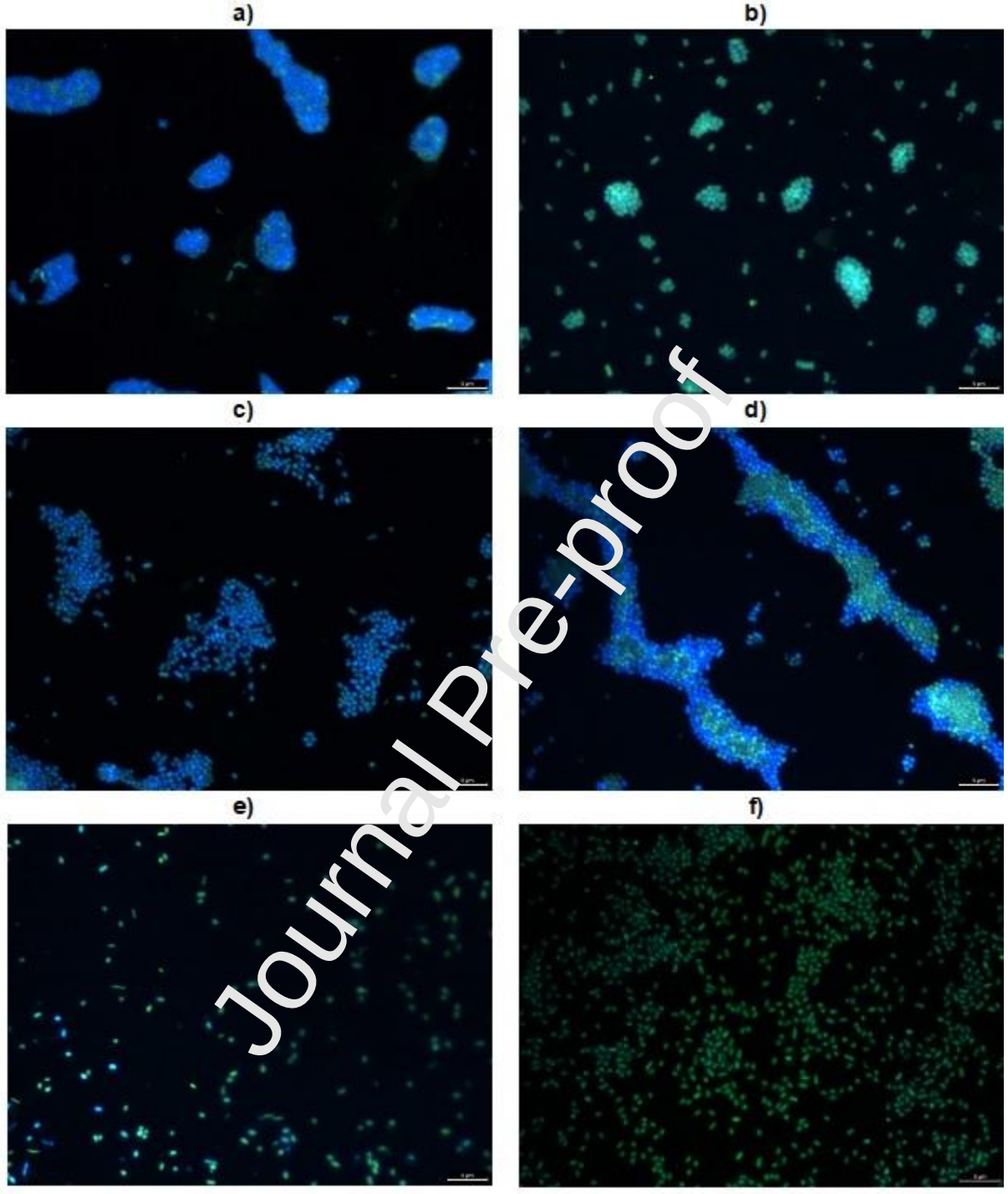


Figure 2

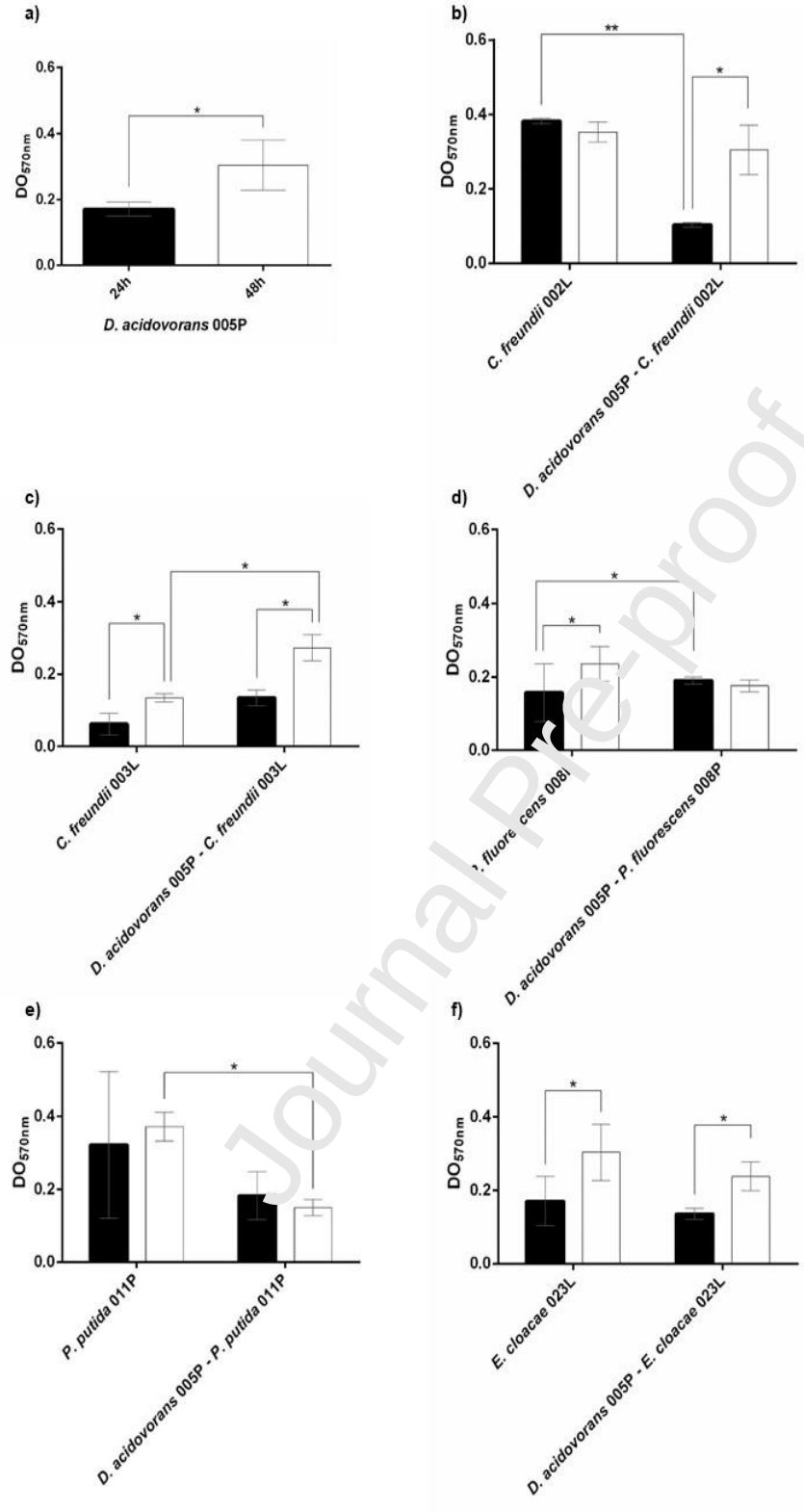


Figure 3

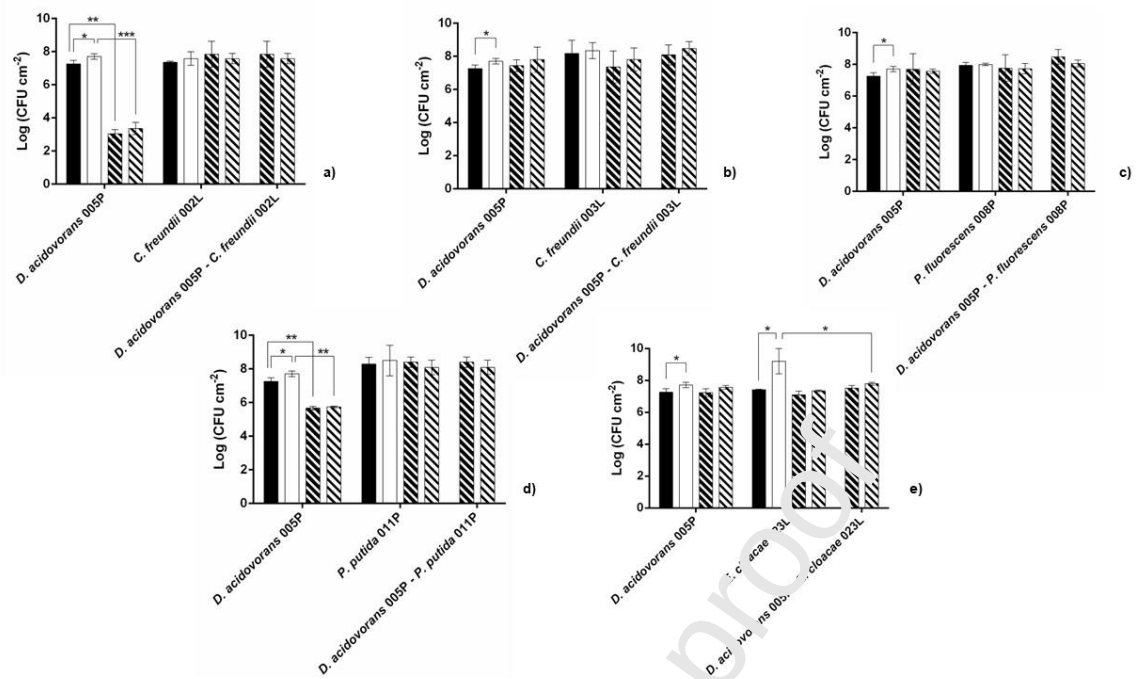


Table 1. Positive autoaggregation results over time (2, 24 and 48 h) among the 38 strains tested in sterile distilled water.

	2 h	24 h	48 h
Acinetobacter sp. 622P/B	0 / 1	1	1
Acinetobacter sp. 023P	0 / 1	1	1
Acinetobacter sp. 021P/Aci	0 / 1	1	1
D. acidovorans[a] 005P	1	1 / 2	1 / 2
D. acidovorans 009P	0	1	1
D. acidovorans 644BP	0	1	1
D. acidovorans 545BP	0 / 1	1	1
D. acidovorans 500PP	1	1	1
C. freundii[b] 002L	0 / 1	1	1

[a]*Delftia acidovorans;* [b]*Citrobacter freundii*

Table 2. Positive coaggregation results over time (2, 24 and 48 h) among 115 pairs tested in sterile distilled water.

	2 h					
	D. acidovorans[a] 005P	*C. freundii*[b] 002L	*C. freundii*[b] 003L	*P. fluorescens*[c] 008P	*P. putida*[d] 011P	*E. cloacae*[e] 023L
D. acidovorans 005P	**1**	**1**	**2**	**1**	**1 / 2**	**1**
C. freundii 002L		**0 / 1**	0	0	0	0
C. freundii 003L			0	0	0	0
P. fluorescens 008P				0	0	0
P. putida 011P					0	0
E. cloacae 023L						0
	24 h					
	D. acidovorans 005P	*C. freundii* 002L	*C. freundii* 003L	*P. fluorescens* 008P	*P. putida* 011P	*E. cloacae* 023L
D. acidovorans 005P	**1 / 2**	**2**	**3**	**2**	**2**	**2**
C. freundii 002L		**1**	0	0	0	0

C. freundii 003L			0	0	0	0
P. fluorescens 008P				0	0	0
P. putida 011P					0	0
E. cloacae 023L						0

			48 h			
	D. acidovorans 005P	*C. freundii* 002L	*C. freundii* 003L	*P. fluorescens* 008P	*P. putida* 011P	*E. cloacae* 023L
D. acidovorans 005P	**1 / 2**	**2**	**2**	**2**	**2**	**2**
C. freundii 002L		**1**	0	0	0	0
C. freundii 003L			0	0	0	0
P. fluorescens 008P				0	0	0
P. putida 011P					0	0
E. cloacae 023L						0

[a]*Delftia acidovorans;* [b]*Citrobacte freundii;* [c]*Pseudomonas fluorescens;* [d]*Pseudomonas utida;* [e]*Enterobacter cloacae*

Table 3. The reversal of autoaggregation and coaggregation using simple sugars.

	D (+) - Galactose	D (+) - Lactose	N-acetyl-D-glucosamine	D (+) - Fucose
D. acidovorans[a] 005P - *D. acidovorans* 005P	+	+	+	+
D. acidovorans 005P - *C. freundii*[b] 002L	-	+	+	-
D. acidovorans 005P - *C. freundii* 003L	+	-	+	+
D. acidovorans 005P - *P. fluorescens*[c] 008P	-	-	-	-
D. acidovorans 005P - *P. putida*[d] 011P	-	-	-	-
D. acidovorans 005P - *E. cloacae*[e] 023L	-	+	-	-

++ Complete disaggregation; + partial disaggregation; - no disaggregation.

[a]*Delftia acidovorans;* [b]*Citrobacter freundii;* [c]*Pseudomonas fluorescens*; [d]*Pseudomonas putida;* [e]*Enterobacter cloacae*

Table 4. The reversal of autoaggregation and coaggregation using heat and protease treatment.

		D. acidovorans[a] 005P		*C. freundii*[b] 002L		*C. freundii* 003L		*P. fluorescens*[c] 008P		*P. putida*[d] 011P		*E. cloacae*[e] 023L	
		UT	T	UT	T	UT	T	UT	T	UT	T	UT	T
		Heat Treatment											
D. acidovorans 005P	UT	**1**	1	**2**	1/2	**2**	1	**1**	0/1	**1/2**	1	**1**	1
	T	1	0	1	0	0/1	0	0	0	0/1	0	0	0
		Protease Treatment											
D. acidovorans 005P	UT	**1**	1	**2**	1	**2**	1	**1**	0/1	**1/2**	0/1	**1**	0/1
	T	1	0	1	0	0/1	0	0	0	0	0	0	0

Bold numbers indicate untreated control scores. UT, the untreated partner; T, the treated partner

[a]*Delftia acidovorans;* [b]*Citrobacter freundii;* [c]*Pseudomonas fluorescens*; [d]*Pseudomonas putida;* [e]*Enterobacter cloacae*

Table 5. Bacterial growth rate and doubling time. The means ± SD of three independent experiments with three replicates are illustrated. [#]statistically significant difference compared to *D. acidovorans* 005P ($P < 0.05$).

	Growth rate (h^{-1})	**Doubling time (h)**
D. acidovorans[a] 005P	0.21 ± 0.00	3.40 ± 0.04
C. freundii[b] 002L	0.14 ± 0.00[#]	5.00 ± 0.19[#]
C. freundii 003L	0.13 ± 0.03[#]	5.33 ± 1.03[#]
P. fluorescens[c] 008P	0.21 ± 0.02	3.25 ± 0.25
P. putida[d] 011P	0.14 ± 0.01[#]	5.08 ± 0.42[#]
E. cloacae[e] 023L	0.19 ± 0.00	3.48 ± 0.06

[a]*Delftia acidovorans;* [b]*Citrobacter freundii;* [c]*Pseudomonas fluorescens*; [d]*Pseudomonas putida;* [e]*Enterobacter cloacae*

CRediT authorship contribution statement

Ana Afonso: conceptualization, methodology, validation, formal analysis, investigation, writing – original draft, funding acquisition.

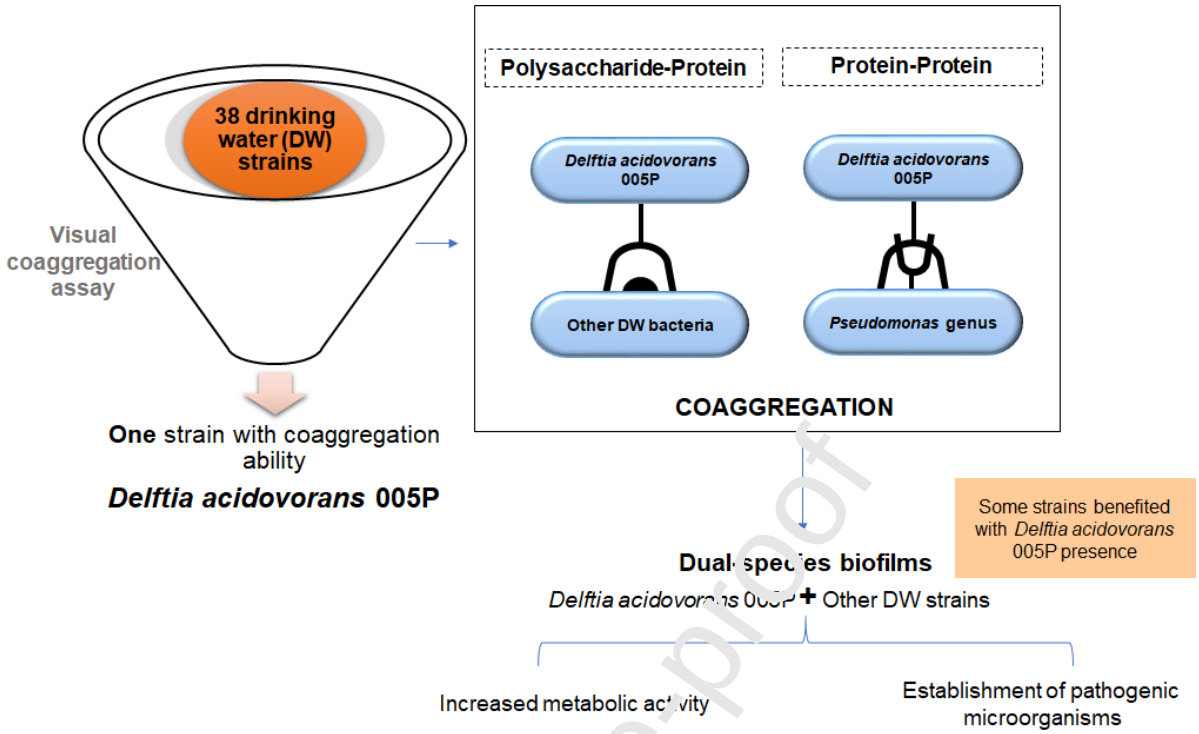
Inês Bezerra Gomes: investigation, writing – review & editing.

Maria José Saavedra: conceptualization, methodology, resources, writing – review & editing, supervision.

Lúcia Simões: conceptualization, methodology, resources, writing – review & editing, supervision.

Manuel Simões: conceptualization, methodology, resources, writing – review & editing, supervision, project administration, funding acquisition.

Graphical abstract



Highlights

- A total of 38 bacterial strains were studied for their coaggregating ability
- Coaggregation of *Delftia acidovorans* is described for the first time
- Polysaccharide-protein and protein-protein interactions were involved in coaggregation
- Multispecies biofilm formation benefited from the presence of *D. acidovorans*
- The production of public goods by *D. acidovorans* is suggested