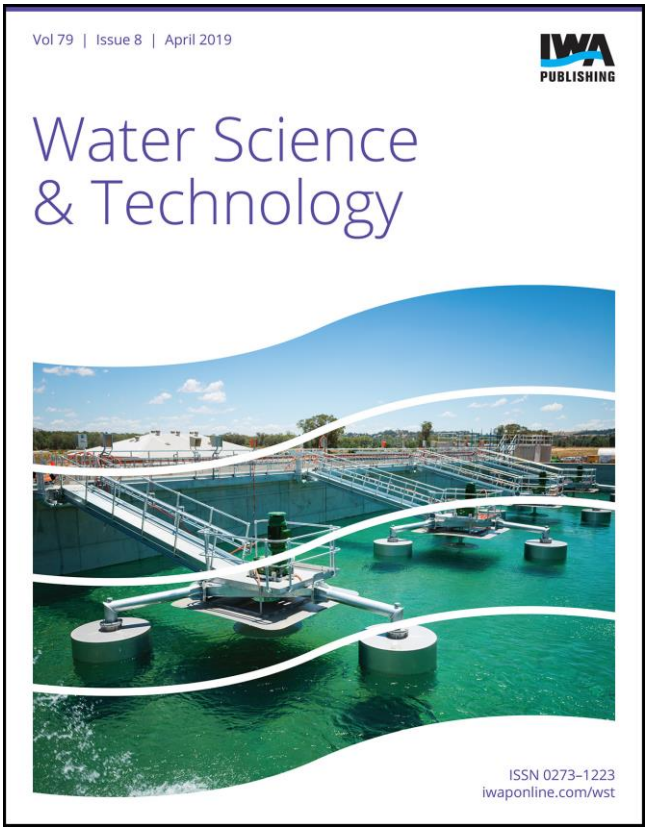

Impact of biotic interactions on the survival of emerging pathogen *Acinetobacter baumannii* in aquatic media

Svjetlana Dekić, Jasna Hrenović, Holger Herlyn, Maria Špoljar and Tomislav Ivanković

ABSTRACT

Acinetobacter baumannii is an opportunistic pathogen causing infections in immunocompromised patients. Recent studies recorded its persistence in a variety of abiotic conditions, but data regarding the biotic interactions with other microorganisms are limited. The aim was to assess the interaction of clinically relevant *A. baumannii* with common faecal bacteria *Escherichia coli* and *Enterococcus faecium*. Additionally, the interaction with a bdelloid rotifer *Adineta vaga* as a potential agent for biological control of *A. baumannii* was examined. Experiments were conducted in nutrient-poor spring water (SW) and nutrient-rich diluted nutrient broth (DNB) at 22 °C. *A. baumannii* coexisted with *E. coli* and *E. faecium* in both media, suggesting the absence of inter-bacterial competition in long-term survival. No difference in the survival of pandrug-resistant, extensively drug-resistant or antibiotic sensitive isolates of *A. baumannii* was observed. Rotifers contributed to the removal of all tested bacteria, particularly in SW. Rotifers were able to remove 5.5 ± 1.3 log CFU/mL of *A. baumannii* in SW and 3.5 ± 1.7 log CFU/mL in DNB. Additionally, no intracellular growth of *A. baumannii* inside *A. vaga* was detected. In wastewater treatment plants and drinking water facilities, grazing by rotifers might be useful for the removal of emerging human pathogens such as *A. baumannii* from water.

Key words | *Acinetobacter baumannii*, bacteria, inter-bacterial interaction, rotifers

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INTRODUCTION

Acinetobacter baumannii is an opportunistic bacterial pathogen causing infections in hospitals and veterinary clinics as well as outside the hospital setting (Roca *et al.* 2012; Dexter *et al.* 2015; Ewers *et al.* 2017). In immunocompromised patients it can cause pneumonia, meningitis, urinary tract, bloodstream and wound infections (McConnell *et al.* 2013). Its occurrence in intensive care units and operating rooms is extremely concerning due to its resistance to multiple disinfectants and antibiotics (Ivanković *et al.* 2017). *A. baumannii* is top-ranked on the WHO list of the most dangerous pathogens for which new treatment measures are urgently needed. The increasing concern is the occurrence of carbapenem-resistant *A. baumannii* isolates in the hospital setting (WHO 2017). Carbapenems are last resort beta-lactam antibiotics used to treat infections caused by multidrug-resistant bacteria. In Croatia, carbapenem resistance of *A. baumannii* isolates has increased drastically from 10% in 2008 to 86% in 2016 (CAMS 2017). Furthermore, *A. baumannii* has been proven to survive in adverse environmental conditions such as starvation, desiccation (Espinal *et al.* 2012; Bravo *et al.* 2016), lack of oxygen (Higgins *et al.* 2018), extreme temperatures and pH regimes (Dekic *et al.* 2018).

A. baumannii was considered to be an exclusively hospital bacterium until its recovery from untreated hospital wastewaters (Ferreira *et al.* 2011; Zhang *et al.* 2013), wastewater treatment plants (Hrenovic *et al.* 2016; Higgins *et al.* 2018) and rivers (Girlich *et al.* 2010; Seruga Music *et al.* 2017). These records have confirmed the dissemination of clinically relevant *A. baumannii* from the hospital setting into the natural environment and raised the question of its survival capability. Previous studies have recorded the successful survival of pure cultures of *A. baumannii* in autoclaved spring water, seawater and wastewater treatment plant effluent for 50 days (Hrenovic *et al.* 2016; Kovacic *et al.* 2017; Dekić & Hrenović 2018).

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These findings imply that *A. baumannii* is a resilient bacterium capable of surviving in various environments and abiotic conditions.

Bacterial competition and interaction with other prokaryotic microorganisms and eukaryotes is generally poorly investigated (Arndt 1993; Lapinski & Tunnacliffe 2003). In natural environments, different bacterial species as well as metazoans (i.e. rotifers, cladocerans) are in mutual relationships, and more studies should be directed toward inter-species interactions. Two studies reported the capability of two strains of *A. baumannii* to outcompete other strains of *A. baumannii*, as well as *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* via the type VI secretion system within several hours at 37°C (Carruthers *et al.* 2013; Repizo *et al.* 2015). However, this mechanism of inter-bacterial competition is not applicable to other *A. baumannii* strains (Repizo *et al.* 2015), and not applicable to environmental conditions.

Since there have been numerous reports of clinically relevant *A. baumannii* in wastewaters (Ferreira *et al.* 2011; Zhang *et al.* 2013; Hrenovic *et al.* 2016; Higgins *et al.* 2018), where faecal bacteria are abundant, the aim of this study was to examine the inter-bacterial competition of clinically relevant *A. baumannii* with common faecal bacteria *E. coli* and *Enterococcus faecium*. In the aquatic environment and during the wastewater treatment process in activated sludge, bacteria come into contact with bacterivores such as rotifers. Rotifers are ubiquitous eukaryotes commonly found in diverse aquatic ecosystems. Bdelloid rotifers are microphagous and can ingest organic particles smaller than 15 μm by filtering the suspension or scraping biofilms (Wallace *et al.* 2006; Kuczyńska-Kippen 2018). They are an important part of activated sludge in wastewater treatment plants, thus taking part in water purification and floccule formation (Lapinski & Tunnacliffe 2003; Kocerba-Soroka *et al.* 2013). However, data regarding the biotic interactions between *A. baumannii* and rotifers are completely lacking. Therefore, another aim was to examine the potential of a bdelloid rotifer *Adineta vaga* as a biological agent for the removal of clinically relevant *A. baumannii* from the aquatic environment.

Experiments were conducted in microcosms containing simulated nutrient-poor or nutrient-rich water at 22°C. To our knowledge, this is the first report on the influence of biotic factors on the long-term survival of *A. baumannii* in simulated environmental conditions, which are applicable for predicting the behavior of this emergent pathogen in the environment.

MATERIALS AND METHODS

Characteristics of bacterial isolates

A. baumannii environmental isolates EF7 (Goic-Barisic *et al.* 2017), EF11 (Higgins *et al.* 2018), and one clinical isolate OB4138 (Seruga Music *et al.* 2017) were used in the experiments. Environmental isolates were recovered from effluent of the wastewater treatment plant for Zagreb, Croatia. Clinical isolate was recovered from a patient suffering from hospital-acquired pneumonia in the Special Hospital for Pulmonary Diseases, Zagreb. The isolates were grouped according to their antibiotic susceptibility profile into several categories: pandrug-resistant (EF7), extensively drug-resistant (OB4138) and sensitive to all tested antibiotics (EF11). Isolates EF7 and OB4138 expressed resistance to carbapenems.

E. coli and *E. faecium* isolated from the effluent of the wastewater treatment plant in Zagreb, Croatia were used for inter-bacterial competition assay. Wastewater was sampled aseptically and transferred to the laboratory within 1 hour. The sample was serially diluted in the physiological solution and filtered through membrane filters (pore size 0.45 μm). The filters were then transferred to selective plates. *E. coli* was cultivated on EC X-GLUC agar (Biolife) at 44 °C/48 h. *E. faecium* was cultivated on Slanetz-Bartley agar (Biolife) at 37 °C/72 h and confirmed on bile esculin azide agar (Biolife) at 44 °C/4 h. Identification of species was confirmed by MALDI TOF MS (Matrix-assisted laser desorption/ionization mass spectrometry, software version 3.0, Microflex LT, Bruker Daltonics). Antibiotic susceptibility profile was determined by the disk diffusion method and Vitek2 system. The results of antibiotic susceptibility testing were interpreted according to EUCAST criteria for clinical isolates of *E. coli* or *E. faecium* (EUCAST 2018). Both isolates were susceptible to all tested antibiotics.

Inter-bacterial competition assay

Interaction of *A. baumannii* with *E. coli* and *E. faecium* was monitored during 7 weeks in autoclaved commercially available spring water (SW) and nutrient broth (Biolife) diluted with distilled water 1:100 (DNB) at room temperature (22 ± 2 °C). SW represented nutrient-poor oligotrophic water, while DNB simulated nutrient-rich eutrophic water such as wastewater. The physio-chemical characteristics of the tested water media are presented in Dekic *et al.* (2018). Three *A. baumannii* isolates (EF7, EF11, OB4138), *E. coli*, and *E. faecium* were grown on CHROMagar *Acinetobacter*

(42 °C/24 h), EC X-Gluc (44 °C/48 h) and Slanetz-Bartley agar (37 °C/72 h), respectively. The bacteria were suspended separately in a test tube containing 10 mL of physiological solution. One mL of each of the *A. baumannii* isolates was inoculated separately into the test tubes containing 40 mL of SW or DNB and mixed with 1 mL of *E. coli* suspension (separate microcosms of *E. coli* with EF7, EF11, OB4138). The procedure was repeated separately with *E. faecium* instead of *E. coli* (separate microcosms of *E. faecium* with EF7, EF11, OB4138). Test tubes were rotated at 3 rpm using Stuart Tube Rotator SB3. The *A. baumannii* control presented here is the mean value of the abundance of pure cultures (without interactions) of all three *A. baumannii* isolates, since all isolates had similar behaviour. *E. coli* and *E. faecium* control are mean values of a pure culture of *E. coli* or *E. faecium* without interactions with *A. baumannii*. Abundance of *A. baumannii*, *E. coli* and *E. faecium* was measured at 0, 1, 2 and every 7 days as colony forming units (CFU) grown on CHROMagar Acinetobacter (42 °C/24 h), EC X-Gluc (44 °C/48 h) and Slanetz-Bartley agar (37 °C/72 h), respectively. All experiments were conducted in duplicate.

Grazing assay

Bdelloid rotifer *A. vaga* was isolated from the clonal culture and used to examine the influence of grazing (the term used here for ingesting bacteria by filtering the suspension) on the removal of *A. baumannii* during 7 weeks. One *A. baumannii* isolate (OB4138) was selected for this experiment because it had the growth curve closest to the mean value of pure cultures of all three *A. baumannii* isolates. Prior to the beginning of the experiments, rotifers were fed with sterile fish food and rinsed to prevent input of heterotrophic bacteria. Grazing influence was monitored in Shott bottles containing 50 mL of SW and DNB at room temperature (22 ± 2 °C). Three microcosms were set up as follows: (1) microcosm 1 – rotifers with *A. baumannii*; (2) microcosm 2 – rotifers with *A. baumannii* and *E. coli*; (3) microcosm 3 – rotifers with *A. baumannii* and *E. faecium*. Initial abundance of rotifers was 20 animals per mL. Rotifer abundance was analysed by light microscopy (Olympus CX21, magnification 100×). One mL from each of the microcosms was serially diluted in physiological solution and inoculated onto selective plates. Abundance of *A. baumannii*, *E. coli* and *E. faecium* was measured on CHROMagar *Acinetobacter* (42 °C/24 h), EC X-Gluc (44 °C/48 h) and Slanetz-Bartley agar (37 °C/72 h), respectively. Rotifer and bacterial abundance was measured at 0, 1, 3 and further every 7 days. All experiments were conducted in duplicate.

Statistical analysis

Abundance of bacteria and rotifers was expressed as log CFU/mL and log N/mL, respectively. Bacterial removal by rotifers was expressed as reduction of log CFU/mL (initial log CFU/mL – final log CFU/mL). Statistical analysis was carried out using Statistica 13.3 (TIBCO Software, Inc.). Comparisons between samples were conducted using Factorial ANOVA and the Duncan post hoc test. Correlations between variables were estimated using Spearman's rank test ($p < 0.05$). In order to account for multiple testing, *p*-values were transformed into false discovery rates (FDRs) according to the Benjamini-Hochberg procedure. The significance level applied is FDR < 0.05.

RESULTS

Inter-bacterial competition assay

The results of the interaction of *A. baumannii* with *E. coli* and *E. faecium* are presented in Figure 1. There was no multiplication of the bacteria in SW, whereas multiplication occurred in DNB medium. All three *A. baumannii* isolates (EF7, EF11, OB4138) had similar performance without statistically significant difference in all microcosms regardless of the tested medium.

After 50 days of contact, no statistically significant difference in SW between the average abundance of *A. baumannii* in microcosm with *E. coli* and *A. baumannii* control was evident (FDR = 0.443), while in DNB such difference was statistically significant (FDR = 0.040). However, the reduction of *A. baumannii* abundance in microcosm with *E. coli* in DNB is negligible in practice, since it was reduced for only 1.0 ± 0.5 log CFU/mL. Additionally, there was no statistically significant correlation of the abundance of *A. baumannii* and *E. coli* in SW (R = −0.135, FDR = 0.732), while in DNB statistically significant positive correlation (R = 0.409, FDR = 0.072) was present.

Furthermore, no statistically significant difference between the average final abundance of *A. baumannii* in microcosm with *E. faecium* and *A. baumannii* control in both SW (FDR = 0.135) and DNB (FDR = 0.082) was recorded. *A. baumannii* abundance had a statistically significant positive correlation (R = 0.808, FDR = 0.000) with *E. faecium*

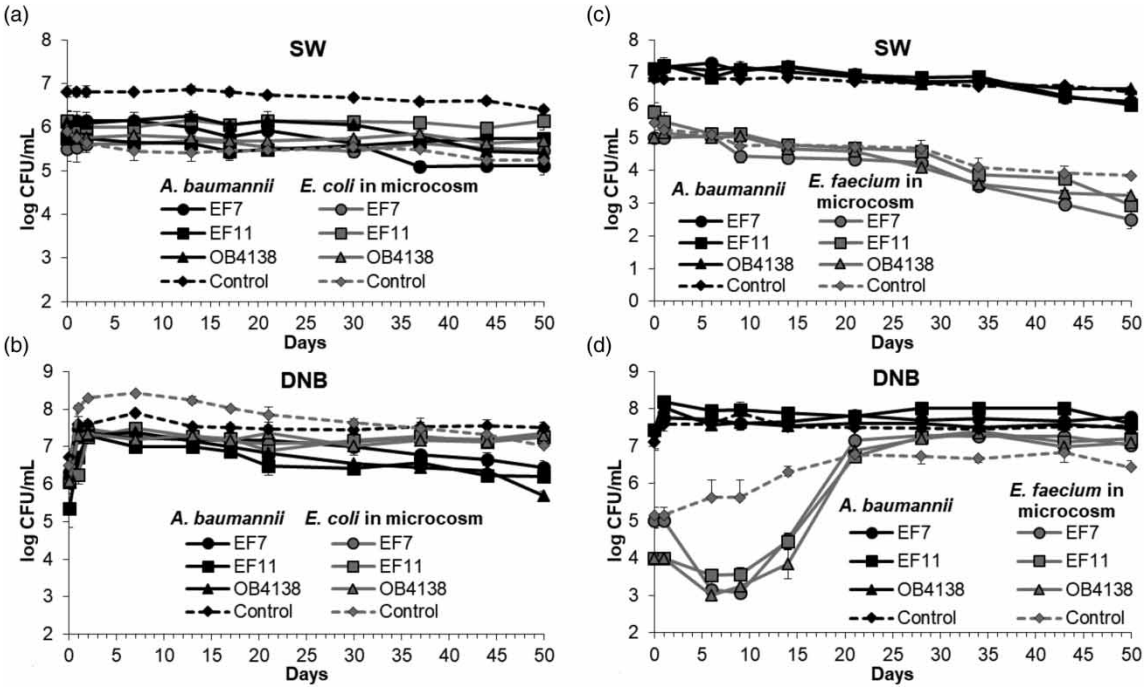

Figure 1 | Abundance (mean ± SD) of *A. baumannii* in microcosm interactions with *E. coli* (a), (b) and *E. faecium* (c), (d) in two water media. SW – commercially available spring water; DNB – nutrient broth diluted with distilled water (1:100); *A. baumannii* control – abundance (mean ± SD) of three pure cultures of *A. baumannii* without interactions are shown; *E. coli* control – abundance (mean ± SD) of pure culture of *E. coli* without interactions are shown; *E. faecium* control – abundance (mean ± SD) of pure culture of *E. faecium* without interactions are shown.

abundance in SW. *E. faecium* performed differently than *A. baumannii* in DNB therefore no significant correlation between them was detected (R = 0.083, FDR = 0.850). In the first 5 days of exposure, the average abundance of *E. faecium* decreased from 4.3 ± 0.5 to 3.2 ± 0.3 log CFU/mL. After 10 days, the abundance of *E. faecium* started to increase and resulted in the final 7.1 ± 0.1 log CFU/mL.

Grazing assay

The results of the grazing assay are presented in Figure 2. Statistically significant correlations among rotifers *vs.* bacteria and bacterium *vs*. bacterium are presented in Table 1. In both tested media (SW and DNB) *A. baumannii*, *E. coli* and *E. faecium* were statistically negatively correlated with rotifers in all three microcosms (Table 1). Furthermore, the abundance of *A. baumannii* was statistically positively correlated in both tested media with the abundance of *E. coli* in microcosm 2 and with *E. faecium* in microcosm 3 (Table 1).

The removal of *A. baumannii* by rotifers in SW was 6.6, 6.7 and 4.5 log CFU/mL, whereas in DNB the values were 1.4, 4.7 and 2 log CFU/mL in microcosms 1–3, respectively (Figure 3). As compared to control, the average contribution of rotifers to the removal of bacteria was as follows: *A. baumannii* 5.5 ± 1.3 log CFU/mL in SW and 3.5 ± 1.7 log CFU/mL in DNB; *E. coli* 5.2 ± 0.7 log CFU/mL in SW and 3.4 ± 0.8 log CFU/mL in DNB; *E. faecium* 1.2 ± 0.8 log CFU/mL in SW and 7.0 ± 0.7 log CFU/mL in DNB.

A. baumannii was statistically more efficiently removed (FDR = 0.000) in SW than in DNB. *E. coli* followed the same trend. However, *E. coli* disappeared from the microcosm after 28 days of exposure, while *A. baumannii* persisted up to day 50 (Figure 2). In DNB *A. baumannii* and *E. coli* persisted even after 50 days. In microcosm 3 *E. faecium* performed differently. The removal rate of *E. faecium* was statistically higher in DNB (FDR = 0.000) (6.3 log CFU/mL) than in SW (3.6 log CFU/mL).

DISCUSSION

The occurrence of *A. baumannii* outside the hospital setting such as in wastewaters (Ferreira *et al.* 2011; Zhang *et al.* 2013), wastewater treatment plants (Hrenovic *et al.* 2016; Higgins *et al.* 2018) and in rivers (Girlich *et al.* 2010; Seruga Music *et al.* 2017) demonstrated the successful survival of *A. baumannii* in different aquatic environments.

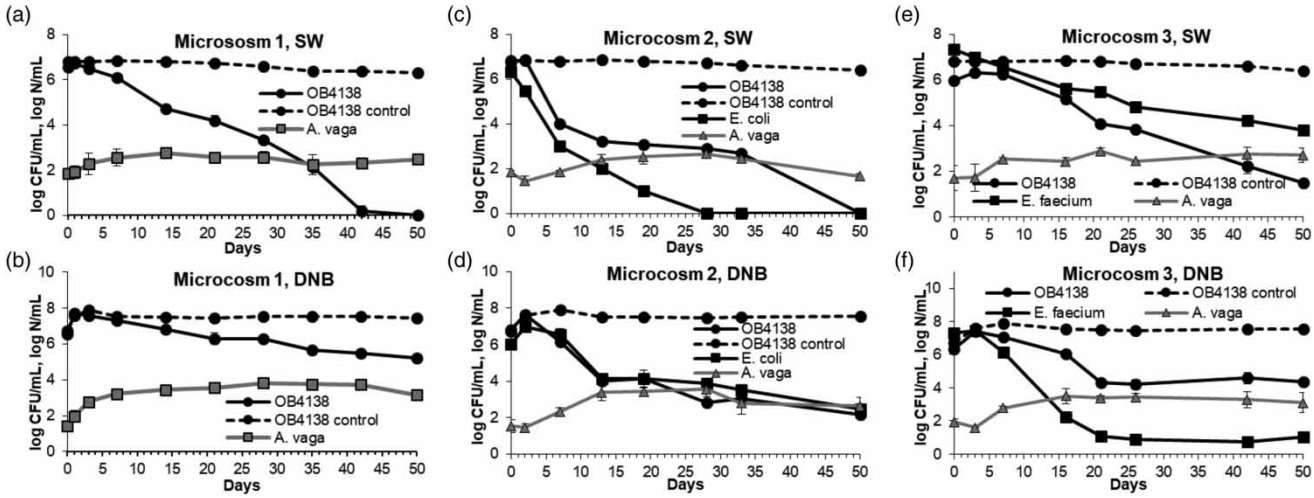

Figure 2 | Abundance (mean ± SD) of bacteria and rotifer *A. vaga* in: microcosm 1 (*A. baumannii* and *A. vaga* (a), (b)); microcosm 2 (*A. baumannii*, *E. coli* and *A. vaga* (c), (d)); microcosm 3 (*A. baumannii*, *E. faecium* and *A. vaga* (e), (f)); in two water media. For the grazing assay, one *A. baumannii* isolate was selected (OB4138). SW – commercially available spring water; DNB – nutrient broth diluted with distilled water (1:100); *A. baumannii* control – abundance (mean ± SD) of pure culture of *A. baumannii* isolate OB4138 without interactions are shown.

Table 1 | Trends in abundance variations shown by Spearman R correlations ($p < 0.05$) in: microcosm 1 (*A. baumannii* and *A. vaga*); microcosm 2 (*A. baumannii*, *E. coli* and *A. vaga*); microcosm 3 (*A. baumannii*, *E. faecium* and *A. vaga*) in two water media. SW – commercially available spring water; DNB – nutrient broth diluted with distilled water (1:100). Statistical significance is marked with asterisk as follows: * 5%, ** 1%, ***0.1% level significance

Microcosm	Water medium	Parameter	Spearman R value	FDR value
1	SW	*A. baumannii* + *A. vaga*	−0.279	0.135
	DNB	*A. baumannii* + *A. vaga*	−0.552	0.002**
2	SW	*A. baumannii* + *A. vaga*	−0.427	0.108
		E. coli + *A. vaga*	−0.536	0.037*
		A. baumannii + *E. coli*	0.949	0.000***
	DNB	*A. baumannii* + *A. vaga*	−0.645	0.012*
		E. coli + *A. vaga*	−0.574	0.025*
		A. baumannii + *E. coli*	0.923	0.000***
3	SW	*A. baumannii* + *A. vaga*	−0.580	0.017*
		E. faecium + *A. vaga*	−0.704	0.006**
		A. baumannii + *E. faecium*	0.914	0.000***
	DNB	*A. baumannii* + *A. vaga*	−0.634	0.011*
		E. faecium + *A. vaga*	−0.653	0.012*
		A. baumannii + *E. faecium*	0.900	0.000***

Additionally, *A. baumannii* was proven to survive under a wide array of abiotic conditions such as starvation, desiccation (Espinal *et al.* 2012; Bravo *et al.* 2016), lack of oxygen (Higgins *et al.* 2018), extreme temperatures and pH conditions (Dekic *et al.* 2018). The survival of *A. baumannii* in aquatic habitats is understudied so far, especially with respect to its performance under competition with other microorganisms or predation by eukaryotes. Therefore, we herein studied biotic interactions among *A. baumannii*, *E. coli* and *E. faecium* directly isolated from wastewater, instead of type strains. Furthermore, the interaction of rotifers with the mentioned bacteria was assessed to provide a better insight into the possible scenarios in a natural environment. Faecal indicators *E. coli* and *E. faecium* are common species in wastewaters, and their presence did not hinder *A. baumannii* to grow in such environments, which could pose a risk for public health. *A. baumannii* and *E. coli* performed similarly in the experiment in both tested media (SW and DNB), whereas *E. faecium* performed differently in DNB possibly due to slower multiplication than *A. baumannii*. Enterococci successfully survive and persist in fresh and marine environments; however, no observation of their multiplication in these environments was recorded (Gilmore *et al.*

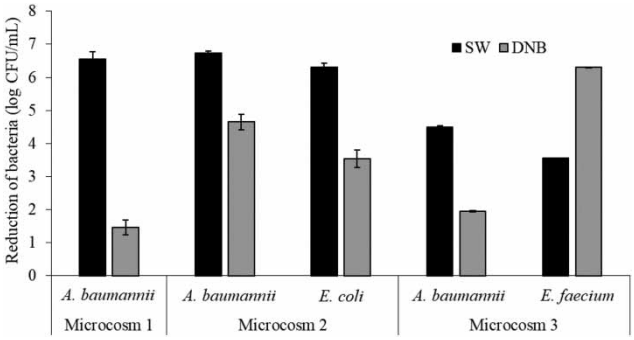

Figure 3 | Bacterial removal by rotifers expressed as reduction of log CFU/mL (initial log CFU/mL – final log CFU/mL) in: microcosm 1 (*A. baumannii* and *A. vaga*); microcosm 2 (*A. baumannii*, *E. coli* and *A. vaga*); microcosm 3 (*A. baumannii*, *E. faecium* and *A. vaga*) in two water media. Reduction of *A. baumannii* and *E. coli* is statistically significantly higher in SW, while the reduction of *E. faecium* is statistically significantly higher in DNB. SW – commercially available spring water; DNB – nutrient broth diluted with distilled water (1:100); average values and SD are shown.

2014). In this study, the multiplication of *E. faecium* was recorded in nutrient-rich water with and without the presence of *A. baumannii*. There was no difference between the survival of pandrug-resistant (EF7), extensively drug-resistant (OB4138) and sensitive isolates (EF11) of *A. baumannii*. All isolates showed similar performance, suggesting that antibiotic susceptibility profile in the absence of antibiotics is not significant in competitive interactions with other bacteria, as well as that clinical isolates behave the same as environmental isolates of *A. baumannii*.

There are limited data regarding the interaction of *A. baumannii* with other microorganisms. Lastoria *et al.* (2014) analysed the bacteria causing healthcare-associated bloodstream infections in Brazil from 2005 to 2010. The incidence of infections caused by *A. baumannii* was negatively correlated with incidence rates of infections caused by *Staphylococcus aureus* and *Enterobacter* spp., as well as several less common Gram-negative bacteria. The authors suggested that competition between pathogens is an important factor driving hospital outbreaks and that infection control policies should take these interactions into account. According to recent studies (Carruthers *et al.* 2013; Repizo *et al.* 2015), some strains of *A. baumannii* with an active type VI secretion system may outcompete *E. coli*. However, none of the examined isolates in this study outcompeted *E. coli*.

In freshwater ecosystems, rotifers, as ubiquitous and common bacterivorous organisms, impact the abundance of bacteria (Fiałkowska & Pajdak-Stós 2004; Kocerba-Soroka *et al.* 2013). In nutrient-poor water, rotifers removed all *A. baumannii* and *E. coli*, whereas in nutrient-rich water bacteria still persisted even after 50 days. Apparently, in SW the absence of organic matter prevents bacterial multiplication and together with constant rotifer grazing caused depletion of viable bacteria. In nutrient-rich water, bacteria multiplied in spite of grazing by rotifers, which resulted in overall lower bacterial removal rates. According to this study, rotifers were more efficient in the removal of *A. baumannii* and *E. coli* in nutrient-poor water, while *E. faecium* was more efficiently removed in the nutrient-rich medium. Slower multiplication of bacteria such as *E. faecium* leads to their higher removal rates by rotifers. The results indicate that the type of water media together with removal by rotifers influence the abundance of viable bacteria in water. According to the results, rotifers were able to remove 5.5 ± 1.3 log CFU/mL of *A. baumannii* in nutrient-poor and 3.5 ± 1.7 in nutrient-rich water. Grazing by rotifers could offer a new prospect for the removal of pathogenic bacteria from nutrient-poor water such as drinking water, as well as from nutrient-rich waters such as those in wastewater treatment plants.

Cateau *et al.* (2011) have recorded the interaction of *A. baumannii* with free-living amoeba that are common bacterivorous inhabitants of tap water and swimming pools. *A. baumannii* was able to survive and grow intracellularly in *Acanthamoeba castellanii* and *A. culbertsoni*. Free-living amoebae have been proven to protect intracellular bacteria from unfavourable environmental conditions, thus promoting their persistence and dissemination (Cateau *et al.* 2011). Dekić *et al.* (2018) have studied the potential of *A. baumannii* to colonize freshwater fish. No accumulation of *A. baumannii* in freshwater fish *Poecillia reticulata* was found in a laboratory experiment, indicating that freshwater fish do not promote the persistence of *A. baumannii*. In the presented study, no survival of *A. baumannii* inside rotifer *A. vaga* was detected. Namely, the whole content of the tube from microcosm 1 (Figure 2(a)) after 50 days of contact was vortexed, filtered and inoculated onto selective medium. The growth of *A. baumannii* colonies was not detected, although rotifers were present in microcosm. Thus, rotifer *A. vaga* is a promising biological agent for the removal of *A. baumannii* from various aquatic environments.

CONCLUSIONS

Clinically relevant *A. baumannii* coexisted with faecal indicator bacteria *E. coli* and *E. faecium* in both nutrient-poor and nutrient-rich water, which suggests its long-term survival in oligotrophic and eutrophic aquatic environments. No difference in the survival of pandrug-resistant,

extensively drug-resistant or antibiotic sensitive isolates of *A. baumannii* was observed, suggesting that the bacterial antibiotic susceptibility profile in the absence of antibiotics does not influence the survival in biotic interactions. Rotifers were successful in removal of *A. baumannii* particularly in nutrient-poor water (reduction of 5.5 ± 1.3 log CFU/mL). In wastewater treatment plants and drinking water facilities, grazing by rotifers might be useful for the removal of emerging human pathogens such as *A. baumannii* from water.

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