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Assessment of predatory bacteria and prey interactions using culture-based methods and EMA-qPCR

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ABSTRACT

Traditional culture-based enumeration methods were compared with the ethidium monoazide quantitative polymerase chain reaction (EMA-qPCR) technique to assess *Bdellovibrio*-and-like-organisms (BALOs) predator-prey interactions. Gram-negative [*Pseudomonas* spp. and *Klebsiella pneumoniae* (*K. pneumoniae*)] and Gram-positive [*Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecium* (*E. faecium*)] organisms were employed as prey cells, while a *Bdellovibrio bacteriovorus* strain (PF13) was used as the predator. The co-culture experiments were also compared in diluted nutrient broth (DNB) and HEPES buffer. In both media, *K. pneumoniae* (maximum log reduction of 5.13) and *Pseudomonas fluorescens* (*P. fluorescens*) (maximum log reduction of 4.21) were sensitive to predation by *B. bacteriovorus* PF13 as their cell counts and gene copies were reduced during all the co-culture experiments, while the concentration of *B. bacteriovorus* PF13 increased. The concentration of *B. bacteriovorus* PF13 also increased in the presence of *S. aureus* (HEPES buffer) and *E. faecium* (DNB), indicating that the predator interacted with these Gram-positive prey in order to survive. Moreover, as no predator plaques were produced in the co-culture experiments with *P. aeruginosa* (DNB and HEPES buffer), *S. aureus* (DNB and HEPES buffer) and *E. faecium* (HEPES buffer), EMA-qPCR proved to be beneficial in monitoring the concentration of *B. bacteriovorus*. In conclusion, the cell counts and/or EMA-qPCR analysis for the HEPES buffer and DNB assays were successfully employed to monitor the predation of *P. fluorescens* and *K. pneumoniae* by *B. bacteriovorus,* while *E. faecium* was sensitive to predation in DNB and *S. aureus* was sensitive to predation in HEPES buffer.

1. Introduction

Predatory bacteria such as *Bdellovibrio, Micavibrio* and *Bacteriovorax* spp. play important roles as "ecological balancer species" in the natural environment (Jurkevitch et al., 2000; Allen et al., 2014; Iebba et al., 2014). These Gram-negative bacteria are collectively referred to as *Bdellovibrio*-and-like-organisms (BALOs) and flourish in a variety of aquatic and terrestrial habitats such as bulk soil (Jurkevitch et al., 2000), the rhizosphere of plants (Jurkevitch et al., 2000), rivers (Sar et al., 2015), estuaries (brackish water) (Schoeffield and Williams, 1989), fish ponds (Chu and Zhu, 2010), water and wastewater treatment plants (Feng et al., 2016; Yu et al., 2017) and they have been isolated from the human gut (Iebba et al., 2013). They thrive by preying on other Gram-negative bacteria either in an epibiotic or periplasmic manner (Kadouri et al., 2013; Avidan et al., 2017).

It is however, challenging to investigate the interaction of the predator with the prey cells in their natural habitat and BALO predator-prey interactions are predominantly studied using culture-based methods. Diluted nutrient broth (DNB) is the most widely used medium to assess bacterial predator-prey interactions and while it contains lower concentrations of nutrients in comparison to full strength nutrient broth (usually 1/10 strength of nutrient broth), HEPES buffer suspension does not contain any nutrients and only provides the cations (magnesium and calcium) required by predatory bacteria for effective predation (Koval, 2006; Rotem et al., 2014). Numerous pitfalls have however, been associated with the accuracy of the methods employed to investigate predator-prey interactions. Firstly, BALOs vary significantly in their prey range and the prey cells employed in isolation experiments may not be specific for the predatory bacteria being investigated (Koval, 2006; Williams and Piñeiro, 2006; Zheng et al., 2008). Additionally, the environmental strains of prey cells may not be amenable to cultivation in the laboratory, which further hinders the discovery and investigation of new or unique BALOs and their prey interactions (Williams and Piñeiro, 2006; Zheng et al., 2008; Rotem et al., 2014). Moreover, while the enumeration of the predator cells using plaque forming units (PFU) on double-layer agar overlays is cost-

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effective, some BALOs have difficulty preying on bacteria in soft agar, which may subsequently hinder plaque formation and influences the accuracy of the plaque counts obtained during co-culture experiments (Koval, 2006). The enumeration of the predatory bacteria is also not a time-effective process, as the plaques may only become visible after 2–7 days of incubation (Koval, 2006; Jurkevitch, 2006).

Accurate methods of investigating and studying the BALO-prey cell interactions are thus required and the use of molecular-based techniques, specifically viability qPCR which incorporates Ethidium Monoazide Bromide (EMA) or Propidium Monoazide (PMA), may be advantageous as the growth and degradation of the predator and prey cells can be monitored directly in co-culture experiments. Viability dyes such as EMA or PMA, effectively reduce the amplification of DNA from cells with compromised membranes (presumed non-viable) or extracellular DNA and therefore can be utilised to monitor the gene copies (GC) from predominantly viable cells (Cenciarini-Borde et al., 2009; Seinige et al., 2014). The use of viability dyes could thus account for the viable but non-culturable (VBNC) community as these cells have intact membranes and their DNA will subsequently be quantified with qPCR after viability dye treatment. Moreover, as some BALOs may have difficulty forming plaques on double-layer agar overlays, viability qPCR could be employed to monitor their growth in co-culture experiments. This technique could thus potentially aid in accurately monitoring and quantifying both predator and prey cells during co-culture experiments in a time-effective manner as results can be generated within 24 h (Cenciarini-Borde et al., 2009; Seinige et al., 2014).

The aim of the current study was thus to compare the standard culture-based methods, employed to monitor the interaction of *Bdellovibrio* spp. with different prey cells, with the molecular-based EMA-qPCR method. A secondary aim was to investigate and compare the interactions of the isolated *Bdellovibrio* spp. with opportunistic pathogenic bacteria in DNB and HEPES buffer. To achieve these aims *Bdellovibrio* spp. were isolated from wastewater as literature has indicated that these bacteria are abundant in wastewater treatment plants (Yu et al., 2017). The potential of the isolated predatory bacteria to prey on Gram-negative organisms was assessed using American Type Culture Collection (ATCC) isolates of *Pseudomonas aeruginosa* (*P. aeruginosa*), *Pseudomonas fluorescens* (*P. fluorescens*) and *Klebsiella pneumoniae* (*K. pneumoniae*) as prey in co-culture experiments. As contradictory evidence on the ability of BALOs to prey on Gram-positive bacteria has been presented (Dashiff et al., 2011; Iebba et al., 2014; Monnappa et al., 2014), the Gram-positive prey, *Staphylococcus aureus* (*S. aureus*) ATCC 25925 and a clinical *Enterococcus faecium* (*E. faecium*) isolate were included in this study.

2. Materials and methods

2.1. Wastewater sample collection and processing

A wastewater sample (1 L) was collected from the influent point of the Stellenbosch Wastewater Treatment Plant (GPS co-ordinates: 33°59′21.13″S 18°47′47.75″E) in a sterile 1 L Schott bottle. Twenty millilitres (20 mL) of the sample was incubated at 30 °C for 1 h with shaking at 200 rpm. The incubated aliquot was subsequently centrifuged at 500 rpm for 10 min, whereafter the supernatant was filtered through a 1.2 µm cellulose nitrate filter (47 mm; Sartorius Biolab Products, Kimix, South Africa) (Feng et al., 2016). The filtrate was utilised for the subsequent *Bdellovibrio* spp. isolation experiments.

2.2. Bdellovibrio-and-like-organisms isolation

Pseudomonas spp. [*P. aeruginosa* ATCC 27853, *P. fluorescens* ATCC 13525 and *Pseudomonas protogens* (*P. protogens*) ATCC 17386] were selected as prey bacteria for the isolation of *Bdellovibrio* spp. in the current study as research has indicated that *Pseudomonas* spp. are sensitive to predation (Dashiff et al., 2011; Feng et al., 2016). The method as outlined by Feng et al. (2016) was used for the isolation of the *Bdellovibrio* spp.

2.3. Bdellovibrio-and-like-organisms purification

To purify the putative BALOs, plaques with varying diameters were removed from the agar using a sterile blade. The plaques were inserted into 70 mL DNB (pH 7.2) [0.1 g/L of Lab Lemco Powder (Oxoid, Hampshire, England), 0.2 g/L of yeast extract (Biolab, Midrand, South Africa), 0.5 g/L peptone bacteriological (Biolab), 0.5 g/L sodium chloride (NaCl; Kimix, Cape Town, South Africa), 3 mM magnesium chloride ($MgCl_2$; Oxoid) and 2 mM calcium chloride ($CaCl_2$; Biolab)] which contained 1 mL of the respective *Pseudomonas* spp. prey cell cultures (10^9 cells/mL) (Feng et al., 2016; Yu et al., 2017). The inoculums were incubated at 30 °C at 200 rpm and were monitored for a reduction in OD every 24 h (Yu et al., 2017). When the incubation medium was cleared (OD $<$ 0.20) (Im et al., 2014), serial dilutions (10^{-2} to 10^{-8}) were prepared, whereafter 500 µL of each dilution was mixed with 500 µL fresh prey cells in 5 mL molten soft-top agar which was then poured onto DNB agar plates (Yu et al., 2017). The plates were incubated at 30 °C. This process was repeated five times to obtain pure BALO strains (Yu et al., 2017).

2.4. DNA extractions and conventional PCR for BALOs identification

To confirm the isolation of BALOs, the isolates and *Pseudomonas* spp. co-cultures were subjected to DNA extractions using the boiling method as previously described by Ndlovu et al. (2015). The DNA extracts were then subjected to conventional PCR for the identification of *Bdellovibrio* spp., using the primers Bd347F – GGAGGCAGCAGTAGGG AATA and Bd549R –GCTAGGATCCCTCGTCTTACC (Van Essche et al., 2009). Each PCR reaction consisted of 1X Green GoTaq® Reaction Buffer (Promega Corp, Madison, USA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.9 µM of each primer, 1 U GoTaq® Flexi DNA Polymerase (Promega Corp, Madison, USA) and 5 µL of DNA in a final volume of 25 µL. The cycling parameters consisted of a denaturation step at 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 60 °C for 30 s and a final elongation step at 72 °C for 10 min. Each PCR assay included a sterile milliQ negative control.

The PCR products were electrophoresed on an agarose gel (1.5%) stained with ethidium bromide (0.5 µg/mL) in 1X tris acetate ethylenediaminetetraacetic acid (TAE) buffer, for 1 h at 80 V. The products were visualised using the Vilber Lourmat gel documentation system (Vilber Lourmat, Collégien, France) to confirm the presence of the desired amplicon (202 bp). Representative PCR products (n = 9) were cleaned, sequenced and the sequence data analysed as outlined in Waso et al. (2016). The DNA sequences of representative isolates that showed > 97% similarity (< 3% diversity) to *Bdellovibrio* spp. were recorded and the DNA obtained from the isolates identified as *Bdellovibrio* spp. were subsequently utilised as positive control DNA in all the molecular-based assays.

2.5. Comparison of culture-based and molecular methods to assess predation

2.5.1. Predator stock lysate

Isolate PF13 (isolated by co-culturing with *P. fluorescens* ATCC 13525 and designated as isolate number 13) was identified as a *Bdellovibrio bacteriovorus* (*B. bacteriovorus*) strain by sequencing analysis (as described above) and was selected for all predation assays as this isolate consistently produced plaques on *Pseudomonas* prey. For the predation assays, a predator stock lysate of *B. bacteriovorus* PF13 was prepared as described by Dashiff et al. (2011). The stock lysate was subsequently used as the predator inoculum in all the predation assays. In addition, 10 mL of the predator stock lysate was filtered through a 0.22 µm Nylon filter (47 mm; Starlab Scientific, Kimix, South Africa)

three times, to remove all the *B. bacteriovorus* cells from the suspension. The absence of the predator from the resulting filtrate was confirmed by double-layer agar overlays as described in the preceding sections. This filtrate then served as the inoculum for the predation negative control in all predation assays as described by Kadouri et al. (2013).

2.5.2. Predation assays

In order to determine whether *B. bacteriovorus* PF13 was able to prey on opportunistic pathogens, *B. bacteriovorus* PF13 was co-cultured with *P. fluorescens* ATCC 13525, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 333305, *S. aureus* ATCC 25925 and a clinical isolate of *E. faecium* (designated as *E. faecium* Clinical). These isolates were obtained from the Water Resource Laboratory Culture Collection at the Department of Microbiology (Stellenbosch University).

To compare the efficiency of *B. bacteriovorus* PF13 to reduce the concentration of the above-mentioned prey cells in co-culture, the experiments were performed in both DNB and HEPES buffer [25 mM, pH 7.2; supplemented with 3 mM $MgCl_2$ (Oxoid) and 2 mM $CaCl_2$ (Biolab)] (Shemesh and Jurkevitch, 2004). All the prey strains were grown in 100 mL LB at 37 °C for 24 h. The prey cell cultures were then centrifuged at 8 000 rpm for 15 min, whereafter the pellet was washed and resuspended in either DNB or supplemented HEPES buffer (OD_{600} = 1.00). For each co-culture experiment, 98 mL of DNB or HEPES buffer was subsequently inoculated as described by Yu et al. (2017). For each set of co-culture flasks, a predation negative control was included, which consisted of 98 mL of DNB/HEPES buffer inoculated with 1 mL of the respective prey cell cultures and 1 mL of filtered predator stock lysate (which lacked predator cells). The co-cultures and predation negative controls were incubated at 30 °C for 120 h with shaking at 200 rpm. These experiments were repeated three times.

2.5.2.1. Culture-based analysis of the co-culture experiments. Three millilitre (3 mL) aliquots were collected from each co-culture flask and the predation negative control at 0, 48, 96 and 120 h. Serial dilutions (10^{-2} to 10^{-9}) were prepared from each aliquot and 100 µL of each dilution was spread plated onto LB agar to determine the colony forming units (CFU)/mL of the respective prey cell cultures, in duplicate. In addition, soft-top agar overlays were prepared as described above, to enumerate the PFU of *B. bacteriovorus* PF13 in co-culture with the various prey cells.

2.5.2.2. Ethidium monoazide treatment and DNA extractions of the co-culture experiments. For the molecular analysis of the co-culture experiments, 500 µL of each 3 mL time series aliquot (0, 48, 96 and 120 h) was EMA treated as described by Reyneke et al. (2017), whereafter the EMA-treated aliquots were subjected to DNA extractions using the Zymo Research Soil Microbe DNA Miniprep™ kit as per the manufacturer's instructions.

2.5.2.3. Quantitative real-time PCR. Quantitative real-time PCR was performed to quantify the GC of *B. bacteriovorus* PF13 in co-culture with *P. fluorescens* ATCC 13525, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 333305, *S. aureus* ATCC 25925 and *E. faecium* Clinical and to quantify the GC of each of the respective prey cells in co-culture for each time point (0, 48, 96 and 120 h) in both the DNB and HEPES buffer. All qPCR assays were performed using the LightCycler® 96 Instrument (Roche Diagnostics, Mannheim, Germany) and the FastStart Essential DNA Green Master (Roche Diagnostics, Mannheim, Germany) as outlined in Waso et al. (2018). All the qPCR primers and cycling parameters are outlined in Table 1.

For GC quantification, a standard curve was included (in duplicate) for each of the respective qPCR assays. Each standard curve was generated by performing conventional PCR (with the primers and cycling parameters as outlined in Table 1) on positive control DNA extracted (using the Zymo Research Soil Microbe MiniPrep™ kit as per manufacturer's instructions) from *P. aeruginosa* ATCC 27853, *K. pneumoniae*

Table 1
The primers and cycling parameters utilised for the quantification of *B. bacteriovorus* PF13, *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *E. faecium* with the EMA-qPCR assays.

Organisms	Primer	Primer Sequences (5' – 3')	qPCR Cycling Parameters	Conventional PCR Cycling Parameters	Gene (product size in bp)	Melting Peak (°C)	Reference
Bdellovibrio spp.	Bd347F	GGAGGCAGCAGTAGGGAATA	2 min at 95 °C; 50 cycles of 15 s at 95 °C and 60 s at 60 °C; high resolution melting of 60 s at 95 °C, 60 s at 40 °C, 1 s at 65 °C and 1 s at 97 °C	2 min at 95 °C; 50 cycles of 15 s at 95 °C and 60 s at 60 °C; final elongation of 10 min at 72 °C	16S rRNA (202)	84.30 ± 1.00	Van Essche et al., 2009
	Bd549R	GCTAGGATCCCTCGTCTTACC					
Enterococcus spp.	ECST784F	AGAAATTCCAAACGAACTTG	10 min at 95 °C; 50 cycles of 15 s at 95 °C and 60 s at 60 °C; high resolution melting of 60 s at 95 °C, 60 s at 40 °C, 1 s at 65 °C and 1 s at 97 °C	5 min at 95 °C; 30 cycles of 30 s at 94 °C, 60 s at 59 °C and 60 s at 72 °C; final elongation of 10 min at 72 °C	23S rRNA (75)	79.15 ± 1.00	Frahm and Obst, 2003
	ENC854R	CAGTGCTCTACCTCCATCATT					
Klebsiella spp.	gyrA-A	CGCGTACTATACGCCATGAACGTA	10 min at 95 °C; 50 cycles of 60 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C; high resolution melting of 60 s at 95 °C, 60 s at 40 °C, 1 s at 65 °C and 1 s at 97 °C	3 min at 95 °C; 35 cycles of 60 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C; final elongation of 10 min at 72 °C	*Gyrase A* (383)	87.70 ± 1.00	Brisse and Verhoef, 2001
	gyrA-C	ACCGTTGATCACTTCGGTCAGG					
Pseudomonas spp.	PS1	ATGAACAACGTTCTGAAATTC	10 min at 95 °C; 50 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C; high resolution melting of 60 s at 95 °C, 60 s at 40 °C, 1 s at 65 °C and 1 s at 97 °C	10 min at 95 °C; 50 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C; final elongation of 10 min at 72 °C	*oprI* (249)	88.70 ± 1.00	Bergmark et al., 2012
	PS2	CTGCGGCTGGCTTTTTCCAG					
Staphylococcus spp.	PanStaphF	CAATGCCACAAACTCG	10 min at 95 °C; 45 cycles of 30 s at 95 °C, 30 s at 61 °C and 30 s at 72 °C; high resolution melting of 60 s at 95 °C, 60 s at 40 °C, 1 s at 65 °C and 1 s at 97 °C	10 min at 95 °C; 45 cycles of 30 s at 95 °C, 30 s at 61 °C and 30 s at 72 °C; final elongation of 10 min at 72 °C	*tuf* (462)	81.50 ± 1.00	Sakai et al., 2004
	PanStaphR	GCTTCAGCGTAGTCTA					

ATCC 333305, *E. faecium* Clinical isolate, *S. aureas* ATCC 25295 and *B. bacteriovorus* PF13. The conventional PCR mixture for *Bdellovibrio* spp. consisted of 1X Green GoTaq® Reaction Buffer (Promega Corp, Madison, USA), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.9 µM of each primer, 1 U GoTaq® Flexi DNA Polymerase (Promega Corp, Madison, USA) and 5 µL of DNA in a final volume of 25 µL. For *E. faecium* the same PCR mixture was used with the exception that 2 mM $MgCl_2$, 0.2 µM of each primer, 1.5 U GoTaq® Flexi DNA Polymerase and 2 µL of DNA were added. For *K. pneumoniae* the same PCR mixture was used with the exception that 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.3 µM of each primer and 1.5 U GoTaq® Flexi DNA Polymerase were added. For *Pseudomonas* spp. and *S. aureus* the same PCR mixture was used with the exception that 2 mM $MgCl_2$, 0.1 mM of each dNTP, 0.1 µM (*Pseudomonas* spp.) and 1.0 µM (*S. aureus*) of each primer and 1.5 U GoTaq® Flexi DNA Polymerase were added. The standard curve for each target organism was then generated as outlined by Waso et al. (2018).

Lastly, high resolution melting curve analysis was included for all of the SYBR Green qPCR assays in order to verify the specificity of the assays. The temperature was thus increased from 40 to 97 °C at a rate of 0.2 °C/s with continuous fluorescent signal acquisition of 5 readings/°C to generate the melting curves.

2.6. Data and statistical analyses

All the qPCR performance characteristics were analysed using the Roche LightCycler® 96 Software Version 1.1 and Microsoft Excel 2016. In addition, the lower limit of detection (LLOD) was determined as the actual GC/µL values consistently and accurately detected per qPCR assay for the standard with the lowest GC. All GC numbers were converted to GC/mL using the following modified equation (excluding the compensation for sample filtration) (Eq. (1)) as described by Rajal et al. (2007):

$$\left(\frac{\text{mL Original Sample}}{\text{mL DNA eluted}}\right) \times (\text{mL used per qPCR assay}) = \text{mL original sample per qPCR assay} \quad (1)$$

All graphs were generated and data analyses were performed using GraphPad Prism 7.04 (2018). Two-way Analysis of Variance (ANOVA) and unpaired t-tests using the Holm-Šídák method were utilised to determine whether there were significant differences between the concentrations of the prey cells detected (using culture-based enumeration and EMA-qPCR quantification) in the co-culture experiments versus the initial inoculum and predator-free controls. Significance was observed as $p < 0.05$.

3. Results

3.1. Bdellovibrio spp. isolation

In total 55 putative BALO strains were isolated from the wastewater sample collected from the Stellenbosch Wastewater Treatment Plant. Representative PCR products ($n = 9$) were sequenced to confirm the detection of *Bdellovibrio* spp. Seven of the representative PCR products were identified as *B. bacteriovorus* (Table 2), while *B. bacteriovorus* isolate PF13 was selected for all predation assays as this isolate produced numerous and large plaques when cultured on a lawn of *P. fluorescens* ATCC 13525 prey.

3.2. Predation by B. bacteriovorus PF13 on Pseudomonas spp

The ability of the predator *B. bacteriovorus* PF13 to attack *P. fluorescens* and *P. aeruginosa* was assessed in DNB and HEPES buffer using culture-based methods [spread plates (CFU/mL) and double-layer agar overlays (PFU/mL)] and EMA-qPCR (GC/mL) (qPCR performance characteristics summarised in the Supplementary Material Table A1).

Table 2
Sequencing results obtained for the representative PCR products sequenced for presumptive BALO strains identification.

BALO Isolate	BLAST result	Sequence Similarity (%)	Accession Number
PP1[a]	*B. bacteriovorus* W, complete genome	99	CP002190.1
PP9[a]	*B. bacteriovorus* strain SOIR-1 16S Ribosomal RNA gene	100	MG230309.1
PP17[a]		100	
PF13[b]		100	
PF17[b]		100	
PF20[b]		100	
PA8[c]	*B. bacteriovorus* W, complete genome	97	CP002190.1

[a] *P. protogens* 17386 used as prey.
[b] *P. fluorescens* ATCC 13525 used as prey.
[c] *P. aeruginosa* ATCC 27835 used as prey.

For the prey bacteria, the cell counts (CFU/mL) and gene copies (GC/mL) obtained after predation were compared to the cell counts and GC obtained in the initial inoculum and the predator-free control. Similarly, for the predatory bacteria, the PFU/mL and GC/mL were compared to the plaque counts and GC obtained in the initial inoculum to determine if the concentrations of these bacteria changed significantly in co-culture.

As indicated in Fig. 1a and Table 3, in comparison to the predator-free control, the *P. fluorescens* ATCC 13525 cell counts were reduced significantly by 3.24 logs ($p = 0.00057$) after 120 h of predation in DNB. Correspondingly, the plaque counts of *B. bacteriovorus* PF13 increased by 5.44 logs at the expense of *P. fluorescens* ATCC 13525 and the maximum cell density was reached after 96 h of predation (Table 4). In contrast to the culture-based enumeration of *P. fluorescens* ATCC 13525 in co-culture with *B. bacteriovorus* PF13 in DNB, the EMA-qPCR results indicated that the GC of *P. fluorescens* ATCC 13525 fluctuated. However, overall, a log reduction of 1.24 was observed for the GC recorded for the predator-free control versus the GC recorded at the end of predation at 120 h (Fig. 1b; Table 3). In accordance with this decrease, the GC of *B. bacteriovorus* PF13 increased by 4.33 logs and reached the maximum GC concentration after 48 h of predation (Table 4).

When *P. fluorescens* ATCC 13525 was exposed to the predator in HEPES buffer, the cell counts were also significantly reduced (4.21 log reduction; $p = 0.0128$) after 120 h of predation (Fig. 1c; Table 3). Correspondingly, the plaque counts for *B. bacteriovorus* PF13 increased by 5.01 logs to reach a maximum cell density at 48 h (Table 4). The GC of *P. fluorescens* ATCC 13525 also decreased significantly by 1.64 logs ($p < 0.0001$) after 120 h of co-culture with *B. bacteriovorus* PF13 in HEPES buffer (Fig. 1d; Table 3). In accordance with this decrease, the GC of *B. bacteriovorus* PF13 increased by 2.72 logs and reached the maximum concentration after 48 h of co-culture with *P. fluorescens* ATCC 13525 (Table 4).

For the predation assays performed in DNB using *P. aeruginosa* ATCC 27853 as prey, in comparison to the predator-free control group, the cell counts of the prey organism were significantly reduced by 1.85 logs ($p = 0.0081$) after 120 h of predation (Fig. 1a; Table 3). However, it should be noted that *B. bacteriovorus* PF13 did not produce visible plaques on the double-agar overlays when using *P. aeruginosa* ATCC 27853 as prey in either the DNB or HEPES buffer trials. In contrast to the culture-based enumeration of *P. aeruginosa* ATCC 27853 in co-culture with *B. bacteriovorus* PF13 in DNB, the EMA-qPCR results indicated that the *P. aeruginosa* ATCC 27853 GC only decreased by 0.159 logs after 120 h of predation (Fig. 1b; Table 3). In accordance with this decrease in the prey concentration, the EMA-qPCR results indicated that the GC of *B. bacteriovorus* PF13 increased by 0.744 logs and reached a maximum concentration after 48 h of co-culture (Table 4).

Comparatively, for the HEPES buffer predation assays, the cell

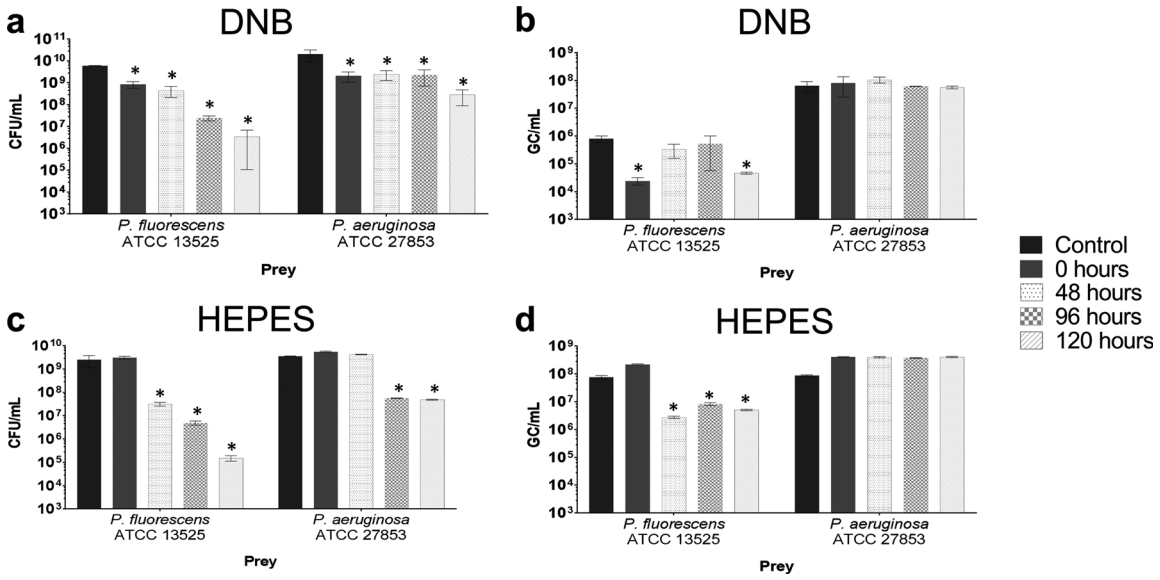

Fig. 1. Enumeration in CFU/mL using culturing (a and c) and quantification in GC/mL using EMA-qPCR (b and d) of *P. fluorescens* ATCC 13525 and *P. aeruginosa* ATCC 27853 co-cultured with *B. bacteriovorus* PF13 in DNB and HEPES buffer. (*) the cell counts or GC were significantly reduced ($p < 0.05$) as compared to the control and/or initial concentration.

counts of *P. aeruginosa* ATCC 27853 were also significantly reduced by 1.87 logs ($p = 0.0002$) after 120 h of predation (Fig. 1c; Table 3). However, for the HEPES buffer EMA-qPCR analysis of *P. aeruginosa* ATCC 27853 in co-culture with *B. bacteriovorus* PF13, after 120 h of predation, the GC of *P. aeruginosa* ATCC 27853 were equal to the initial concentration (4.06×10^8 GC/mL) (Fig. 1d; Table 3). Correspondingly, the GC of *B. bacteriovorus* PF13 did not increase, but decreased by 0.035 logs after 120 h of co-culture with *P. aeruginosa* ATCC 27853 in HEPES buffer (Table 4).

3.3. Predation by B. bacteriovorus PF13 on Klebsiella pneumoniae

The results showed that in DNB, the *K. pneumoniae* ATCC 333305 cell counts were significantly reduced by 3.58 logs ($p < 0.0001$) after 120 h of predation (Fig. 2a; Table 3). Correspondingly, the plaque counts of *B. bacteriovorus* PF13 increased by 3.85 logs to reach the maximum predator concentration after 48 h (Table 4). The EMA-qPCR analysis then indicated that the GC of *K. pneumoniae* ATCC 333305 also decreased significantly by 3.65 logs ($p = 0.0103$) after 120 h of predation in DNB (Fig. 2b; Table 3). In accordance with this decrease in the GC of *K. pneumoniae* ATCC 333305, the GC of *B. bacteriovorus* PF13 increased by 4.68 logs to the maximum GC concentration after 96 h of predation (Table 4).

In comparison, when *K. pneumoniae* ATCC 333305 was exposed to the predator in HEPES buffer, the cell counts were reduced significantly after 120 h of predation (5.13 log reduction; $p < 0.0001$) (Fig. 2c; Table 3). Accordingly, the plaque counts for *B. bacteriovorus* PF13 increased by 5.26 logs and reached the maximum cell density after 48 h of co-culture (Table 4). The molecular analysis also indicated that the GC of *K. pneumoniae* decreased significantly, by 3.29 logs ($p < 0.0001$) after 120 h of co-culture (Fig. 2d; Table 3). In accordance with this decrease, the GC of *B. bacteriovorus* PF13 increased by 4.22 logs and reached the maximum concentration after 96 h (Table 4).

3.4. Predation by B. bacteriovorus PF13 on Staphylococcus aureus

For the DNB trials using *S. aureus* ATCC 25925 as prey, the cell counts were reduced by 0.272 logs after 120 h of predation (Fig. 3a; Table 3). However, similar to the results observed for the *P. aeruginosa* trials, *B. bacteriovorus* PF13 did not produce plaques in the double-layer agar overlays when *S. aureus* was used as prey in either the DNB or HEPES buffer trials. In contrast to the culture-based enumeration of *S. aureus* ATCC 25925 in DNB, the EMA-qPCR results indicated that the GC of *S. aureus* ATCC 25925 increased (albeit not significantly) by 0.553 logs after 120 h of predation (Fig. 3b; Table 3). In addition, the GC of *B. bacteriovorus* PF13 increased significantly by 4.18 logs ($p < 0.0001$) to a maximum concentration after 96 h of predation (Table 4).

In comparison, when *S. aureus* ATCC 25925 was exposed to the predator in HEPES buffer, the cell counts were reduced significantly by 1.80 logs ($p < 0.0001$) after 120 h (Fig. 3c; Table 3). Accordingly, the EMA-qPCR analysis indicated that the concentration of *S. aureus* ATCC 25925 decreased by 1.27 logs ($p < 0.0001$) (Fig. 3d; Table 3), while the concentration of *B. bacteriovorus* PF13 increased by 2.66 logs after 120 h of predation (Table 4).

3.5. Predation by B. bacteriovorus PF13 on Enterococcus faecium

Upon exposure to the predator in DNB, the *E. faecium* cell counts were significantly reduced by 2.71 logs ($p = 0.0002$) after 120 h of predation (Fig. 4a; Table 3). In accordance with the reduction in cell counts of *E. faecium*, the *B. bacteriovorus* PF13 concentration increased by 3.57 logs to the highest concentration after 48 h of predation (Table 4). The results from the EMA-qPCR analysis also indicated that the concentration of *E. faecium* decreased (0.642 log) after 120 h of co-culture (Fig. 4b; Table 3), while the concentration of *B. bacteriovorus* PF13 increased by 3.92 logs after 120 h (Table 4).

For the HEPES buffer trials, however, the cell counts of *E. faecium* were only reduced by 0.193 logs after 120 h of predation (Fig. 4c; Table 3). Additionally, in contrast to the results obtained for the DNB trials, *B. bacteriovorus* PF13 did not produce any visible plaques on the double-layer agar overlays during the HEPES trials with *E. faecium* as prey (Table 4). The EMA-qPCR analysis then indicated that the concentration of *E. faecium* decreased (albeit not significantly) by 0.318 logs (Fig. 4d; Table 3) and correspondingly the concentration of *B. bacteriovorus* PF13 increased by 0.449 logs after 120 h of co-culture (Table 4).

4. Discussion

Pseudomonas fluorescens and *P. aeruginosa* were employed as prey cells in the current study as various studies have indicated that *Pseudomonas* spp. are sensitive to predation by *B. bacteriovorus* (Dashiff

Table 3
Summary of the results of the cell counts (CFU/mL) and gene copies (GC/mL) of the different prey cells after exposure to the predatory bacteria, *B. bacteriovorus* PF13, in DNB and HEPES buffer.

Prey	DNB						HEPES Buffer					
	Culture-based Analysis			EMA-qPCR			Culture-based Analysis			EMA-qPCR		
	Control CFU/mL[a]	Predated CFU/mL	Log Change	Initial GC/mL[b]	Predated GC/mL	Log Change	Control CFU/mL[a]	Predated CFU/mL	Log Change	Initial GC/mL	Predated GC/mL	Log Change
Pseudomonas fluorescens	5.91×10^{9}	3.40×10^{6}	−3.24	8.06×10^{5} [c]	4.60×10^{4}	−1.24	2.48×10^{9}	1.51×10^{5}	−4.21	2.19×10^{8}	5.02×10^{6}	−1.64
Pseudomonas aeruginosa	2.02×10^{10}	2.83×10^{8}	−1.85	8.11×10^{7}	5.63×10^{7}	−0.159	3.53×10^{9}	4.80×10^{7}	−1.87	4.06×10^{8}	4.06×10^{8}	0.00
Klebsiella pneumoniae	1.44×10^{9}	3.80×10^{5}	−3.58	1.11×10^{8}	2.50×10^{4}	−3.65	2.72×10^{9}	2.00×10^{4}	−5.13	4.01×10^{8}	2.06×10^{5}	−3.29
Staphylococcus aureus	1.59×10^{9}	8.50×10^{8}	−0.272	1.18×10^{6}	4.22×10^{6}	+0.553	1.25×10^{9}	2.00×10^{7}	−1.80	4.96×10^{8}	2.64×10^{7}	−1.27
Enterococcus faecium	6.74×10^{9}	1.31×10^{7}	−2.71	5.17×10^{8}	1.18×10^{8}	−0.642	5.40×10^{8}	3.46×10^{8}	−0.193	1.43×10^{9}	6.88×10^{8}	−0.318

[a] Cell counts in CFU/mL of the non-predatory control samples after 120 h.
[b] Initial inoculation concentration of the prey cells in GC/mL quantified using EMA-qPCR.
[c] Concentration in GC/mL of *P. fluorescens* in the control sample after 120 h.

Table 4
Summary of the results of the cell counts (PFU/mL) and gene copies (GC/mL) of *B. bacteriovorus* PF13 after co-culture with the different prey cells in DNB and HEPES buffer.

Prey	DNB						HEPES Buffer					
	Culture-based Analysis			EMA-qPCR			Culture-based Analysis			EMA-qPCR		
	Initial PFU/mL	Maximum PFU/mL	Log Change	Initial GC/mL	Maximum GC/mL	Log Change	Initial PFU/mL	Maximum PFU/mL	Log Change	Initial GC/mL	Maximum GC/mL	Log Change
Pseudomonas fluorescens	2.53×10^{5}	6.95×10^{10}	+5.44	2.77×10^{3}	5.99×10^{7}	+4.33	1.80×10^{4}	1.83×10^{9}	+5.01	7.06×10^{3}	3.68×10^{6}	+2.72
Pseudomonas aeruginosa	ND	ND	ND	4.54×10^{3}	2.52×10^{4}	+0.744	ND	ND	ND	2.05×10^{3}	1.89×10^{3}	−0.035
Klebsiella pneumoniae	2.53×10^{5}	1.78×10^{9}	+3.85	1.24×10^{3}	5.95×10^{7}	+4.68	1.75×10^{4}	3.20×10^{9}	+5.26	3.22×10^{3}	5.32×10^{7}	+4.22
Staphylococcus aureus	ND	ND	ND	8.17×10^{2}	1.25×10^{7}	+4.18	ND	ND	ND	3.76×10^{3}	1.72×10^{6}	+2.66
Enterococcus faecium	2.53×10^{5}	9.45×10^{8}	+3.57	1.17×10^{3}	9.55×10^{6}	+3.92	ND	ND	ND	9.88×10^{6}	2.78×10^{7}	+0.449

ND – Not Determined as *B. bacteriovorus* PF13 did not produce visible plaques on the double-layer agar overlays for these prey cells.

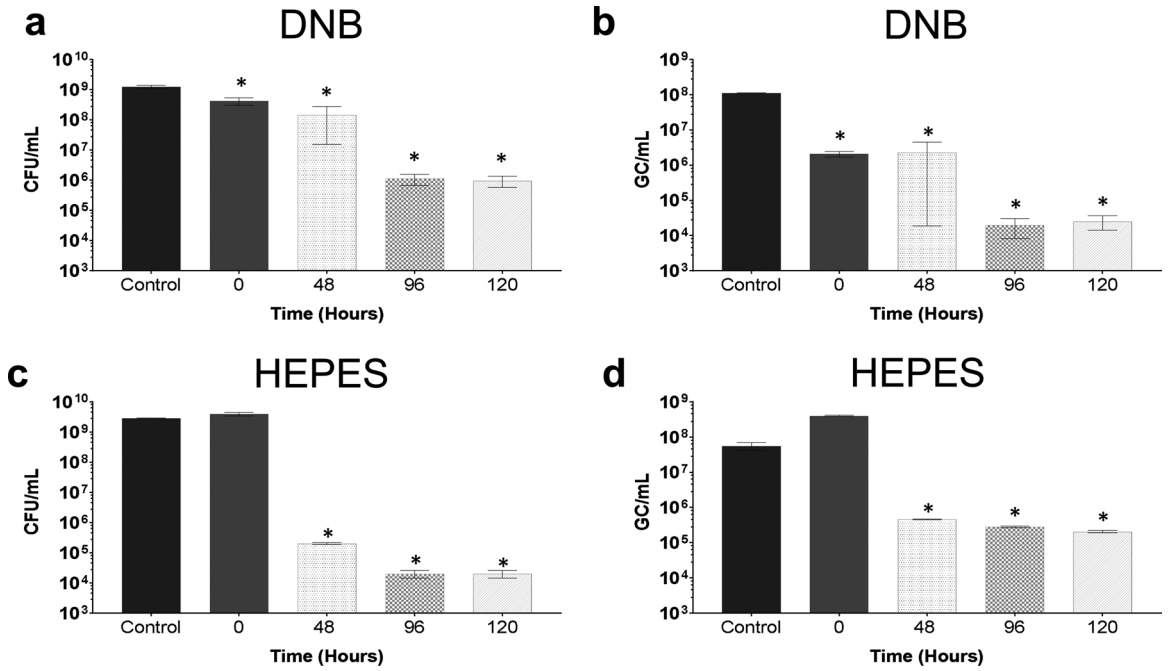

Fig. 2. Enumeration in CFU/mL using culturing (a and c) and quantification in GC/mL using EMA-qPCR (b and d) of *K. pneumoniae* ATCC 333305 co-cultured with *B. bacteriovorus* PF13 in DNB and HEPES buffer. (*) the cell counts or GC were significantly reduced ($p < 0.05$) as compared to the control and/or initial concentration.

et al., 2011; Kadouri et al., 2013; Shanks et al., 2013). Based on the prey cell counts, predator plaque counts and GC for *P. fluorescens* and *B. bacteriovorus* in co-culture, it was evident that *B. bacteriovorus* PF13 was able to replicate at the expense of *P. fluorescens* ATCC 13525 in both the DNB and HEPES buffer. However, for *P. aeruginosa* variable results for the cell counts and GC were observed. The cell counts recorded for *P. aeruginosa* indicated that the concentration of this organism was significantly reduced ($p < 0.05$) after 120 h of co-culture with the predatory bacterium in DNB and HEPES buffer. These findings were in accordance with a previous study by Dashiff et al. (2011) where *P. aeruginosa* ATCC BAA427 was sensitive to predation by *B. bacteriovorus* in DNB, with a 1.00 log reduction in the cell counts observed for co-culture experiments after 48 h of incubation. Kadouri et al. (2013) reported higher log reduction values, where the concentration of *P. aeruginosa* GB771 was reduced by 3.96 and 3.07 logs in DNB, using *B. bacteriovorus* 109 J and HD100, respectively, indicating that different predator strains may have variable effects on different prey strains of the same species. While the EMA-qPCR analysis conducted in the current study confirmed that the GC of *P. aeruginosa* in co-culture with *B. bacteriovorus* PF13 in DNB were reduced, the reduction recorded (0.159

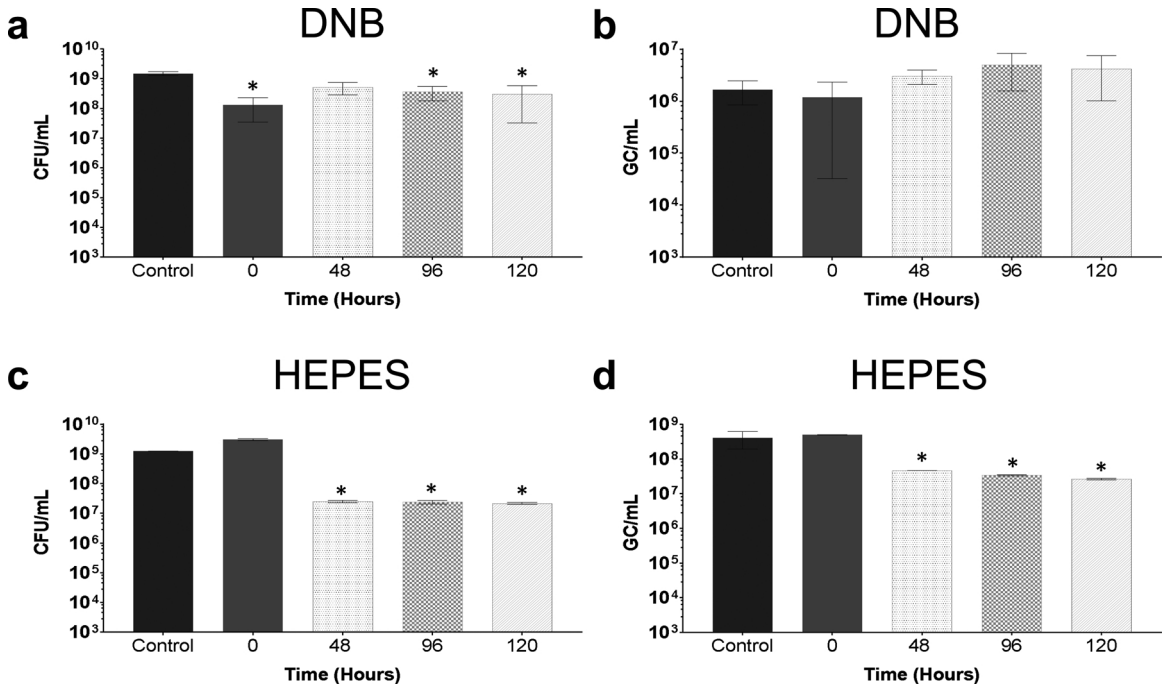

Fig. 3. Enumeration in CFU/mL using culturing (a and c) and quantification in GC/mL using EMA-qPCR (b and d) of *S. aureus* ATCC 25925 co-cultured with *B. bacteriovorus* PF13 in DNB and HEPES buffer. (*) the cell counts or GC were significantly reduced ($p < 0.05$) as compared to the control and/or initial concentration.

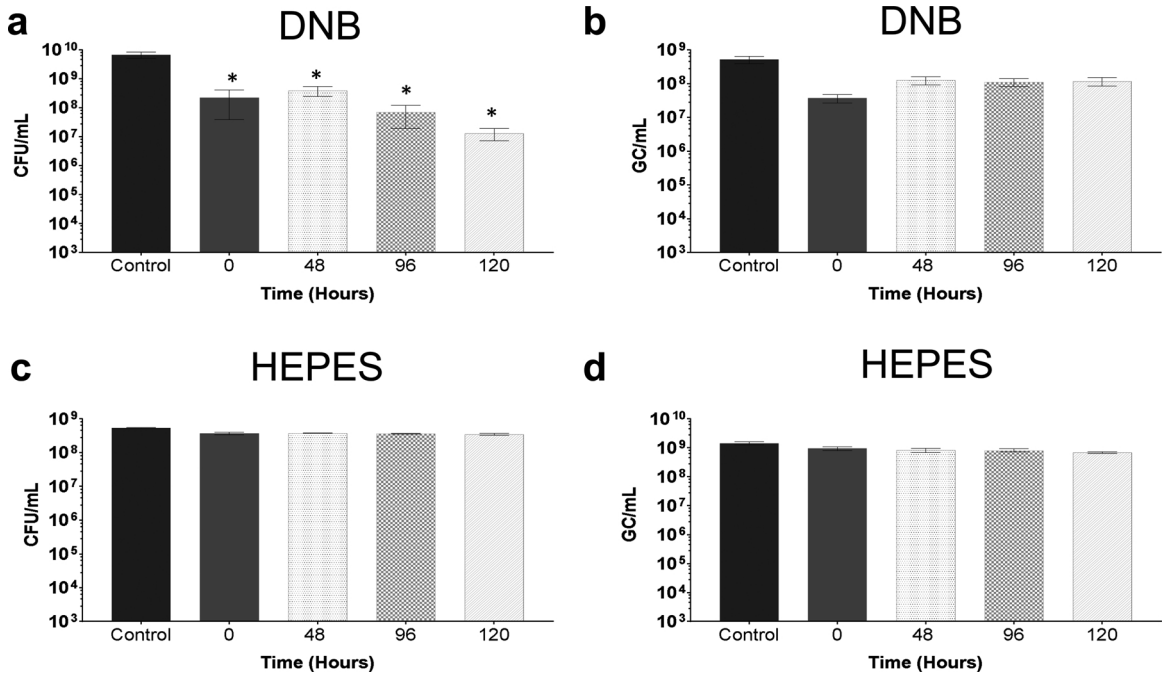

Fig. 4. Enumeration in CFU/mL using culturing (a and c) and quantification in GC/mL using EMA-qPCR (b and d) of *E. faecium* Clinical co-cultured with *B. bacteriovorus* PF13 in DNB and HEPES buffer. (*) the cell counts or GC were significantly reduced ($p < 0.05$) as compared to the control and/or initial concentration.

logs) was much lower than the log reduction observed for the cell counts (1.85 log reduction). Moreover, in HEPES buffer, the EMA-qPCR results indicated that the concentration of *P. aeruginosa* after 120 h of predation, was equal to the initial inoculum concentration. Accordingly, the *B. bacteriovorus* PF13 GC increased (0.744 logs) in the DNB, but decreased by a negligible margin (0.035 logs) in the HEPES buffer trials. For *P. aeruginosa*, the reductions recorded using the culture-based enumeration techniques did thus not correlate with the molecular analysis. It is well known that bacteria enter a VBNC state under unfavourable conditions, which can be induced if cells are stressed as a result of light, temperature, high or low salinity, pressure and low nutrient levels (Ramamurthy et al., 2014). Lambert et al. (2010) also showed that *B. bacteriovorus* can induce a stress response in *E. coli* with genes involved in osmotic stress, amino acid and carbon starvation and toxin efflux pumps significantly upregulated within 15 min of exposure to predatory bacteria. It is thus hypothesised that in the current study, a subpopulation of the *P. aeruginosa* cells entered a VBNC state upon exposure to the predator, which may account for the reduction in the cell counts observed using the culture-based methods, while the GC, which enumerates cell viability, were not significantly reduced after 120 h of predation in the DNB or HEPES buffer. Moreover, the *P. aeruginosa* strain employed in the current study may not have been the ideal prey for the *B. bacteriovorus* strain isolated from wastewater. Pantanella et al. (2018) noted that mutants of *B. bacteriovorus* could arise in the absence of preferred or ideal prey as a potential survival strategy. These mutants could alter the secretion of hydrolytic enzymes such as proteases and access nutrients available in culture media or break down prey cells (releasing the cellular contents into the culture medium), to subsequently grow or survive axenically. The *B. bacteriovorus* strain employed in the current study may thus have altered its expression of lytic enzymes in order to survive, which in turn could have influenced the culturability of the *P. aeruginosa* prey. However, this hypothesis should be assessed in future studies by screening for mutations in the host-independent (*hit*) locus which has been associated with the host-independent phenotype of *Bdellovibrio* spp. (Capeness et al., 2013).

Based on the results obtained for the trials using *K. pneumoniae* as prey, it was evident that *B. bacteriovorus* PF13 could effectively utilise the *K. pneumoniae* cells as a nutrient source and for replication as the cell counts and GC of *K. pneumoniae* were significantly reduced after 120 h of co-culture, in both the DNB and HEPES buffer trials. Correspondingly, the *B. bacteriovorus* PFU and GC significantly increased in the presence of *K. pneumoniae* in DNB and HEPES buffer. These results were in accordance with results reported by Dashiff et al. (2011), where planktonic cell counts in DNB were reduced by 2, 2 and 4 logs for *K. pneumoniae* ATCC 33495, ATCC BAA1705 and ATCC BAA1706, respectively, while the concentration of six different *K. pneumoniae* clinical isolates were each reduced by 5 logs in the presence of *B. bacteriovorus*. However, similar to the results observed for the *P. fluorescens* ATCC 13525, the *K. pneumoniae* cells were not completely eradicated, even after 120 h of co-culture with *B. bacteriovorus* PF13 in DNB and HEPES buffer, as cell counts and GC were still detected. This observation is in accordance with literature indicating that prey cell populations exhibit an inherent plastic phenotypic resistance towards predation (Shemesh and Jurkevitch, 2004; Kadouri et al., 2013; McNeely et al., 2017). This resistance mechanism is not specific towards a predator strain and is a general phenotypic adaptation observed in residual prey populations after exposure to a predatory bacterium (Shemesh and Jurkevitch, 2004). Once the predator is removed or the predator concentration is reduced, the prey population returns to a predation sensitive phenotype (Shemesh and Jurkevitch, 2004).

The results for *S. aureus* indicated that while a negligible decrease in cell counts was recorded after 120 h of co-culture with *B. bacteriovorus* PF13 in DNB, a significant decrease in prey cell counts was recorded for the co-culture experiments conducted in HEPES buffer. Accordingly, while the EMA-qPCR results indicated that the GC of *S. aureus* were reduced in HEPES buffer, the GC of *S. aureus* increased in DNB. It was however, interesting to note that the GC of *B. bacteriovorus* PF13 increased significantly in co-culture with *S. aureus* in DNB and the HEPES buffer, respectively. Iebba et al. (2014), reported that in the presence of Gram-positive prey such as *S. aureus* cells, *B. bacteriovorus* produces non-secreted hydrolytic enzymes which may include proteases, glycanases and DNases, which differ from the enzymes produced in the presence of Gram-negative prey, subsequently enabling the predator to utilise Gram-positive organisms as a nutrient source. Furthermore, Monnappa et al. (2014) confirmed that host-independent *B.*

bacteriovorus produce a range of lytic enzymes such as proteases and nucleases that are effective in dispersing *S. aureus* and *S. epidermis* biofilms. *Bdellovibrio bacteriovorus* also switches from a periplasmic predation strategy in the presence of Gram-negative prey, to an epibiotic predation strategy in the presence of *S. aureus* cells (Iebba et al., 2014; Pantanella et al., 2018). Based on the results obtained for the *B. bacteriovorus* PF13 and *S. aureus* ATCC 25925 co-cultures in HEPES buffer; the predator used in the current study may thus have been able to produce hydrolytic enzymes or switch its predation strategy in order to utilise the *S. aureus* cells as a nutrient source and for replication. For the DNB trials however, the *S. aureus* cell counts were not significantly reduced, while the GC of *S. aureus* increased. Thus, in DNB the predatory bacteria could have secreted lytic enzymes to access the nutrients suspended in the media in order to survive and grow, while they were not actively preying on the *S. aureus* cells. Additionally, the *S. aureus* cell counts were only reduced by 0.272 logs in DNB, compared to a 1.80 log recorded for the HEPES buffer trials, suggesting that *B. bacteriovorus* does not directly attack the *S. aureus* cells in the DNB and rather utilises the nutrients in this medium for growth.

Based on predominantly the culture-based analysis results obtained for the trials where *E. faecium* was utilised as prey, it is hypothesised that *B. bacteriovorus* PF13 benefitted from being co-cultured with *E. faecium* in DNB as the concentration of the predator increased, while the concentration of the prey decreased. In addition, plaque formation of *B. bacteriovorus* PF13 was observed when *E. faecium* was utilised as prey during the DNB trials, indicating that in DNB *B. bacteriovorus* PF13 preys on *E. faecium*. Contrastingly, the *E. faecium* concentration in HEPES buffer was marginally reduced with a corresponding increase in the predator concentration, albeit not as great as the increase observed for the DNB trials, while plaques were also not observed during the HEPES buffer trials. Limited information is however, available on the interaction of *B. bacteriovorus* with *Enterococcus* spp. (Jurkevitch, 2006). Dashiff et al. (2011) however, indicated that *E. faecalis* is not sensitive to predation by *B. bacteriovorus* or *M. aeruginosavorus*. Thus, in contrast to available literature, preliminary results (in DNB media) obtained in the current study suggests that the *B. bacteriovorus* strain isolated from wastewater is able to interact with *E. faecium* as a potential nutrient source, however additional research is required to corroborate these results. This may include the use of gene expression analysis targeting predation specific genes (encoding for flagella, pili and various lytic enzymes) of *B. bacteriovorus,* to determine if these genes are similarly upregulated when *B. bacteriovorus* is exposed to *E. faecium* as compared to Gram-negative prey.

While both culture-based and molecular techniques can be used to assess viable microbial cells, molecular techniques are more accurate if VBNC cells are present (Delgado-Viscogliosi et al., 2009; Reyneke et al., 2017). Although limited information on the use of molecular techniques to assess the interaction of *B. bacteriovorus* with potential prey cells is available, qPCR has been used to assess the interaction of *B. bacteriovorus* HD100 with *P. aeruginosa* and *S. aureus* in DNB (Pantanella et al., 2018). The viability dye EMA was thus combined with qPCR in the current study in order to distinguish between live and dead microbial cells and was particularly beneficial during the predation trials, where *P. aeruginosa*, *S. aureus* and *E. faecium* were used as prey and *B. bacteriovorus* PF13 did not produce plaques on the double-layer agar overlays (with the exception of the trials conducted in DNB with *E. faecium* as prey). However, while EMA effectively suppresses the signal from extracellular DNA or DNA from cells with compromised membranes, it has been reported that this viability dye can penetrate the intact membrane of live cells, while high concentrations of EMA may also be cytotoxic to viable cells, which may influence the accuracy with which live cells are quantified (Nocker et al., 2006; Fittipaldi et al., 2012). Conflicting conclusions have also been made regarding the effect of the membrane composition of bacterial cells (complex structure of the outer membrane of Gram-negative bacteria versus the thick peptidoglycan layer of Gram-positive bacteria) on the efficacy of viability dye treatment (Nocker et al., 2006; Flekna et al., 2007; Fittipaldi et al., 2012). However, an in-depth analysis by Reyneke et al. (2017) on the application of EMA, PMA and DNase in combination with qPCR to detect viable cells indicated that EMA-treatment was effective for certain Gram-positive (*E. faecalis*) and Gram-negative organisms (*P. aeruginosa* and *S. typhimurium*). Additionally, Reyneke et al. (2017) optimised the concentration of EMA to detect viable cells of Gram-negative and Gram-positive bacteria and found that a final concentration of 6 μM EMA (concentration of EMA applied in the current study) was optimal.

5. Conclusions

Corresponding results were generally obtained for the culture-based and EMA-qPCR analysis and it can be concluded that EMA-qPCR can be used to monitor the interaction of *B. bacteriovorus* with various prey cells in different media. Additionally, while variable results were obtained specifically with regards to the interaction of *B. bacteriovorus* with Gram-positive prey, these variations were dependent on the specific prey cells used and the media employed to assess these interactions.

HEPES buffer or DNB could also be employed to monitor the predation of *P. fluorescens* and *K. pneumoniae* by *B. bacteriovorus,* while predation on *E. faecium* can be monitored in DNB and predation on *S. aureus* can be monitored in HEPES buffer. Results from this study thus indicated that it may be noteworthy to assess the activity of newly isolated *B. bacteriovorus* strains on prey species in both DNB and HEPES buffer, in order to fully investigate the predator-prey interactions in nutrient poor (DNB) and nutrient deficient (HEPES buffer) conditions. However, for *P. aeruginosa*, conflicting results were obtained for the plate counts versus the EMA-qPCR results during the DNB and HEPES buffer trials. Further analysis is thus required to determine whether the *P. aeruginosa* strain employed in the current study entered a VBNC state during the co-culture trials and co-culture experiments with different *P. aeruginosa* strains may need to be conducted.

Importantly, *P. fluorescens*, *K. pneumoniae*, *E. faecium* (in DNB) and *S. aureus* (in HEPES buffer) were found to be sensitive to predation in varying degrees in the current study. This is significant as these organisms are associated with human disease, which supports the notion of employing predatory bacteria as live antimicrobials or biocontrol agents to combat pathogenic microbial species.

Authors' contributions

Conceived and designed the experiments: MW WK. Performed experiments: MW. Analysed the data: MW WK. Contributed reagents/materials/analysis tools: WK. Compiled the manuscript: MW WK. Edited the manuscript: MW WK SK. All authors approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the

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