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Predatory bacteria in the haemolymph of the cultured spiny lobster *Panulirus ornatus*

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Graphical abstract

Predator *Halobacteriovorax* sp. Hbv and prey *Vibrio* sp. Vib in spiny lobster.

Abstract

Bdellovibrio and like organisms (BALOs) are Gram-negative obligate predators of other bacteria in a range of environments. The recent discovery of BALOs in the circulatory system of cultured spiny lobster *P. ornatus* warrants more investigation. We used a combination of co-culture agar and broth assays and transmission electron microscopy to show a *Halobacteriovorax* sp. strain Hbv preyed upon the model prey bacterium *Vibrio* sp. strain Vib. The haemolymph microbiome of juvenile *P. ornatus* was characterised following injection of phosphate buffered saline (control) or prey and/or predator bacteria for 3 d. The predator Hbv had no effect on survival compared to the control after 3 d. However, when compared to the prey only treatment group, lobsters injected with both prey and predator showed significantly lower abundance of genus *Vibrio* in the haemolymph bacterial community composition. This study indicates that predatory bacteria are not pathogenic and may assist in controlling microbial population growth in the haemolymph of lobsters.

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Keywords: *Bdellovibrio* and like organisms; predatory bacterium *Halobacteriovorax*; prey bacterium *Vibrio*; haemolymph; cultured spiny lobster.

Abbreviations: BALO, *Bdellovibrio* and like organism; Hbv, *Halobacteriovorax* sp.; KEGG, Kyoto Encyclopedia of Genes and Genomes; OTU, operational taxonomic unit; PBS, phosphate buffered saline; Vib, *Vibrio* sp.

The sequences generated were deposited in the NCBI Sequence Read Archive under BioProject accession PRJNA678039.

Two supplementary figures are available with the online version of this article.

INTRODUCTION

Bdellovibrio and like organisms (BALOs) are a group of Gram-negative bacteria that prey on other Gram-negative bacteria. This group consists of families *Bdellovibrionaceae*, *Bacteriovoracaceae*, *Halobacteriovoraceae* and *Peredibacteraceae* [1, 2]. BALOs are both small and motile, conferring a physical advantage over other predatory / bacteriovorous microorganisms including bacteriophages that are smaller but nonmotile [3] and protozoans which are larger and less motile [4]. BALOs exhibit either epibiotic or periplasmic modes of predation: epibiotic predators divide by binary fission while attached to prey while periplasmic predators divide by synchronous septation inside prey [5].

BALOs act as natural top-down population control mechanisms for bacterial communities in a range of aquatic and terrestrial environments [6–9] and within organs (e.g. gill, shell) of aquatic animals [10, 11]. There are fewer reports of BALOs within animals compared to those from the environment, however they have been detected in the gut of sturgeon [12] and snakehead fish [13]. These BALOs (*Bacteriovorax* sp. and *Bdellovibrio bacteriovorus*) were shown to protect their hosts and other aquatic animal species against infections caused by a number of aquatic bacterial pathogens [13–15]. It is because of this biocontrol potential that BALOs have been suggested as an alternative treatment to antibiotics and thereby mitigating associated ecological threats of antibiotic resistance [16].

Various aspects of disease management and health are currently being investigated for the ornate spiny lobster *P. ornatus* as closed life cycle aquaculture production of this species reaches the commercialisation phase under UTAS-Nexus Aquasciences Pty. Ltd. (UNA). An aspect of health was to establish baseline microbiomes of healthy lobsters. Two studies from this project have employed next generation sequencing to perform the first extensive characterisation of the haemolymph [17] and gut [18] microbiomes of healthy juvenile *P. ornatus*. We revealed that BALO members were present at low relative abundances in the haemolymph (*Bacteriovorax* 0.4–0.8%, *Bdellovibrio* 0.3–4.4%) and hindgut (*Bacteriovorax* 0.03–8.0%, *Bdellovibrio* 0.1%) of cultured juvenile *P. ornatus* [17, 18]. This is consistent with reported abundances of BALOs in the natural environment (e.g. water and terrestrial), which comprise less than 0.2% of total bacteria [2]. Still, prior to this project there was no record of BALOs residing in the circulatory system of animals. Lobsters have an open circulatory system where bacteria in the water may enter via injured integument, gills and gut [19, 20], and particularly during moulting [21].

BALOs are not commonly studied as they cannot be isolated by routine culture methods. Besides suitable nutrients and conditions, the isolation of BALOs require a high concentration of prey bacteria. The finding of BALOs in lobster haemolymph may infer a functional importance. The aim of this study was to screen for and characterise BALOs and examine their effect on the haemolymph microbiome and immune response of cultured *P. ornatus* juveniles.

METHODS

Prey *Vibrio* (Vib)

Vibrio sp. strain Vib was used as prey bacterium in this study. This strain was isolated on marine agar 2216 (Difco Laboratories Inc., MI, USA) from the haemolymph of a juvenile *P. ornatus* exhibiting lethargy and minimal response during handling at the Institute for Marine and Antarctic Studies (IMAS), Tasmania, Australia. The strain was identified by sequencing of the 16S rRNA gene. Sanger sequencing included a 20 µl PCR reaction: 10 µl of 2×MyTaq HS mix (Bioline Pty. Ltd., NSW, Australia), 400 nM each of 27F (5′ – AGAGTTTGATCMTGGCTCAG – 3′) and 1492R primers (5′ – GGTTACCTTGTTACGACTT – 3′) and 1 µl of extracted nucleic acids; using PCR programme: 95 °C for 3 min; 30 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s; and a final extension of 72 °C for 3 min. The isolate (10^8 cell ml^{-1}) was stored in 25% (v/v) glycerol at −80 °C. Subcultures of Vib were grown in a modified marine broth consisting of 0.5% peptone, 0.3% yeast extract and 3.5% Instant Ocean sea salt (Aquarium Systems, France) at 28 °C with shaking (100 r.p.m.) overnight. Bacteria were harvested by centrifugation at 8000 ***g*** for 5 min and the cell pellet was resuspended in autoclaved seawater adjusted to 35 ppt with Instant Ocean sea salt.

Predator *Halobacteriovorax* (Hbv)

Sea water was sampled at IMAS. Two hundred and fifty millilitres of water was passed through a 3 µm filter, centrifuged at 16000 ***g*** for 20 min and the concentrated suspension was further centrifuged at 1000 ***g*** for 5 min to separate algae from bacteria. The supernatant was cultured with harvested Vib (~10^8 cell ml^{-1}) in modified Luria broth (0.1% Luria broth base [Sigma-Aldrich Co., MO, USA] in autoclaved sea water) at 28 °C with shaking (100 r.p.m.). The broth co-culture was monitored daily at optical density 700 nm from opaque (0.7) until clear (0.1). The enriched co-culture was used in a double layer agar plating method, consisting of a base layer (modified Luria broth with 1.2% agar) and overlay layer (modified Luria broth with 0.6% agar with co-culture and ~0.5×10^{10} Vib cells). Plates were incubated at 28 °C and observed for plaques over 7 d. Individual plaques were subcultured in modified Luria broth with Vib. To avoid losses of the predator strain during processing, no attempts were made to isolate Hbv from Vib in subcultures by filtration.

Amplification of *Bacteriovoracaceae*-specific 16S rRNA

Broth co-cultures were initially centrifuged at low speed (1000 ***g*** for 5 min) to remove debris, followed by high speed centrifugation (16100 ***g*** for 15 min) to concentrate bacteria. The pellet was vortexed in 200 µl of lysis buffer (7.8 M urea, 0.5% sodium dodecyl sulphate), heated at 55 °C for 10 min and incubated on ice for 10 min. The lysate was vortexed with 100 µl of 7.5 M ammonium acetate for 30 s and centrifuged at 14000 ***g*** for 5 min (4 °C). The supernatant was inverted (40 times) with 300 µl of isopropanol with 0.02 µg μl^{-1} pink co-precipitant and centrifuged at 16000 ***g*** for 10 min. The

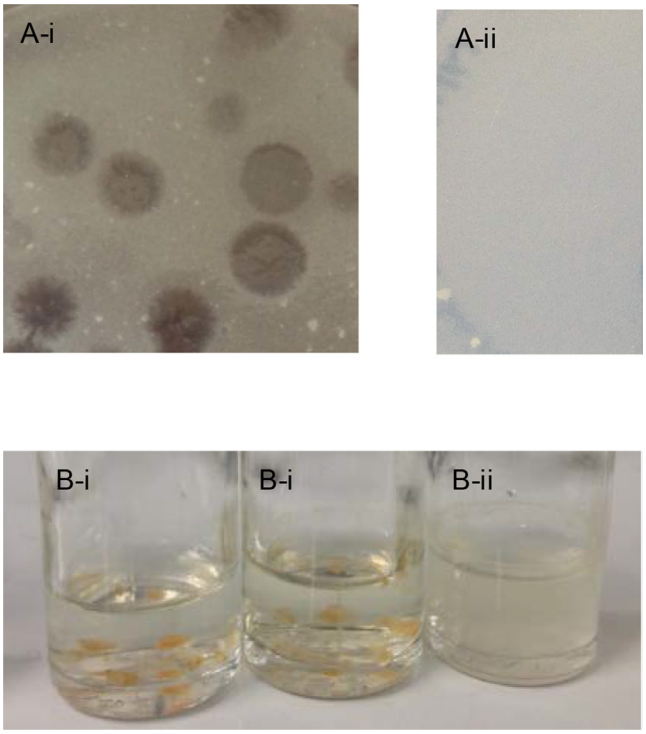

Fig. 1. Cultivation of *Halobacteriovorax* sp. Hbv. (a) Double layer agar plate of [i] Hbv and *Vibrio* sp. Vib and [ii] Vib only. (b) Broth of [i] Hbv and Vib and [ii] Vib only.

pellet was washed with 500 µl of 60% ethanol twice and resuspended in 50 µl of molecular grade water.

A semi-nested PCR was performed using universal bacterial primers and *Bacteriovoracaceae*-specific primers. Both PCR 10 µl mixtures contained 5 µl of 2×MyTaq HS mix, 400 nM each of 63F (5′ - CAGGCCTAACACATGCAAGTC 3′) [22] or Bac676F (5′ – ATTTCGCATGTAGGGGTA – 3′) [7] for primary (63F and Bac1442R) and secondary (Bac676F and Bac14442R) PCRs respectively, and Bac1442R primer (5′ – GCCACGGCTTCAGGTAAG – 3′) and 2 µl of nucleic acids (primary PCR) or 1:10 diluted primary PCR products (secondary PCR). The PCR thermal cycling programme was conducted at 95 °C for 1 min, 25 cycles (primary PCR) or 30 cycles (secondary PCR) of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 15 s using CFX Connect Real-Time System (Bio-Rad Laboratories Inc., USA). PCR products were examined on a 1.5% agarose gel. For purification, PCR amplicons were mixed with equal volumes of 19% polyethylene glycol and 2 µl of Polyacryl Carrier (Molecular Research Centre Inc., OH, USA), incubated at room temperature for 15 min and centrifuged for 16100 ***g*** for 20 min. The pellet was rinsed with 70% ethanol and centrifuged at 16100 ***g*** for 15 min, twice. The pellet was resuspended in 25 µl of buffered water (0.05% Triton X-100, 10 mM TRIS pH 7) and quantified using a Qubit fluorometer (Invitrogen, Life Technologies, VIC, Australia). Purified PCR products and *Bacteriovoracaceae*-specific primers (Bac676F and Bac1442R) were sent to Australian Genome Research Facility (AGRF, QLD, Australia) for Sanger sequencing. The results were compared with other sequences in the Ribosomal Database Project and National Centre for Biotechnology Information (≥95% identity).

Transmission electron microscopy of Hbv

Multiple broth co-cultures from 1 to 6 d old were used to observe the different stages of the Hbv life cycle. The co-cultures were centrifuged at 1000 ***g*** for 5 min. The supernatant was deposited on Formvar/carbon grid. The sample was negatively stained with 1% uranyl acetate and examined with a Hitachi HT7700 electron microscope at 80 kV.

Hbv and Vib injection in lobster

Experimental design

Panulirus ornatus were cultured from hatch at IMAS as previously described [17]. Five juveniles per treatment (67.7±3.4 g; n=15 ♀ and 5 ♂) were placed in 4×50 litre tanks within a recirculating system (temperature 28 °C; dissolved oxygen 98%; pH 8; salinity 35 ppt). Lobsters were injected with 100 µl of either [1] phosphate buffered saline, pH 7.4 [PBS group] [2]; Vib (1×10^8 cell ml^{-1}) [prey group] [3]; Hbv (1.04×10^8 cell ml^{-1}) [BALO group]; or [4] Hbv and Vib (1.52×10^8 cell ml^{-1}, combined) [prey +BALO group]. The inoculum concentrations were selected after a pilot study showed that bacterial inoculums between 10^5–10^8 cell ml^{-1} did not cause lobster mortality. The prey and predator inoculums were harvested as described above but resuspended in PBS, pH 7.4. The predator bacteriolytic ability of the harvested inoculums were confirmed *in vitro* by adding an equal volume of modified Luria broth before incubation at 28 °C and colony enumeration using the drop plate technique on marine agar. Injection and sampling were made between the basis and the ischium of lobster pereiopods. Approximately 160 µl of haemolymph was sampled from each animal prior to injection (0 d) and additionally 1, 2 and 3 d after injection using a chilled syringe (23G needle) pre-filled with an equal volume of anticoagulant (400 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, 40 mM EDTA) (modified from [23]). All lobsters were in intermoult phase during sampling.

Bacterial analyses

Three hundred microlitres of anticoagulated haemolymph from each animal was centrifuged at 500 ***g*** for 10 min (4 °C) and the supernatant was further centrifuged at 16000 ***g*** for 10 min (4 °C) to concentrate bacteria from the plasma. All but 30 µl of the supernatant was removed and 300 µl of lysis buffer was added before storage at −20 °C.

Thawed plasma samples were added with 5 µl of proteinase K (Bioline), heated at 55 °C for 20 min (vortexed every 5 min) and put on ice for 10 min. Samples were added with 200 µl of 7.5 M ammonium acetate, vortexed for 30 s and centrifuged at 14000 ***g*** for 5 min (4 °C). Six hundred microlitres of isopropanol with 0.02 µg µl^{-1} pink co-precipitant was mixed with the supernatant and incubated for 15 min before centrifugation at 16000 ***g*** for 30 min. The pellet was rinsed with 500 µl of 60% ethanol twice and resuspended in 30 µl of buffered water.

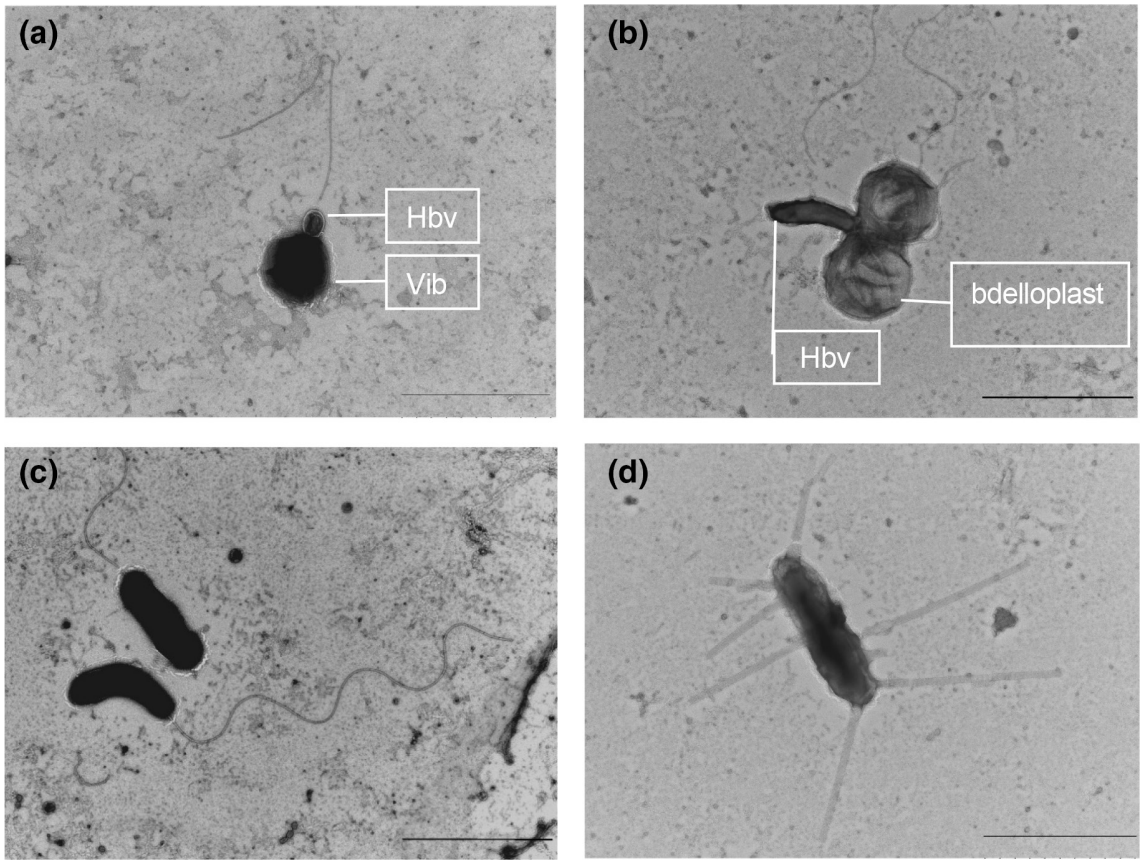

Fig. 2. Transmission electron micrographs of life cycle of *Halobacteriovorax* sp. Hbv. (a): Attached to prey *Vibrio* sp. Vib. (b): Hbv and bdelloplast. (c): Free swimming cells with flagella. (d): Cell with multiple pili. All scale bars: 2 µm.

To analyse bacterial diversity, a nested PCR approach was employed due to the occurrence of nonspecific PCR products in early assays. The amplicon library was constructed from all the plasma sampled on days 1 and 3 from each treatment group. The primary PCR mixture contained 5 µl of 2×MyTaq HS mix, 200 nM each of 27F and 1492R 16S rRNA gene primers and 1 µl of plasma extract. A thermal cycling programme of 95 °C for 3 min, 20 cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s was conducted using a C1000 Thermal Cycler (Bio-Rad Laboratories Inc., USA). The secondary PCR consisting of 5 µl of 2×MyTaq HS mix, 400 nM each of 27F and 1100R (5′ – AGGGTTGCGCTCGTTG – 3′) and 1 µl of primary PCR product was ran at 95 °C for 3 min, 28 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. PCR products were purified using SureClean (Bioline) according to manufacturer's instructions and quantified using Qubit. Twenty microlitres suspension containing 2 ng µl^{-1} of PCR amplicons of each plasma and extraction control was sent to AGRF for amplicon diversity profiling 27F - 519R (Illumina MiSeq, USA). The sequences were deposited in the NCBI Sequence Read Archive under BioProject accession PRJNA678039.

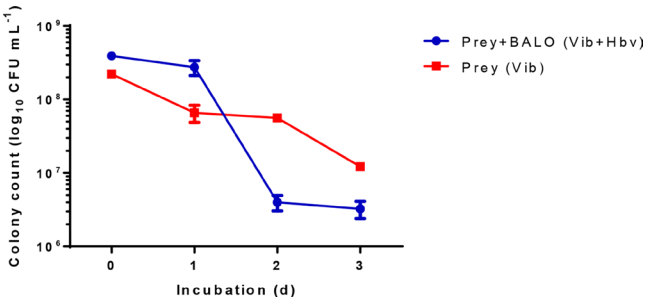

Fig. 3. Enumeration of *Vibrio* sp. Vib on marine agar (c.f.u. ml^{-1}) in modified Luria broth for 3 d. Each dot represents mean±SEM, *n*=5.

Data analyses

Paired-end amplicon reads were aligned using PEAR and trimmed from primers using Geneious 8.1.7 [24]. Chimaeras were removed using UCHIME and nonchimera files were processed in CloVR pipeline [25] which assigned operational taxonomic units (OTUs) to known taxa based on Greengenes database using RDP Bayesian classifier with 0.8 confidence threshold. OTUs that were in the extraction controls were removed from the OTU table (CloVR output) before being uploaded to MicrobiomeAnalyst [26] to examine alpha and

Table 1. Sampling depth, richness and alpha diversity indices for haemolymph sequence libraries of juvenile *P. ornatus*

Treatment group	Lobster no.	Sampling day	Sampling depth								Richness estimators		Diversity indices	
			Filtered sequences	Obs. OTUs	Good's coverage (%)	Phylum	Class	Order	Family	Genus	Chao1	ACE	Shannon	Simpson
PBS	1	1	35026	106	99.5	4	8	13	17	20	114	116	2.79	0.89
		3	142	(excluded)										
	2	1	23813	113	98.7	4	6	13	16	18	129	131	2.33	0.84
		3	28426	76	98.7	4	6	11	13	10	81	86	1.32	0.55
	3	1	26650	45	99.6	4	9	14	17	13	56	53	1.85	0.76
		3	62964	99	99.3	3	6	10	13	11	108	105	1.88	0.77
	4	1	33287	28	99.8	4	7	9	12	12	39	44	0.69	0.33
		3	3178	48	93.1	3	4	7	8	6	53	56	2.49	0.87
	5	1	10630	45	98.7	3	4	6	9	6	79	70	0.36	0.12
		3	34899	13	99.9	4	6	9	10	8	41	32	0.01	0.003
Prey (Vib)	1	1	28536	42	99.7	4	7	15	19	16	61	60	0.37	0.15
		3	22030	19	99.8	4	7	13	13	8	28	26	0.03	0.01
	2	1	6808	69	95.8	4	7	14	18	15	78	81	1.56	0.53
		3	23596	17	99.9	3	4	7	10	9	32	43	0.02	0.003
	3	1	36698	23	99.9	3	6	12	16	11	34	40	0.01	0.002
		3	7476	14	99.6	4	6	11	13	8	15	16	2.48	0.90
	4	1	26351	60	99.7	4	3	12	16	16	65	68	1.77	0.74
		3	34959	5	99.9	2	5	4	4	4	5	5	0.02	0.01
	5	1	40342	41	99.9	3	5	10	15	14	52	54	1.45	0.71
		3	63435	18	99.9	3	4	10	11	10	25	30	0.01	0.001
BALO (Hbv)	1	1	44010	86	99.7	4	7	16	21	19	114	111	0.80	0.27
		3	39747	20	99.9	4	6	12	14	10	23	25	0.01	0.002
	2	1	34226	62	99.5	4	8	12	14	15	76	79	0.96	0.49
		3	44736	50	99.9	4	8	11	14	14	53	54	1.51	0.70
	3	1	27240	108	99.5	4	7	14	18	18	128	131	2.37	0.86

Continued

Table 1. Continued

Treatment group	Lobster no.	Sampling day	Sampling depth								Richness estimators		Diversity indices	
			Filtered sequences	Obs. OTUs	Good's coverage (%)	Phylum	Class	Order	Family	Genus	Chao1	ACE	Shannon	Simpson
		3	36925	125	99.2	4	5	10	14	19	140	150	2.70	0.87
	4	1	17722	24	99.9	4	6	9	13	13	30	36	1.01	0.59
		3	45975	16	99.9	4	7	11	12	9	17	19	0.50	0.30
	5	1	24526	98	99.6	3	5	8	13	18	109	112	2.32	0.82
		3	22117	14	99.9	5	7	10	11	10	20	29	2.38	0.88
Prey+BALO	1	1	52343	146	98.7	4	6	13	18	23	150	155	2.85	0.90
(Vib+Hbv)		3	47464	44	99.8	5	6	8	10	10	62	51	1.06	0.52
	2	1	37763	102	99.1	5	7	11	16	16	117	118	2.12	0.81
		3	62597	42	99.9	4	7	12	12	11	44	46	1.26	0.67
	3	1	15848	26	99.7	5	6	7	11	12	32	45	0.54	0.25
		3	244	(excluded)										
	4	1	58564	39	99.9	5	6	12	15	15	54	60	0.31	0.10
		3	40699	21	99.9	4	5	10	13	14	51	56	0.63	0.43
	5	1	48254	24	99.9	4	7	13	17	16	30	33	0.83	0.53
		3	25901	65	99	5	8	13	18	17	84	88	1.17	0.53

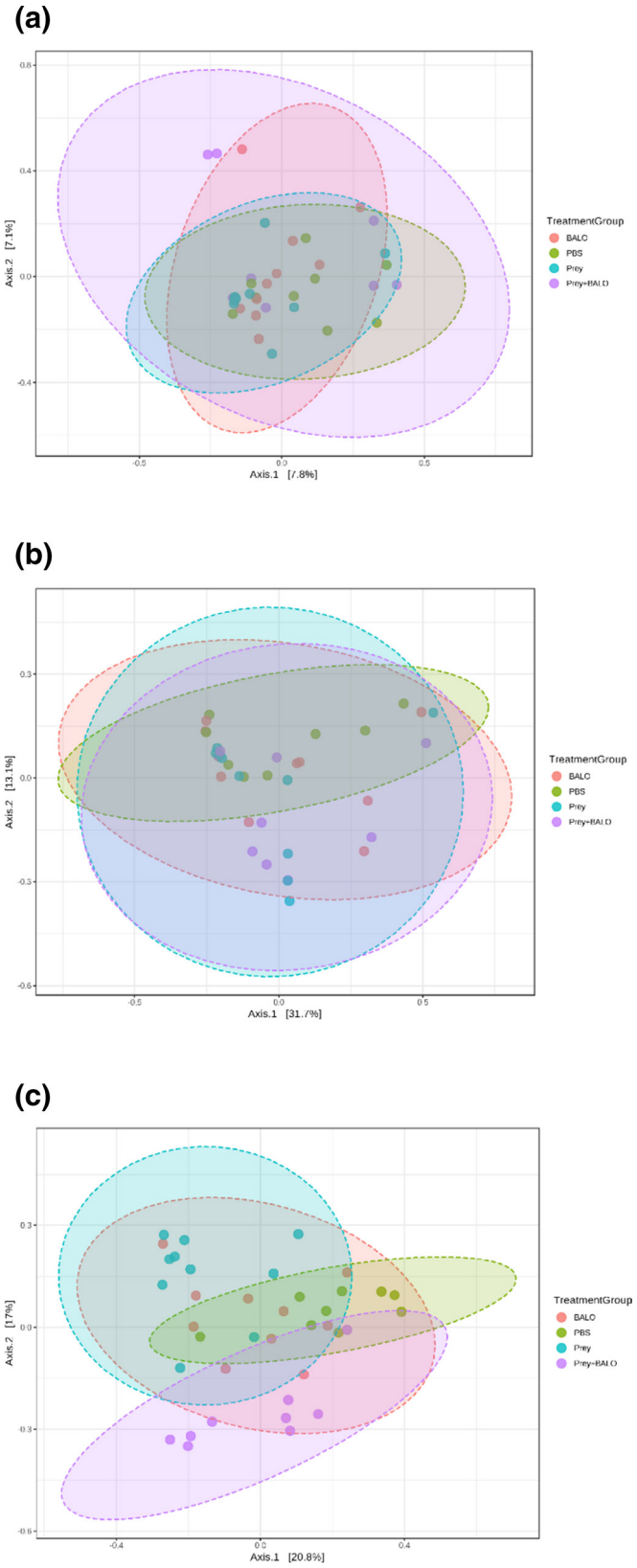

Fig. 4. (a) Bray Curtis, (b) weighted UniFrac and (c) unweighted UniFrac principal coordinate analysis plots showing similarity in haemolymph sequence libraries of *P. ornatus* juveniles.

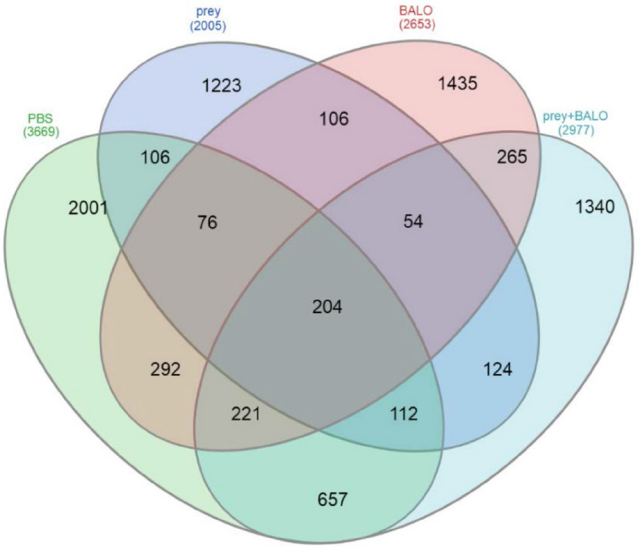

Fig. 5. Venn diagram showing shared and unique OTUs in haemolymph libraries of *P. ornatus* juveniles.

beta diversity, core microbiome, relative abundance and functional potential. Low abundance OTUs (≤2 count) with 10% or lower prevalence in samples were removed. Two samples with low reads were excluded from analysis. Good's coverage was calculated by [1 - (number of singleton reads / total number of reads)]×100%. The beta diversity was analysed by Bray Curtis, weighted and unweighted UniFrac distance based principal coordinate analysis at OTU level and PERMANOVA. Stacked bars of relative abundance of OTUs at phylum, class and family levels were generated. The functional potential of OTUs was predicted using PICRUSt [27] and presented as a functional diversity profile from the sum of abundance of each OTU for each KEGG metabolism normalised by category size. The functional association analysis was used to compare the KEGG pathways across treatment groups. The differential abundance of OTUs was compared among treatment groups using DAME [28]. A Venn diagram with unique and shared OTUs of each treatment group was drawn using InteractiVenn [29]. For all statistical analyses, a *P* value of ≤0.05 was considered significant.

RESULTS

Cultivation and identification of Hbv

Clear plaques appeared on double layer agar plates containing Hbv and Vib, whereas control plates with only Vib exhibited an opaque lawn of confluent bacterial growth (Fig. 1a). Plaque diameters increased with incubation duration, ranging from 1.5 mm after 5 d to 6 mm at 7 d. Broth cultures of individual plaques cleared within approximately 3 d compared to control broth that remained cloudy (Fig. 1b). Sanger sequencing of cleared broth (approximately 766 bp) shared 95% similarity with *Halobacteriovorax marinus* (GenBank accession number CP017414.1). The Vib sequence (876 bp) shared 99% similarity with *Vibrio coralliilyticus* (Genbank accession number CP031472.1).

Life cycle of Hbv

Halobacteriovorax cells were 1.6 to 2.3 µm long and 0.3 to 0.6 µm wide with a single polar flagellum. Different stages of Hbv life cycle were observed, including attachment to prey Vib (Fig. 2a), Hbv and bdelloplast (Fig. 2b), free swimming cells (Fig. 2c) and cells with multiple pili (Fig. 2d).

Hbv and Vib injection in lobster

In vitro bacterial culture

In vitro broth co-cultures were used to confirm that Hbv retained the ability to lyse Vib following inoculum preparation. The number of Vib colonies on marine agar decreased approximately two orders of magnitude within 2 d (Fig. 3).

Bacterial diversity

Forty haemolymph sequence libraries yielded a total of 657757 filtered reads with a mean of 16443 reads per sample. Observed OTUs ranged from 13 to 146 and Good's coverage ranged from 93.1–99.9% (Table 1). There were no significant differences ($P>0.05$) in the observed OTUs, richness estimators (Chao1, ACE) and diversity indices (Shannon, Simpson) when lobster treatment groups were compared (Table 1).

The two axes of principal coordinate analysis based on Bray Curtis, weighted UniFrac and unweighted UniFrac plot explained 14.9, 44.8 and 37.8% of the variations in abundance of OTUs among different samples (Fig. 4). This variation was not related to treatment groups except for unweighted UniFrac where there was some separation along the first axis. The PCoA results were supported by PERMANOVA. When the Bray Curtis index ($P=0.183$), weighted UniFrac ($P=0.180$) and unweighted UniFrac ($P=0.002$) distance matrices were analysed statistically using PERMANOVA, only the last showed significant difference among the four types of treatment groups.

Sequencing of all the haemolymph libraries based on OTUs grouped by phylum indicated that the core microbiome consisted of Proteobacteria and Bacteroidetes (Fig. S1, available in the online version of this article). The Venn diagram showed that the four treatment groups shared 204 OTUs (2% of total OTUs) belonging to phyla Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria (Fig. 5). The haemolymph of PBS group shared the highest number of OTUs (i.e. 1194; 15% of total OTUs) with that of prey +BALO group, while the haemolymph of prey group shared the least OTUs (i.e. 440; 5% of total OTUs) with that of BALO group. The haemolymph libraries of prey group had the highest percentage of unique OTUs (1223; 61% of prey group).

The top three phyla in the haemolymph of lobsters were Proteobacteria, Bacteroidetes and Firmicutes (Fig. 6). The three most represented classes in the haemolymph of juveniles were Gammaproteobacteria, Alphaproteobacteria and Bacilli. *Rhodobacteraceae*, *Pseudoalteromonadaceae*, *Enterobacteriaceae* and *Flavobacteriaceae* were predominant families in

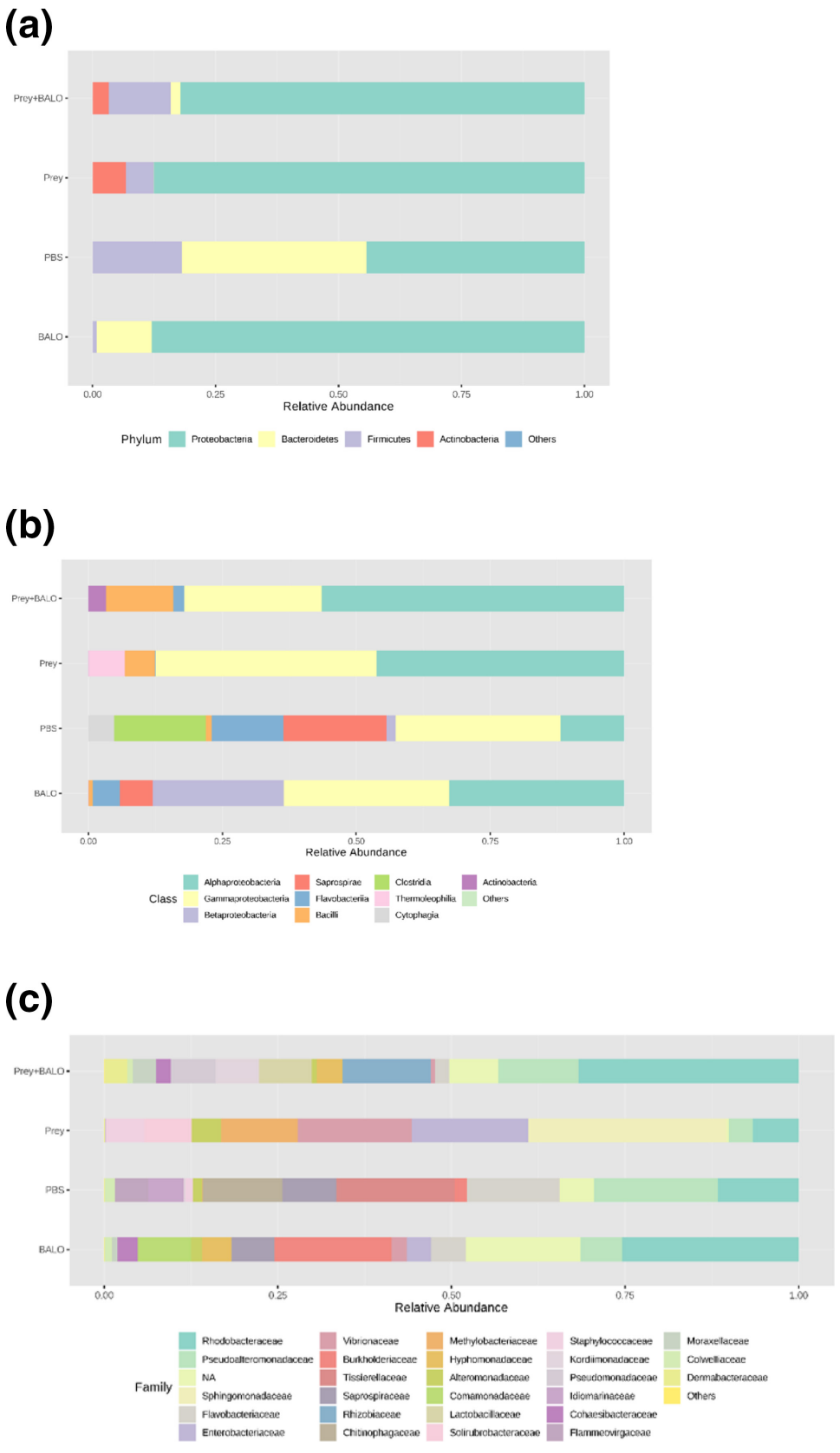

Fig. 6. Relative abundance of OTUs in haemolymph libraries of juvenile *P. ornatus* at (a) phylum, (b) class and (c) family levels.

the haemolymph libraries. Family *Rhizobiaceae* (11.4±11.1%; *P*=0.006) was significantly more abundant in the haemolymph of prey +BALO lobsters than the other treatment groups. The haemolymph of prey lobsters had significantly more of genus *Vibrio* (13.7±10.0%; *P*=0.003) but less of genus *Pseudoalteromonas* (9.2±9.0%; *P*=0.015) represented than other treatment groups. The haemolymph of BALO animals had significantly higher abundance of genus *Tenacibaculum* (4.1±2.2%; *P*=0.003) than the prey +BALO and prey groups. Additionally, the genus *Octadecabacter* (10.1±5.4%; *P*<0.001) was significantly more abundant in the haemolymph libraries of BALO lobsters compared to the prey and PBS groups. However, the abundance of injected Hbv was not significantly different among treatment groups.

PICRUSt was used to predict gene families found in lobster haemolymph communities and metabolic functional profiles were consequently applied using KEGG (Fig. S2). The four most predicted functions were metabolism of other amino acids, amino acid metabolism, metabolism of cofactors and vitamins and carbohydrate metabolism. There was no significant difference (*P*>0.05) in potential functions across treatments.

DISCUSSION

The present study is the first to report on the inoculation and effect of a BALO in the circulatory system of crustacean species. In our short term experiments, Hbv injected singularly at approximately 10^7 cells per animal had no apparent pathogenic effect towards *P. ornatus* juveniles following observations that survival and bacterial community diversity or predicted function did not significantly change when compared to control (PBS injected) animals. This discovery is consistent with other studies of BALO injection in rats [30], zebrafish [31] and New York worms [32] which did not incur pathogenic effects.

We used a combination of techniques including *in vitro* co-culture (broth and agar) and transmission electron microscopy to demonstrate Hbv preyed upon the model prey Vib. The suggestion of Hbv as a periplasmic predator is consistent with the report of other marine BALOs, *Halobacteriovorax* sp. PA1 [33] and *Bacteriovorax* sp. DA5 [34]. One of the benefits of periplasmic predation is that internal resources of prey cells can be accessed which reduces the dependence on obtaining nutrients from the environment, where supplies may be variable or depleted. A further advantage of BALOs within prey cells is that they have less exposure to host's immune defences such as opsonins and prophenoloxidase pathway which are induced by lipopolysaccharides [35]. In the present study, the attack phase of Hbv involved using its nonflagellated pole to attach and invade Vib, forming a bdelloplast. From here, Hbv appeared to undergo filamentous growth, septation into progeny cells, and exit of the exhausted prey through pores as reported by Fenton, Kanna [5]. The single polar flagellum of the mature free swimming attack phase Hbv was almost certainly used for directional propulsion via chemotaxis upon detecting high concentrations of Vib prey [2]. Interestingly, the Hbv isolate also harboured multiple pili. Type IV pili have been reported on the nonflagellated pole of *Bdellovibrio* with functions in prey attachment, penetration and replication [36, 37]. Unlike those reports, the isolate in the present study had seven to eight pili randomly distributed on the cell surface. Nevertheless, a variety of morphological forms of BALO isolates can exist due to the complexity of host-dependent and -independent phase of their life cycles [38].

The core haemolymph microbiome of *P. ornatus* juveniles comprised Proteobacteria and Bacteroidetes, with predicted functions primarily associated with the metabolism of amino acids, cofactors and vitamins, and carbohydrates. This is in agreement with an earlier study of older *P. ornatus* (162.7±4.6 g wet weight) juveniles from the same facility [17]. Taken together, these studies suggest a degree of haemolymph microbiome stability throughout development that contribute positive functional roles to host physiology, nutrition and health. The four most common bacterial families found across all treatment groups were *Rhodobacteraceae* (e.g. *Octadecabacter*, *Phaeobacter*, *Tropicibacter*, *Loktanella*, *Ruegeria*, *Nautella*), *Pseudoalteromonadaceae* (e.g. *Pseudoalteromonas*), *Enterobacteriaceae* and *Flavobacteriaceae* (e.g. *Tenacibaculum*). A high prevalence of *Rhodobacteraceae* and *Flavobacteriaceae* was demonstrated also in an earlier study of older *P. ornatus* juveniles [17]. Members of *Rhodobacteraceae* such as *Phaeobacter* spp. and *Ruegeria* spp. can produce antibiotics that inhibit the colonisation and growth of pathogenic bacteria [39]. Antibiotics are also produced *by Pseudoalteromonas* spp. [40], which have been recovered from wild-captured and cultured *P. ornatus* larvae [41]. *Loktanella* spp. have been isolated from nudibranch cerata [42] and sea anemone [43]. Although *Flavobacteriaceae* are recognised for their ability to decompose organic compounds [44], one of its genera *Tenacibaculum* spp. have been associated with diseased fish [45].

Following inoculation of juvenile *P. ornatus* with prey and/or predator, we found very limited detection of *Halobacteriovorax* in amplicon diversity profiling. This could be attributed to amplification biases of the three pairs of PCR primers or possible removal of the bacteria by host immune response (e.g. prophenoloxidase-activating system, antimicrobial proteins, phagocytosis and clottable proteins [35]) before 24 h post-injection. Furthermore, the taxonomic assignment of OTUs could have been confounded by older classification systems present in genomic databases. For example, marine *Bacteriovorax* (*Bacteriovoracaceae*) have recently been reclassified as *Halobacteriovorax* (*Halobacteriovoracaceae*) [1], inferring that *Bacteriovorax* sequences found in the haemolymph in the present study likely includes marine strains. It is possible also that sequenced *Bacteriovorax* and *Vibrio* found in our microbiomic analyses are part of resident microbiota, given both genera were identified in earlier studies of *P. ornatus* [17].

Nonetheless, there were several measured haemolymph parameters of prey +BALO injected juveniles that were significantly different when compared to other treatment groups. This included exhibiting a significantly lower abundance of *Vibrio* compared to the prey only treatment group, which could indicate that the injected Vib was predated by Hbv. This may be due to a top-down regulation in the haemolymph by predatory Hbv, which we demonstrated also caused a 100-fold decrease in Vib after 2 d in *in vitro* cultures. Taken together, this indicates that BALOs may assist lobsters in controlling bacterial population numbers in the haemolymph. In humans, a lower abundance of intestinal BALOs (e.g. *Bdellovibrio bacteriovorus*) were found to be associated with intestinal diseases when compared to healthy individuals [46]. Indeed, the attraction of BALOs as an alternative to antibiotic treatment lies within their broad prey spectrum activity mediated through attachment to the ubiquitous cell surface lipopolysaccharides of Gram-negative bacteria. This makes it exceedingly difficult for a range of bacterial hosts to develop resistance [2].

As our haemolymph microbiome analyses could indicate an effect on Vib populations by Hbv, the use of BALOs as biocontrol agents of specific lobster pathogens warrants further study. A number of animal models (e.g. worms and zebrafish) using BALOs to control human pathogens have shown promising results [31, 32]. Moreover, BALOs have been administered to culture water to successfully treat pathogens of Pacific white shrimp *L. vannamei* [14, 15, 34, 47], black tiger shrimp *P. monodon* [48], goldfish *Carassius auratus* [8], snakehead fish *Ophiocephalus argus* [13], Eastern oyster *Crassostrea virginica* [38] and through feed in Chinese white shrimp *Fenneropenaeus chinensis* [49]. BALOs may be involved in regulating bacterial populations in the natural environment. For example, *Halobacteriovorax* sp. are known to suppress *Vibrio parahaemolyticus* populations in estuarine waters [50] and regulate coral microbiomes and protect corals against pathogens such as *Vibrio coralliilyticus* and *Vibrio harveyii* [33].

This is the first study to demonstrate that the BALO *Halobacteriovorax* sp. Hbv had no negative effect on *P. ornatus* upon injection into the haemolymph. Animals injected with both predator Hbv and prey *Vibrio* sp. Vib showed significant differences in haemolymph bacterial composition and load compared to other treatment groups. Recommendations for future *in vivo* studies include successfully treating definitive pathogens of juvenile lobsters with BALOs, exploration of different methods of BALO administration, and observation of long-term effects of BALO administration over a range of lobster developmental stages. Such studies will be required to explore the use of BALOs as a treatment option for systemic bacterial diseases in lobsters.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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