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Understanding the role of the shrimp gut microbiome in health and disease

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ABSTRACT

With rapid increases in the global shrimp aquaculture sector, a focus on animal health during production becomes ever more important. Animal productivity is intimately linked to health, and the gut microbiome is becoming increasingly recognised as an important driver of cultivation success. The microbes that colonise the gut, commonly referred to as the gut microbiota or the gut microbiome, interact with their host and contribute to a number of key host processes, including digestion and immunity. Gut microbiome manipulation therefore represents an attractive proposition for aquaculture and has been suggested as a possible alternative to the use of broad-spectrum antibiotics in the management of disease, which is a major limitation of growth in this sector. Microbiota supplementation has also demonstrated positive effects on growth and survival of several different commercial species, including shrimp. Development of appropriate gut supplements, however, requires prior knowledge of the host microbiome. Little is known about the gut microbiota of the aquatic invertebrates, but penaeid shrimp are perhaps more studied than most. Here, we review current knowledge of information reported on the shrimp gut microbiota, highlighting the most frequently observed taxa and emphasizing the dominance of Proteobacteria within this community. We discuss involvement of the microbiome in the regulation of shrimp health and disease and describe how the gut microbiota changes with the introduction of several economically important shrimp pathogens. Finally, we explore evidence of microbiome supplementation and consider its role in the future of penaeid shrimp production.

1. Introduction

Gut-inhabiting microbes are recognised as important drivers of several metabolic processes in the host. As such, the characterisation and subsequent manipulation of this microscopic community is an attractive proposition for aquaculture research. Penaeid shrimp aquaculture is an important source of economic gain for many Asian and Latin American countries (Hernández-Rodríguez et al., 2001) and shrimp research has subsequently dominated the field of marine-based invertebrate gut microbiomes. However, in comparison with mammals and terrestrial invertebrates, relatively very little is known about the bacteria living in the gut of aquatic invertebrates such as penaeid shrimp.

In this review, we summarise gut microbiome sequence data from currently available penaeid shrimp studies that utilise a high-throughput sequencing (HTS) approach, in order to investigate the diversity of gut-associated bacteria in shrimp grown under a range of conditions across the world. Proteobacteria were the dominant phylum in most studies, the vast majority of which have been carried out in China (Fig. 1A) (Table 1). Proteobacteria are widespread in aquatic invertebrate gut microbiotas and are often a dominant component of this community in other Crustacea (Hakim, 2015; Holt et al., 2020; Huang, 2014; Meziti, 2010; Rungrassamee, 2013, 2014; Zhang, 2014). The phylum Proteobacteria is highly diverse in terms of physiology, morphology, and genetics. They are Gram-negative, and most are facultative or obligate anaerobes (Stackebrandt et al., 1988). Gammaproteobacteria, the largest class in the phylum, are often described as the most common bacteria in the gut of giant tiger shrimp (*Penaeus monodon*) (Chaiyapechara et al., 2012; Rungrassamee et al., 2013, 2014, 2016) and Pacific white shrimp (*Litopenaeus vannamei*) (Tzuc et al., 2014; Rungrassamee et al., 2016; Zheng et al., 2017). This class, mainly comprising *Vibrio* and *Photobacterium* spp., has also been

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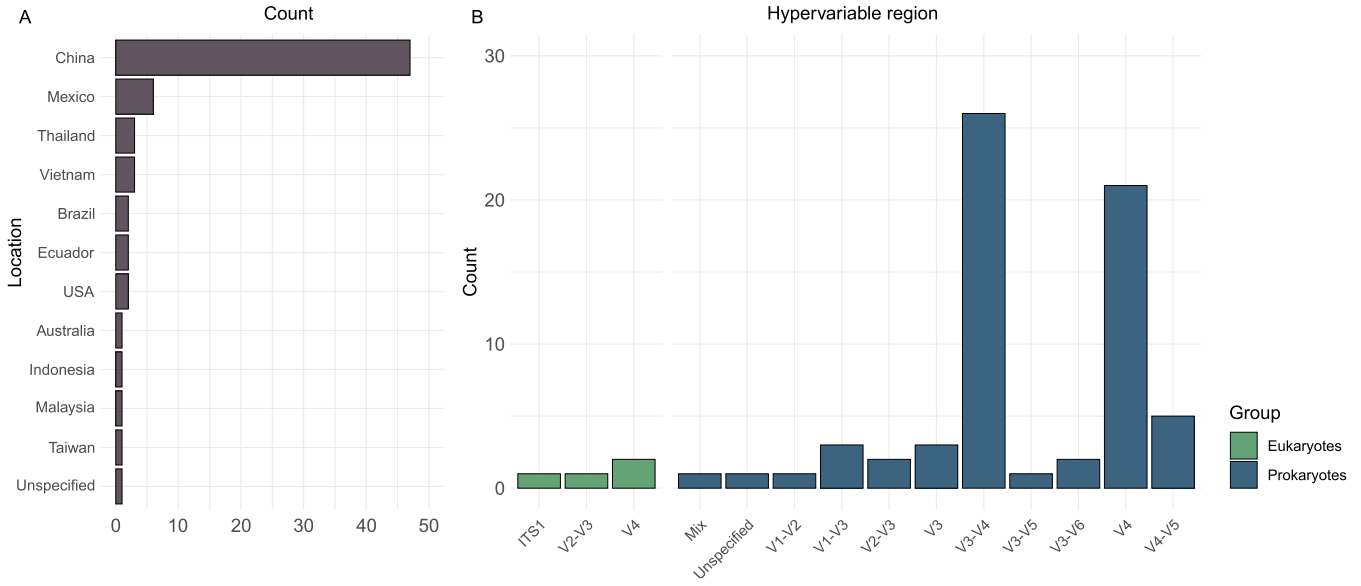

Fig. 1. Summary of high-throughput 16S studies investigating the shrimp gut microbiome. (A) Total number of studies originating from each country. Locations of spatial comparisons are treated as one, unless comparing multiple countries. (B) Ribosomal small subunit hypervariable region/s used in each study. Green bars represent eukaryotic studies. Blue bars represent prokaryotic. Duplicate studies listed in Table 1 removed from both plots.

reported to account for more than 70% of sequences isolated from the guts of wild-caught and domesticated *P. monodon* with the remaining classified sequences attributed to other high-level taxa: Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria (Fig. 2B) (Rungrassamee et al., 2014). Many *Vibrio* spp. produce chitinolytic enzymes (Sugita and Ito, 2006) which may explain their dominance in a chitin-rich environment such as the crustacean gut, providing a niche substrate for their utilisation. However, the enzymatic potential of several *Vibrio* spp. may contribute to negative effects on the carapace of the animal and other health implications, such as tail necrosis, red disease and loose shell syndrome (Liu and Lee, 2002; Jayasree et al., 2006). As such, several *Vibrio* spp. have historically caused large losses to the aquaculture industry, with vibriosis often causing mass mortalities (Lavilla-Pitogo et al., 1998) and seemingly non-pathogenic *Vibrio* having expressed virulence in compromised hosts (Manilal et al., 2010). Despite this, *Vibrio* spp. are often described as the dominant genus within the shrimp gut microbiota and many exist harmoniously with the host. This is an important caveat when considering that therapeutic supplements are often designed to target the *Vibrio* genus.

While the majority of HTS microbiome studies focus on the midgut, or in some cases, an unspecified region of the gut, relatively few describe the community of the foregut and hindgut. The penaeid digestive tract, and the digestive tract of that of all Crustacea, is made up of three sections (Fig. 2A); the foregut, containing the oesophagus and the two chambered stomach; the midgut; which begins at the junction of the hepatopancreas (HP) and traverses the length of the cephalothorax and the majority of the abdomen; and finally the hindgut, containing the rectum and anus. These regions of the gut differ in their cell structure and function (Ceccaldi, 1989). There are few studies describing communities inhabiting the foregut, however Alphaproteobacteria along with Planctomycetales dominated the stomach of healthy Pacific white shrimp (*L. vannamei*) in a study from Vietnam (Chen et al., 2017). Microbial profiles are likely influenced by the longitudinal axis of the gut itself as different morphologies and functions along the gut will induce differential pressures on selection. These internal pressures are perhaps why wild-caught and domesticated *P. monodon* shared similar taxa in the gut despite clear differences in their rearing environment (Rungrassamee et al., 2014) and *L. vannamei* guts from different farms were more similar to each other despite differences in the community structure of their respective rearing waters (Zoqratt et al., 2018). In an earlier study, wild type *L. vannamei* from Mexico were shown to harbour a more diverse bacterial community compared to healthy cultured animals and, unlike *P. monodon*, contained substantial proportions of Cyanobacteria (Fig. 2C) (Cornejo-Granados et al., 2017). The availability and diversity of the diet likely impacts spatial comparisons. Mode and location of feeding may determine the abundance of usable substrate and the subsequent proliferation of microbial taxa within the gut. Furthermore, studies tracking gut composition over development stages have implicated changing in feeding to be the cause of bacterial community changes seen at the family-level throughout development. Although Gammaproteobacteria dominated the gut throughout the different life-stages of *P. monodon* in Thailand (Fig. 2B), there were shifts from a *Photobacterium*-based community to a *Vibrio* –based community between PL and juvenile stages (Rungrassamee et al., 2013). Gammaproteobacteria also dominated the guts of *L. vannamei* at different life-stages in a holding facility in China, with the exception to 2-month old juveniles which mainly harboured Bacteroidetes (Fig. 2C) (Huang et al., 2014). Aquaculture practices, such as indoor- vs pond-based culture can also impact the composition of the microbiome (Landsman et al., 2019a) as can the integration of a multi-trophic aquaculture system, which is also thought to improve productivity (Omont et al., 2020). Overall, the growing wealth of evidence suggests that both environmental and internal, host-associated factors can contribute to the determination of microbial communities and it is often difficult to untangle the direct effects of any one variable.

2. Patterns and processes relating shrimp health to gut microbiota

One of the biggest threats to shrimp aquaculture is the onset of disease and subsequent mortality in cultured stocks (Seibert and Pinto, 2012; Stentiford et al., 2012). Even in cases where the clinical signs of disease are well described, little is known about how the presence of a pathogen may impact or interact with the microbial communities in the gut and subsequently influence the metabolic processes within the host. On the other hand, it is unclear whether changes to the gut microbiome may predispose the gut to invasion by (a) pathogen(s). Changes in gut microbiome structure could also facilitate the progression of enteric pathogens that rely on translocation through the gut epithelia to initiate infection in the target tissue. The notion of a 'one pathogen-one disease' scenario is being increasingly challenged (Dai et al., 2018; Bass et al.,

Table 1
List of papers describing shrimp gut microbiomes using high throughput sequencing. * indicate studies that appear under more than one table subheading. Water and sediment samples are only listed under 'culture environment' comparisons.

Reference	Species	Location	Comparison	Hypervariable region/ Primers	Sequencing Platform	Data Accession
Culture Environment						
Rungrassamee et al., 2014	*Penaeus monodon*	Andaman Sea Surat Thani province, Thailand	Wild Domesticated	V3-V4/338F-518R	454	KF329429–KF334451,KF334452–KF344403,KF344404–KF355928 KF322280–KF325238,KF325239–KF328420,KF328421–KF329428
Oetama et al., 2016	*Penaeus monodon*	Bali Jakarta Bay Pejarakan, Singaraja, Bali	Wild Wild Aquaculture farm	V4/515F-806R	Illumina	SRP059721
Cornejo-Granados et al., 2017	*Litopenaeus vannamei*	Nayarit coast, Mexico Sonora state, Mexico	Wild (healthy and diseased) Cultured (healthy and diseased) Cultured - sediment	V2-4–8 mix, V3-6–7-9 mix/ Unpublished	Ion Torrent	SRR5585664-84. Bioproject: PRJNA387510
Hou et al., 2018a	*Litopenaeus vannamei*	Guangdong Province, China	Water Sediment Shrimp gut	V4/515F-806R	Illumina	SRR5387734
Huang et al., 2018	*Litopenaeus vannamei*	Shandong, China	Middle stage of farming - shrimp gut Middle stage - water Middle stage - sediment Late stage - shrimp gut Late stage - water Late stage - sediment	V4-V5/515F-907R	Illumina	SRP118749
Su et al., 2018*	*Litopenaeus vannamei*	Guangdong Province, China	Mud pond Aquaculture farm Intensive pond	V4/515F-806R	Illumina	SRP129489
Zoqratt et al., 2018	*Litopenaeus vannamei*	Quang Yen, Quang Ninh, Vietnam Sitiawan, Perak, Malaysia	Shrimp gut Water Shrimp gut Water	V3-V4/S-D-Bact-0341-*b*-S-17(F)-S-D-Bact-0785-a-A-21(R)	Illumina	SRP126985. BioProject: PRJNA422950
Deng et al., 2019	*Litopenaeus vannamei*	China	Low stocking density - shrimp gut Low stocking density - water Medium stocking density - shrimp gut Medium stocking density - water High stocking density - shrimp gut High stocking density - water	V3-V4/341F-805R	Illumina	SAMN10462254-SAMN10462265
Fan et al., 2019b	*Litopenaeus vannamei*	Panyu, Guangdong, China	Freshwater cultured - shrimp gut Freshwater cultured - water Marine cultured - shrimp gut Marine cultured - water	V3-V4/338F-806R	Illumina	
Landsman et al., 2019a	*Litopenaeus vannamei*	Minnesota, USA	Indoor-raised Pond-raised Wild-caught	V1-V3/27F-519R	Illumina	SRP185856.Bioproject PRJNA522274
He et al., 2020	*Litopenaeus vannamei*	Haikou, Hainan province, China	'Higher place' culture ponds - shrimp gut Water Effluent	V4-V5/515F-907R	Illumina	SRR9687557-SRR9687559
Huang et al., 2020a	*Litopenaeus vannamei*	Wenzhou, Zhejian Province, China	Shrimp gut Small sized biofloc Medium/large sized biofloc	V4/515FY – 806RB	Illumina	
Omont et al., 2020	*Litopenaeus vannamei*	La Paz, Baja California Sur, Mexico	Shrimp monoculture Shrimp-oyster co-culture	V3/338F-533R	Illumina	PRJNA594718
Growth Stage/Time						

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Table 1 (*continued*)

Reference	Species	Location	Comparison	Hypervariable region/ Primers	Sequencing Platform	Data Accession
Rungrassamee et al., 2013	*Penaeus monodon*	Surat Thani province, Thailand	Postlarvae 15 days Juvenile 1 month Juvenile 2 months Juvenile 3 months	V3-V6/338F-786R	454	JX919344-JX926388 JX916289-JX919343 JX926389-JX939518 JX939519-JX941408
Huang et al., 2014	*Litopenaeus vannamei*	Xiamen, Fujian Province, China	Postlarvae 14 days Juvenile 1 Juvenile 2 Juvenile 3	V3-V5/338F-907R	454	BioProject:PRJNA248559
		Field pond, Zhangzhou, Fujian Province, China	Pond 1 Pond 7			
Zeng et al., 2017	*Litopenaeus vannamei*	Maoming, Guangdong, China	1 (15 dph) 2 (30 dph) 3 (45 dph) 4 (60 dph) 5 (75 dph)	V4/515F-806R	Illumina	SRX2946975
Zheng et al., 2017*	*Litopenaeus vannamei*	Hainan, China	Zoea 1 Zoea 3 Mysis 1 Mysis 3 Postlarva 1 Postlarva 6	V3-V6/341F-1073R	454	SRP080243
Xiong et al., 2017a	*Litopenaeus vannamei*	Ningbo, China	Larvae Postlarvae Juvenile Preadult Adult	V3-V4/341F-806R	Illumina	DRA005256
Gainza et al., 2018	*Litopenaeus vannamei*	El Oro, Ecuador	Nursery Harvest	V2-V3/341F-518R	Ion Torrent	BioProject: PRJNA352369
Su et al., 2018*	*Litopenaeus vannamei*	Guangdong Province, China	Juvenile Adult	V4/515F-806R	Illumina	SRP129489
Xue et al., 2018*	*Litopenaeus vannamei*	Guangdong Province, China	Nauplii 5 Zoea 2 Mysis 1 Postlarvae 1	V4/515F-806R	Illumina	CRA000198
Liu et al., 2019	*Litopenaeus vannamei*	Zhejiang Province, China	ZT strain day 5 ZT strain day 15 ZT strain day 20 ZT strain day 40 ZT strain day 60 ZT strain day 75 ZT strain day 90 ZT strain day 105 PM strain day 5 PM strain day 15 PM strain day 20 PM strain day 40 PM strain day 60 PM strain day 75 PM strain day 90 PM strain day 105	V4/515F_Y-806R_B	Illumina	SRP150920

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Table 1 (*continued*)

Reference	Species	Location	Comparison	Hypervariable region/ Primers	Sequencing Platform	Data Accession
Xiong et al., 2019	*Litopenaeus vannamei*	Xianhshan, Ningbo, China	Larvae Juvenile Adult	V3-V4/341F-806R	Illumina	DRA007714
		Zhannqi, Ningbo, China	Larvae Juvenile Adult			
Garibay-Valdez et al., 2020	*Litopenaeus vannamei*	Sonora, Mexico	Farm Day 0 Day 20 Day 40 Day 60 Day 80	V4/515F-806R	Illumina	
DIET						
Zhang et al., 2014	*Litopenaeus vannamei*	Shenzhen, China	Soybean oil (Diet) Beef tallow Linseed Oil Fish Oil SBL SBF	V4-V5/515F-907R	Illumina	PRJNA253075
Qiao et al., 2017	*Litopenaeus vannamei*	Shenzhen, China	Glucose Sucrose Corn starch	V4-V5/515F-907R	Illumina	PRJNA291010
Niu et al., 2018	*Litopenaeus vannamei*	Quanzhou, China	Control diet 1% *Porphyra haitanensis* 2% 3% 4% 5% 6%	V4/515F-806R	Illumina	
Cheng et al., 2019	*Litopenaeus vannamei*	Pingtung, Taiwan	Control diet *Bacillus subtilis* E20-fermented soybean meal Antimicrobial peptide isolated from *B. subtilis* E20-FSBM	V3-V4/S17-A21	Illumina	
Fan et al., 2019a	*Litopenaeus vannamei*	Shan-Wei, China	Fishmeal (Week 1) Fishmeal (Week 2) Fishmeal (Week 3) Fishmeal (Week 4) Fishmeal (Week 5) Fishmeal (Week 6) Fishmeal (Week 7) Fishmeal (Week 8) Krill meal (Week 1) Krill meal (Week 2) Krill meal (Week 3) Krill meal (Week 4) Krill meal (Week 5) Krill meal (Week 6) Krill meal (Week 7) Krill meal (Week 8)	V3-V4/	Illumina	SRP136220

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Table 1 (*continued*)

Reference	Species	Location	Comparison	Hypervariable region/ Primers	Sequencing Platform	Data Accession
Shao et al., 2019	*Litopenaeus vannamei*	Qingdao, China	Control fishmeal diet 10% fermented soybean meal (FSM) 20% FSM 30% FSM 40% FSM	V3-V4/338F-806R	Illumina	SRP128484
Yuan et al. 2019	*Litopenaeus vannamei*	China	No supplemented copper Copper sulphate Copper amino acid complex from Availa®Cu100 1:1 copper sulphate + copper amino acid complex	V3-V4/338F-806R	Illumina	PRJNA417739
Elizondo-González et al., 2020	*Penaeus vannamei* syn. *Litopenaeus vannamei*	La Paz, Baja California Sur, Mexico	Control diet (P) P + *Ulva lactuca* P + *Ulva clathrata* *Ulva lactuca* *Ulva clathrata*	V4/515F-806R	Illumina	PRJNA417739
Schleder et al., 2020	*Litopenaeus vannamei*	Santa Catarina, Brazil	Control diet 0.5% *Sargassum filipendula*: 1% *Undaria pinnatifida* 0.5S:2U 0.5S:4U	V3-V4/314F-806R	Illumina	
Simon et al., 2020	*Litopenaeus vannamei*	Australia	Control diet (Fishmeal) Novacq™ Krill meal Krill hydrolysate Whole squid	V1-V3/27F-519R	Illumina	
Health/Disease						
Xiong et al., 2015	*Litopenaeus vannamei*	Zhanqum Ningbo, China	Black intestine (Healthy) Red intestine (Sub-healthy) Empty intestine (Diseased)	V4/515F-816R	Illumina	DRA002398
Rungrassamee et al., 2016	*Penaeus monodon*	Shrimp Biotechnology Business Unit (SBBU), Thailand	0 h post exposure 6HPE 12HPE 24HPE 48HPE 72HPE	V3-V4/338F-786R	454	KP944208-KP944681 KP948364-KP948529 KP944682-KP946571 KP946572-KP946691 KP946692-KP948363 KP948530-KP948831
	Litopenaeus vannamei		0HPE 6HPE 12HPE 24HPE 48HPE 72HPE			KP948832-KP951735 KP953299-KP953763 KP951736-KP952247 KP952248-KP952978 KP952979-KP953298 KP953764-KP953903
Chen et al., 2017	*Litopenaeus vannamei*	Ben Tre Province, Vietnam	AHPND - HP AHPND + HP	V3-V4/S17-A21	Illumina	SRP102384
Dai et al., 2017	*Litopenaeus vannamei*	Xiangshan, Ningbo, China	Normal Retarded Overgrown	V2-V3/18S_F82-Euk_R516	Illumina	DRA005322.
Xiong et al., 2017b	*Litopenaeus vannamei*	Xiangshan, Ningbo, China	Normal Retarded Overgrown Water	V3-V4/338F-806R	Illumina	DRA005153
Zheng et al., 2017*	*Litopenaeus vannamei*	Hainan, China	Healthy Diseased	V3-V6/341F-1073R	454	SRP080243

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Table 1 (*continued*)

Reference	Species	Location	Comparison	Hypervariable region/ Primers	Sequencing Platform	Data Accession
Dai et al., 2018	*Litopenaeus vannamei*	Ningbo, China	Healthy – Disease emergence (July 1st) Diseased – Disease emergence (July 1st) Healthy – Disease exacerbation (July 4th) Diseased – Disease exacerbation (July 4th) Healthy – Disease exacerbation (July 10th) Diseased – Disease exacerbation (July 10th)	V3-V4/341F-806R	Illumina	DRA005997
Hou et al., 2018b	*Litopenaeus vannamei*	Guangzhou, China	WFS + Healthy	V4/515F-806R	Illumina	SRR6286523
Le et al., 2018	*Penaeus monodon*	Dong Hai district, Bac Lieu province, Vietnam	Asymptomatic gut Symptomatic gut	V3-V4/338F-806R	Illumina	SAMN06062067- SAMN06062068
Pilotto et al., 2018	*Litopenaeus vannamei*	Florianópolis, Brazil	Healthy Biofloc Healthy Clear seawater WSSV + Biofloc WSSV - Clear seawater	V3-V4/341F-806R	Illumina	
Xiong et al., 2018a	*Litopenaeus vannamei*	Ningbo, China	Healthy larvae Healthy juveniles Healthy adults Diseased adults	V3-V4/341F-806R	Illumina	DRA005782
Xiong et al., 2018b	*Litopenaeus vannamei*	Zhanqi, Ningo, China	Healthy postlarvae Healthy juveniles Healthy adults Disease emergence Diseased exacerbation	V4/3NDf-V4_Euk_R2	Illumina	DRA005998
Yao et al., 2018	*Litopenaeus vannamei*	Ningbo, China	Healthy (sampled at 70 days) Healthy 80 days Healthy 85 days Diseased 70 days Diseased 80 days Diseased 85 days	V3-V4/338F-806R	Illumina	SRP131736
Dai et al., 2019	*Litopenaeus vannamei*	Ningbo, China	Pre-WFS Pre-Healthy WFS Healthy	V4/3NDf-V4_Euk_R2	Illumina	
(Li et al., 2019)	*Litopenaeus vannamei*	Guangdong, China	White faeces Black gill Retarded growth Healthy	ITS1/ITS1F-ITS2	Illumina	PRJNA495902
Wang et al., 2019	*Litopenaeus vannamei*	Maoming, Guangdong Province, China	Control WSSV +	V4/515F-806R	Illumina	SRP145560
Zhou et al., 2019	*Litopenaeus vannamei*	Wenchang, Hainan, China	Healthy Diseased	V3-V4/338F-806R	Illumina	SRP192810
Dai et al., 2020	*Litopenaeus vannamei*	Ningbo, China	Healthy 84 days Diseased 84 days Healthy 87 days Diseased 87 days Healthy 93 days Diseased 93 days	V3-V4/341F-806R	Illumina	DRA005256

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Table 1 (*continued*)

Reference	Species	Location	Comparison	Hypervariable region/ Primers	Sequencing Platform	Data Accession
Huang et al., 2020b	*Litopenaeus vannamei*	Zhuhai, Guangdong, China	Healthy WFS Healthy + PBS transplant Healthy + WFS transplant Healthy + PBS transplant 2 Healthy + WFS transplant 2	V4/515F-806R	Illumina	PRJNA542015
Liang et al., 2020	*Penaeus vannamei* syn. *Litopenaeus vannamei*	Guangdong Province, China	Big General Small Blue	V3-V4/341F-806R	Illumina	
Supplementation						
Sha et al., 2016	*Litopenaeus vannamei*	Qingdao, China	Basal diet *Lactobacillus pentosus* *Enterococcus faecium* *Lactobacillus pentosus* supernatant	V1-V2/8F-338R	Illumina	SRP071046
Duan et al., 2017	*Litopenaeus vannamei*	Shenzhen, China	Control diet 1% dietry poly-β-hydroxybutyrate (PHB) 3% PHB 5% PHB	V4/515F-806R	Illumina	
He et al., 2017	*Litopenaeus vannamei*	Xiamen, China	Control AviPlus ® 0.6 g/kg AviPlus ® 1.2 g/kg		Illumina	
Vargas-Albores et al., 2017	*Litopenaeus vannamei*	Empalme, Sonora, Mexico	Control 30 day Control day Eco-AQUAPROTEC 30 day Eco-AQUAPROTEC 60 day	V3-V4/341F-805R	Illumina	https://www.dropbox.com/home/Helgoland%20Marine%20Research
Liu et al., 2018a	*Litopenaeus vannamei*	Zhanqi, Ningbo, China	Control Microbial agent treatment	V4/515F-806R	Illumina	SRR3944126
Landsman et al., 2019b	*Litopenaeus vannamei*	Minnesota, USA	Shrimp Improvement Systems (genetic line). 43 days pre-treatment control SIS.57 days + BioWish 3P SIS.57 days SIS.71 days + BioWish 3P SIS.71 days Oceanic Institute (genetic line). 43 days pre-treatment control OI.57 days + BioWish 3P OI.57 days OI.71 days + BioWish 3P OI.71 days	V1-V3/27F-519R	Illumina	
Mazón-Suástegui et al., 2019	*Litopenaeus vannamei*		RL8 (Strep) Lac-Strep Bac-Strep Control	V3/338F-533R	Illumina	
Xie et al., 2019	*Litopenaeus vannamei*	Sanya, Hainan province, China.	Control diet 1000 mg/kg probiotics 2000 mg/kg 3000 mg/kg 4000 mg/kg 8000 mg/kg	V4/515F-806R	Illumina	
Gainza and Romero, 2020	*Litopenaeus vannamei*	Huaquillas, El Oro Province, Ecuador	Control diet Mannan oligosaccharides	V2-V3/341F-519R	Ion Torrent	PRJNA352369

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Table 1 (*continued*)

Reference	Species	Location	Comparison	Hypervariable region/ Primers	Sequencing Platform	Data Accession
Stress						
Suo et al., 2017	*Litopenaeus vannamei*	Shenzhen, China	0 μg/L sulphide 425.5 μg/L sulphide 851 μg/L sulphide	V4-V5/	Illumina	SRP091598
Duan et al., 2018	*Litopenaeus vannamei*	China	Control group Ammonia stress Nitrite stress	V4/515F-806R	Illumina	
Duan et al., 2019a	*Litopenaeus vannamei*	Shenzhen, China	Control group Low pH stress High pH stress	V4/515F-806R	Illumina	
Duan et al., 2019b	*Litopenaeus vannamei*	Shenzhen, China	Control group Sulfide stress group	V4/515F-806R	Illumina	
Jiang et al., 2019	*Litopenaeus vannamei*	Yangjiang, Guangdong Province, China	Control group Ammonia stress Sulfide stress Co-stress	V3-V4/341F-806R	Illumina	
Yu et al., 2020	*Litopenaeus vannamei*	Hainan, China	pH 6.5 pH 8.0 pH 9.5	V3-V4/339F-806R	Illumina	PRJNA578594
Qian et al., 2020	*Litopenaeus vannamei*	Wenchang, Hainan, China	0 mg/L Cu exposure 0.1 mg/L Cu exposure 1 mg/L Cu exposure	V3-V4/338F-806R	Illumina	PRJNA596585
Library preparation						
Xue et al., 2018*	*Litopenaeus vannamei*	Guangdong Province, China	Bacterial DNA kit Mollusc DNA kit Stool DNA kit Tissue DNA kit	V4/515F-806R	Illumina	CRA000198
García-López et al., 2020	*Litopenaeus vannamei*	Sinaloa, Mexico	V3 16S rRNA hypervariable region V3V4 16S rRNA hypervariable region V4 16S rRNA hypervariable region	V3/338F-533R V3-V4/341F-806R V4/515F-805r	Illumina	

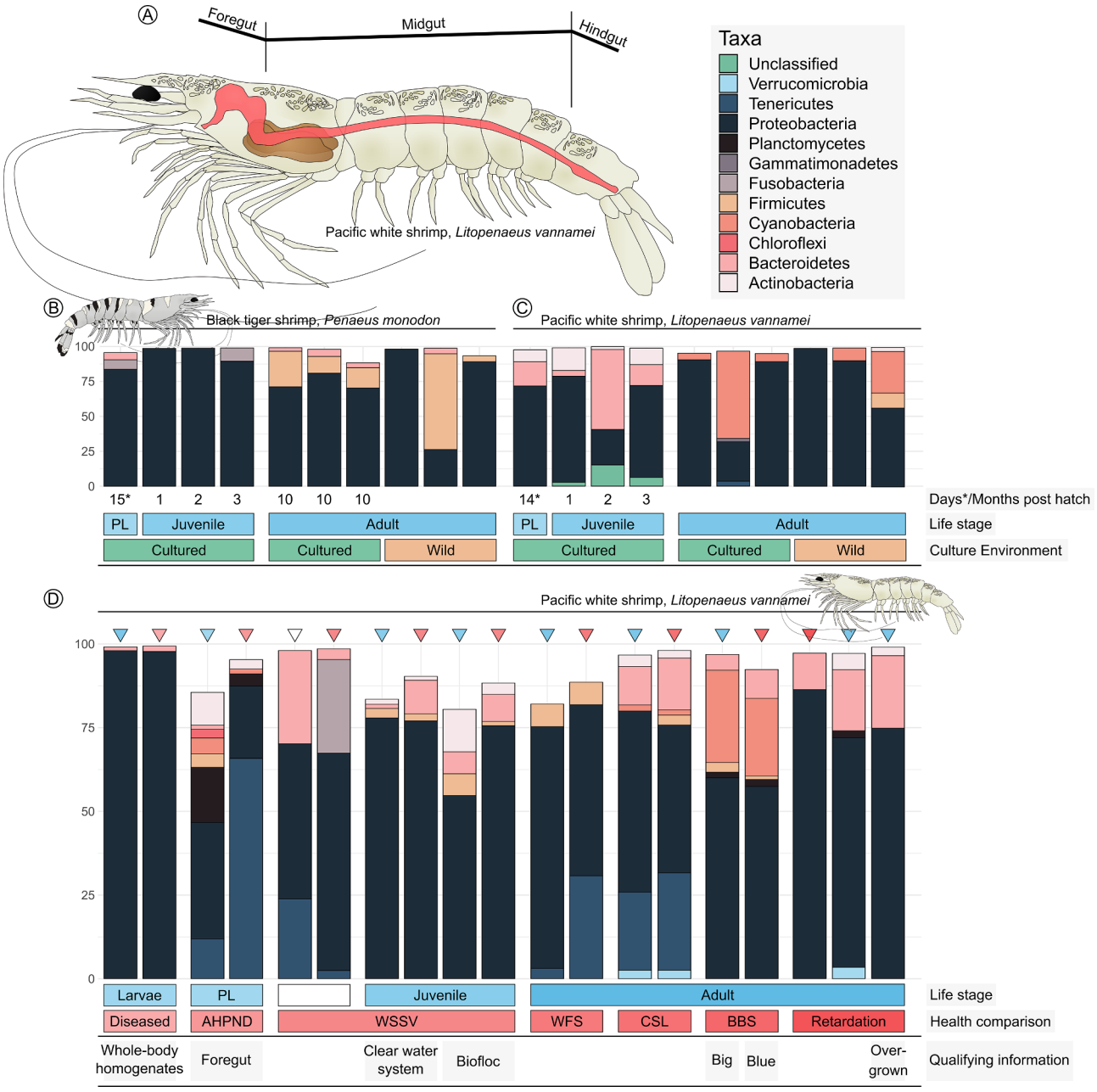

Fig. 2. Overview of the penaeid shrimp gut microbiome in relation to disease, life stage and culture environment. (A) Visual mapping of the tripartite digestive tract. (B) Bacterial gut profiles of black tiger shrimp (*Penaeus monodon*) at increasing life stages (Rungrassamee et al. 2013) and different culture environments (Rungrassamee et al. 2014). (C) Bacterial gut profiles of Pacific white shrimp (*Litopenaeus vannamei*) at increasing life stages (Huang et al., 2014) and different culture environments (Cornejo-Granados et al., 2017). (D) Major bacterial phyla associated with gut changes in Pacific white shrimp (Litopenaeus vannamei) during pathogenesis; including diseased larvae from China (Zheng et al. 2017), Acute Hepatopancreatic Necrosis Disease (AHPND) infected postlarvae (PL) form Vietnam (Chen et al. 2017), White Spot Syndrome Virus (WSSV) infected shrimp from China (Wang et al. 2019), WSSV juveniles in clearwater and Biofloc systems (Pilotto et al. 2018), adults showing symptoms of White Faeces Syndrome (WFS) (Hou et al., 2018), adults with 'cotton shrimp'-like disease (CSL) (Zhou et al. 2019), adults with blue body syndrome (BBS) (Liang et al., 2020) and growth retarded adults from China (Xiong et al. 2017a). Blue triangle accompanying profile indicate healthy animals. Pink triangle indicates corresponding disease. Relative abundance values are taken from studies cited in text. When relative abundances were not stated in manuscript, corresponding bars in original figures were measured as a percentage of the axis scale.

2019; Huang et al. 2020b). The 'pathobiome' concept argues that the interactions between free-living microbes in the environment, host-associated symbionts (including the gut microbiota) and the host itself likely drive both beneficial and detrimental impacts on host health (Bass et al., 2019).

In humans, changes to the gut microbiota have been implicated in a wide range of health conditions. Characterisation of the interplay between the microbiota and the host immune system is becoming increasingly well-defined (Sekirov et al., 2010). Pattern recognition receptors (PRRs) such as Toll-like receptors on the surface of the gut epithelia are in close proximity to microbial associated molecular patterns (MAMPs) of the microbiota such as lipopolysaccharides (Chu and Mazmanian, 2014). Although there are key differences between the vertebrate and invertebrate immune system, the gut microbiota likely has important roles to play in maintaining the health of the shrimp. The presence alone of symbiotic microbiota could itself provide a kind of immunity. A general theory true of all hosts is that space and resources within the gut are ultimately finite and colonisation resistance may

limit the proliferation of pathogenic organisms through competitive exclusion (Lawley and Walker, 2013). Furthermore, colonisation resistance may be further supported through microbiota-derived antimicrobial compounds, which may limit the establishment and proliferation of transient microbes in the digestive tract (Kobayashi and Ishibashi, 1993). A more species-diverse microbiota in the gut may facilitate resistance to a greater degree of potentially problematic colonisers, as there is consequently a larger set of species-species antagonisms. Reducing the abundance of certain bacterial classes within the microbiota can allow previously symbiotic species to become pathogenic (Blumberg and Powrie, 2016).

Because of the links between the gut microbiota and the host immune system, it is often suggested that a reduction in bacterial diversity within the gut or the differential abundance of particular microbial taxa may be responsible for the onset of pathogenesis. However, without follow-up studies involving gut supplementation and/or gnotobiotic organisms (germ free animals and/or organisms that harbour a defined microbial community) it is often impossible to discern between cause and effect. Nevertheless, these correlations should not be dismissed without merit and several studies have described such correlations in shrimp under the affliction of important pathogens which cause massive economic loss (Table 1, Fig. 2D).

3. Changes to the gut microbiome can correlate with the incidence of disease

The following section summarises what is known of the microbiome in relation to key diseases which can impact production. Although we have collated these studies in Fig. 2, it is important to recognise that these samples were analysed independently of each other within their original studies. Therefore, differences in methodologies and/or analyses (for example in the DNA extraction method, the region of the genes sequenced and the bioinformatics approaches used) may in turn bias comparisons between microbiomes associated with different disease studies (Cornejo-Granados et al., 2018; Xue et al. 2018; García-López et al., 2020). The majority of studies sequencing the bacterial gut microbiota utilise the V3-V4 amplicon (Fig. 1B). The V3-V4 amplicon targeting the shrimp gut microbiota produced a larger number of operational taxonomic units (OTUs) compared to V3 and V4 alone (García-López et al., 2020). However, the V4 region, which is the second most used 16S region, is much less variable in sequence length (García-López et al., 2020) and is sometimes preferred on this basis to V3 or both regions together. Unfortunately, short read data were not accessible for all studies shown in Fig. 2 when we attempted a *meta*-analysis to directly compare the results of all health studies.

3.1. Clinical signs of disease in penaeids

In 'diseased' Pacific white shrimp raised in a commercial hatchery in Hainan, China, and characterised by poor growth, inactivity, lack of appetite, empty digestive tracts and/or low survival rate, there were no significant differences in the microbiota when compared to healthy individuals up to and including 18 days post-larvae (Fig. 2D) (Zheng et al., 2017). However, Linear Discriminate Analysis (LDA) Effect Size (LEFSe) highlighted several taxa that were indicative of the disease state (Zheng et al., 2017). Species of the *Nautella* genus (Rhodobacteraceae), which can be pathogenic to algae and brine shrimp (Gardiner, Thomas and Egan, 2015; Zheng et al., 2016) showed the greatest association with diseased individuals and the water in which they were reared. Unlike the shrimp samples themselves, water from healthy and diseased ponds formed distinct clusters when ordinated with non-metric multidimensional scaling (NMDS), therefore environmental DNA (eDNA) assessment of the microbiome within the rearing environment may be a useful indicator of disease in the cultivar. Due to size restrictions, these data were based on whole-body homogenates, however external tissues were cleaned prior to extraction in a bid to remove adherent microorganisms (Zheng et al. 2017).

3.2. Acute hepatopancreatic necrosis disease (AHPND)

Sometimes referred to as Early Mortality Syndrome (EMS), AHPND has been responsible for large production losses of cultured shrimp. The disease results in atrophy of the HP and ultimately necrosis of the HP tubules, and is caused by plasmid-borne toxin-producing genes carried by several species of *Vibrio*, resulting in the production of *Photorhabdus* insect-related (Pir) binary toxins (Lee et al., 2015; Liu et al., 2018b; Restrepo et al., 2018). The incidence of AHPND in *L. vannamei* corresponded to a significant reduction in bacterial diversity of the HP compared to that of healthy individuals (Fig. 2D), with those infected with AHPND showing a reduction in diversity of over 53% within 7 days. Several *Vibrio* clusters were associated with AHPND positive individuals, along with a high abundance of '*Candidatus* Bacilloplasma'-like sequences. By analysing interaction networks within the community, it is suggested that different commensal '*Candidatus* Bacilloplasma' OTUs, which are found in several aquatic invertebrates, interact with the pathogenic *Vibrio* strains and either enhance or inhibit infection (Chen et al., 2017).

3.3. White spot syndrome virus (WSSV)

White spot syndrome virus is the biggest threat to shrimp health worldwide (Stentiford et al., 2009). The double-stranded DNA (dsDNA) virus infects nuclei of mesodermal- and/or ectodermal-derived tissues and results in lethargy of the infected host and a reduction in food intake (Pradeep and Rai, 2012). Although predominantly infecting shrimp, its severe pathogenesis results in a reduction in growth and ultimately high mortality rates in a wide range of cultured species (Stentiford et al., 2009; Bateman et al., 2012). The gut microbiota of *L. vannamei,* obtained from a farm in Maoming, China, was recently shown to be significantly altered in association with WSSV infection (Wang et al., 2019). Individuals infected with WSSV saw a significant increase in Proteobacteria and Fusobacteria in the gut, including potentially pathogenic bacteria belonging to the *Arcobacter* genus, together with a reduction in Bacteroidetes and Tenericutes (Fig. 2D). Despite changes in relative abundances of particular phyla, there was no change in overall bacterial OTU richness and/or diversity of the gut reported in animals infected with WSSV (Wang et al., 2019). It would seem that compositional changes in response to WSSV infection are also impacted by environmental factors in relation to culture environment, which might obscure microbiome changes specifically associated with the disease and/or presence of the virus. When comparing clear seawater and biofloc systems before and after WSSV infection, there were inconsistent changes in phyla abundance and diversity (Pilotto et al., 2018). Furthermore, although Proteobacteria did increase after WSSV challenge in the biofloc system, a decrease in Bacteroidetes was not observed in either culture condition, indicating a degree of disparity between both studies. Evidence suggests that the gut microbiota of shrimp raised in a variably-sized biofloc system have similar bacterial communities to those of only medium-large sized biofloc (Huang et al., 2020a) therefore, the presence of a biofloc could alter any microbiome-mediated resistance to WSSV infection.

3.4. White faeces syndrome (WFS)

White faeces syndrome, characterised by white-golden gut contents and white faecal strings, is a syndromic condition of unknown aetiology. WFS was initially thought to be linked to the presence of the microsporidian *Enterocytozoon hepatopenaei*. Although PCR and *in situ* hybridization has since demonstrated that ponds with high levels of environmental (i.e. host-independent) *E. hepatopenaei* signal often lack characteristic symptoms of the disease in the corresponding stocks (Tangprasittipap et al., 2013), it is also true that white faeces can

contain densely packed *E. hepatopenaei* spores (Tang et al., 2016). Gregarine-like vermiform bodies are also associated with characterisic signs of WFS, through the transformation, sloughing and aggregation of microvilli within the hepatopancreas (Sriurairatana et al., 2014). The cause of this phenomenon is unknown, however it would seem that white faeces is a common characteristic of multiple health conditions, and that EHP may be a necessary but insufficient cause of WFS, at least in some manifestations . When comparing bacterial gut profiles of WFS infected shrimp and asymptomatic individuals, there was an increase in '*Candidatus* Bacilloplasma' (Tenericutes) and *Phascolarctobacterium* (Firmicutes) along with a decrease in *Paracoccus* (Proteobacteria) and *Lactococcus* spp. (Firmicutes), which correlated with a significant reduction in overall diversity of the bacterial community (Fig. 2D) (Hou et al., 2018b). '*Candidatus* Bacilloplasma' is commonly found in the shrimp gut. Considering how well adapted this genus is for living in the gut environment (Kostanjšek et al., 2007), its increased relative abundance in diseased individuals is likely a consequence of the reduction in other taxa, and overall diversity of the gut microbiota. An increase in '*Candidatus* Bacilloplasma' and a reduction in overall richness and diversity in WFS-infected guts has also been confirmed elsewhere, where the probability of disease could be estimated with 99.4% diagnostic accuracy using disease-discriminatory taxa in the gut (Huang et al., 2020b). Furthermore, this study demonstrated that 36.7% of healthy shrimp that received intestinal microbiota transplants (IMTs) from WFS-infected donors eventually became infected with the disease. Conversely, WFS-infected shrimp receiving IMTs from healthy donors recovered from the disease (Huang, et al. 2020b).

White faeces was also associated with changes to the eukaryotic gut community but with somewhat contradicting results. Li et al. (2019) noted Ascomycota and Basidiomycota were abundant in healthy and diseased individuals with an increase in pathogenic *Candida* spp. in individuals exhibiting clinical signs of WFS. Dai et al. (2019) reported an overrepresentation of Ascomycota and Basidiomycota in WFS-infected individuals. Both studies also reported significant differences in non-host eukaryotic (Shannon) diversity associated with WFS.

3.5. 'Cotton shrimp-like' disease (CSL)

Despite no differences when comparing bacterial diversity, estimates of species richness were significantly increased in individuals suffering with a disease referred to as cotton shrimp-like disease, herein referred to as CSL (Zhou et al., 2019). The clinical signs of this disease include reduced growth, associated with atrophy of the HP and an empty digestive tract, inactivity and a soft shell with slightly white, opaque muscle (a definitive characteristic of cotton shrimp disease) (Zhou et al., 2019). The authors note that an increase in *Tenacibaculum* was associated with CSL, along with the presence of Rickettsiaceae, however at very low abundance (~0.03%) (Zhou et al., 2019). Despite a shared clinical sign (white, opaque muscle) with cotton-shrimp disease, CSL is of unknown aetiology, unlike cotton shrimp disease which is primary associated with the presence of several microsporidian genera: *Pleistophora, Thelohania, Perezia, Agmasoma* and *Ameson* (Sprague and Couch, 1971; Overtsreet, 1973; Lightner 1996; Ramasamy et al., 2001; Sokolva et al., 2015; Han et al., 2016). The gut microbiota at the phylum level was reported to be very similar when comparing healthy and CSL-infected individuals (Fig. 2D) (Zhou et al., 2019). However, interspecies interaction was substantially reduced in gut bacterial community networks associated with the disease (Zhou et al., 2019).

3.6. Blue body syndrome (BBS)

Characterised by a blue colouration of the body and internal tissues, 'blue body syndrome' (BBS) or 'blue body disease', reportedly occurs accompanied with slow growth, reduced or no feed intake and thin bodies Liang et al., 2020). The blue shell is the result of low levels of carotenoid astazanthin, a reddish pigment found in several animals (Baticados, 1990), and therefore a microbiota-dependent dietary deficiency is a valid mechanism to explore. Healthy shrimp express more penaeidin, lectin and defensins1 compared to those with BBS. However, no significant and/or substantial differences in gut community composition or alpha diversity were observed when comparing healthy and BBS-positive individuals (Fig. 2D) (Liang et al., 2020). On the contrary, NMDS indicated a significant dissimilarity between the gut microbiota of healthy and BBS individuals, which were more similar to bacterial communities in the water (Liang et al., 2020), perhaps indicating a reduction in the environmental filtering capacity of the infected host.

3.7. Nutritional acquisition and slow growth

The bacterial gut microbiome can impact the growth of the shrimp through the modification of digestive enzyme activity. After rearing larval *L. vannamei* for 70 days in ponds located in Xiangshan, China, body size and weight significantly and positively correlated with amylase, pepsin and lipase activity (Xiong et al., 2017b). Structural equation modelling (SEM) demonstrated how gut community composition of both bacteria and eukaryotes accounted for significant positive effects on enzymatic activity (Dai et al., 2017; Xiong et al., 2017b). Bacterial diversity was significantly reduced in retarded shrimp as the relative abundance of Gammaproteobacteria dramatically increased (Fig. 2D) (Xiong et al., 2017b). Retarded shrimp also harboured less phylogenetically clustered gut communities compared to normal individuals, indicating a reduction in host determinism in the assemblage of bacterial gut communities (Xiong et al., 2017b).

Gut microbiotas are repeatedly noted to be distinct from the bacterial communities of their rearing waters (Harris 1993; Meziti et al., 2012; Xiong et al., 2015; Zhang et al., 2016). This may be explained by deterministic processes, such as environmental filtering, in the colonisation of the shrimp gut during early life stages (Xiong et al., 2017a; Xiong et al., 2018a). The onset of disease, however, can cause compositional shifts to atypical microbiota, often referred to as dysbiosis (Xiong et al., 2015; Zhu et al., 2016; Xiong et al., 2017a; Xiong et al., 2018a). The emergence of disease may also correlate with a reduction in deterministic processes that influence microbiota composition and a more stochastic assembly of gut colonisers (Zhu et al., 2016; Xiong et al., 2017a). Therefore a dysbiosis may indicate (or precede) the presence of a disease (Zhu et al., 2016). Furthermore, considering healthy, sub-healthy, and diseased *L. vannamei*, based on characteristic gross pathology of the gut, the severity of disease correlated with the degree of dysbiosis, and the onset of disease can be modelled based on the composition of the gut microbiota (Xiong et al., 2015; Xiong et al., 2017a). Specifically, the shift in foregut microbiota associated with AHPND was hypothesised to be a result of increased inability of the shrimp to select gut bacteria (a deterministic process) thus increasing the role of stochastic processes shaping gut microbiota assembly (Chen et al., 2017). Furthermore, gut profiles of shrimp challenged with *Vibrio harveyi* showed a lower degree of similarity (20–40% similar DGGE profiles) compared to the uninfected, control group (80% similarity) (Rungrassamee et al., 2016), which we suggest may be the result of a shift to more stochastic determination of the gut flora post-challenge. This shift in ecological processes is not only limited to bacterial community assembly; the eukaryotic microbiota of WFS-infected shrimp showed more stochastic assembly compared to healthy individuals (Dai et al. 2019). We hypothesise that early stochastic outcomes could result in variation in microbiotas between members of a shrimp population which then predispose certain individuals to pathogenesis; a phenomenon which could help explain variations in disease susceptibilities within a population.

4. Improving shrimp production with gut supplementation

In light of the disease-associated compositional changes described in

the studies cited above, it is perhaps unsurprising that manipulating the gut microbiota has been shown to produce a number of positive effects on the shrimp host. The addition of live, beneficial microorganisms (probiotics) have been explored in a range of farmed animals for decades and is now becoming commonplace in shrimp aquaculture. Probiotic supplementation can increase competition in the gut, potentially supporting colonisation resistance against pathogenic microbes (Farzanfar, 2006). Furthermore, supplemental bacteria can directly affect and antagonise pathogens. *Streptomyces* spp., for example, have demonstrated a protective effect in *Artemia*, *P. monodon* and *L. vannamei* when challenged with pathogenic *Vibrio* strains, with an increase in survival reported for all three shrimp species (Das et al., 2010; Augustine et al., 2016; García Bernal et al., 2017; Mazón-Suástegui et al., 2019). Notably, the addition of *Streptomyces* sp. RL8 alone and a combination of *Streptomyces* and *Bacillus* spp. led to an increase in bacterial diversity in the guts of *L. vannamei* and also increased the abundance of antimicrobial-producing gut bacteria (Mazón-Suástegui et al., 2019). Isolation of lactic acid bacteria from wild shrimp guts enabled experiments showing that application of *Lactobacillus plantarum* MRO3.12 can also cause a reduction of *V. harveyi*, a common cause of shrimp mortalities. Shrimp supplemented with *L. plantarum* in their diet showed a significant increase in growth and survival rates, along with an increased abundance of haemocytes and a reduction of *V. harveyi* in the haemolymph (Kongnum and Hongpattarakere, 2012). Infection with *V. harveyi* has also shown to alter the intestinal bacterial profiles of both *P. monodon* and *L. vannamei*. Interestingly, the altered profiles of infected *L. vannamei* reverted back to that of a healthy animal after 72 h post infection. This was not observed with infected *P. monodon*. The ability to regain intestinal normality was noted as a possible explanation for the greater survival rate of *L. vannamei* infected with *V. harveyi* (Rungrassamee et al., 2016).

There is now a range of probiotic complexes that are marketed to the farming industry, however application of general combinations may not be beneficial to the host (Liu et al., 2018b; Landsman et al., 2019b). Firstly, probiotics must be able to survive passage through the gut. Common probiotic mixtures used in shrimp aquaculture often contain bacterial species that are not indigenous to the marine environment and subsequently have limited proliferation potential (Vargas-Albores et al., 2017). Identifying candidate probiotics from shrimp guts themselves, much as in the case of *Lactobacillus plantarum* MRO3.12 above, reduces the uncertainty about survivability in the host environment. Host genetics, however, is an important consideration and constraint on the ability of probiotics to illicit change in the gut microbiome (Landsman et al., 2019b; Liu et al., 2019). Despite probiotics being an attractive alternative to the use of broad-spectrum antibiotics, their use should be tightly monitored. For example, antibiotic resistant genes have been identified in probiotic supplements (Wong et al., 2015), including those often applied to shrimp culture (Uddin et al., 2015). However, the latter study did not identify any genetic elements associated with horizontal gene transfer.

Prebiotic supplementation (inert sources of bacterial nutrition) offers an alternative to using probiotic strains and may also offer benefit to the microbiome by encouraging the proliferation of beneficial microbes within the gut. In an eight-week feeding trial using juvenile *L. vannamei*, mannan oligosaccharide (MOS), one of the most common prebiotics, significantly improved weight gain and growth rate. The prebiotic also significantly increased the length of the microvilli in the intestine which could account for increased surface area for nutrient absorption, subsequently improving growth (Zhang et al., 2012). Although MOS did not significantly improve survival, its addition did significantly increase the activity of phenoloxidase and superoxide dismutase – both important pathways in the invertebrate immune system. In contrast, the application of MOS to an intensive commercial culture of *L. vannamei* did not correspond to increases in growth parameters but did improve survival (Gainza and Romero, 2020). Inulin, a prebiotic oligosaccharide isolated from grain, fruits and vegetables, has also demonstrated positive effects on the gut microbiota. An inulin-enriched diet significantly increased the abundance of lactic-acid bacteria (LAB), which are recognised as beneficial to host health, and correlated to a significant increase in survival of Indian white shrimp post-larvae, *Fenneropenaeus indicus* (Hoseinifar et al., 2015).

Co-application of both pre- and probiotics, termed synbiotics, can stimulate an immune response in *L. vannamei* infected with WSSV, subsequently increasing survival (Li et al., 2009), and could offer a potential alternative to the traditional yet ineffective use of antibiotics to treat the viral infection. Twenty-seven per cent (15/56) of shrimp farmers interviewed in Thailand incorrectly used antibiotics as antiviral preventions and treatments (Holmström et al., 2003) therefore gut supplementation may serve as a more effective means to manage (particularly viral) disease in aquaculture production and prevent unnecessary antibiotic pressures on the environment. Dietary supplementation of the probiotic *Bacillus* PC465, isolated from Chinese white shrimp (*Fenneropenaeus chinensis)* also reduced cumulative mortalities of *L. vannamei* infected with WSSV (Chai et al., 2016) and recent evidence suggests a diet of brown seaweeds impacts the composition of the shrimp gut microbiota and subsequently improves resistance to WSSV infection (Schleder, 2020). As the seaweed was not sterilised, however, it is unclear whether this effect was due to the addition of seaweed itself or the microorganisms that were associated with the seaweed. The addition of the macroalgae *Porphyra haitanensis* was previously associated with improving survival after WSSV challenges (3 and 4% supplement) and increased growth, as a result of increased feed intake (Niu et al., 2018). It is difficult to separate any health/growth benefits arising from ameliorisation of the gut microbiome from simply increased nutritional resources provided by the addition of dietary supplements.

As well as being ineffective in the treatment of several of the abovementioned diseases, antibiotics can have a direct impact on the gut microbiome which may be detrimental to the host. Antibiotic application can decrease colonisation resistance within the gut, alter its microbial composition, and facilitate the emergence of disease (Jernberg et al., 2010). Zeng et al. (2019) showed that the addition of ciprofloxacin and sulphonamide, which are commonly used to treat bacterial diseases in aquaculture, caused a short-term reduction in bacterial richness and diversity of the gut along with a significant increase in antibiotic resistant genes in healthy *L. vannamei*. Antibiotic resistant genes have been detected in aquaculture facilities throughout the world and can persist in bacterial reservoirs even after the initial pressure for their selection (Tamminen et al., 2011). Furthermore, antibiotic resistance genes have been found to be more abundant in adult shrimp compared to juveniles (Su et al. 2018). Phylogenetic analysis suggests resistance genes are transferred from intestinal bacteria to those in the culture environment (Zeng et al., 2019) and horizontal gene transfer can spread resistance between microbes in the environment, including those that are serious human pathogens (Tomova et al., 2015).

5. Recommendations for future microbiome studies

The investigation into the gut microbiome of the aquatic invertebrates is a relatively new discipline. Therefore, any attempts to guide the field into a more consistent and reliable consensus, in terms of the information required for accurate reporting, should be encouraged. Given the increasing number of available sample preparations and bioinformatic tools, it is unrealistic to limit all future studies to one methodology or analytical pipeline. However, that is not to say that these same studies should not include the same level of detail, samples sizes and availability of data that we expect from other, more established fields. For example, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines have established a strong precedent for publishing reliable gene expression datasets by encouraging best experimental practices through a set of

standardised guidelines (Bustin et al., 2009). The Minimum Information required to support a Stimulant Assessment experiment (MISA) guidelines aimed to provide the same for immunostimulant work (Hauton et al., 2015). There are several extensive 'best practice' papers which offer useful instruction for the design and implementation of marker gene microbiome studies (Goodrich et al., 2014; Pollock et al., 2018; Hornung et al. (2019), along with standards for the minimum information about a marker gene sequences (MIMARKS) (Yilmaz et al., 2011). When considering implementing marker gene analysis for microbial profiling (metabarcoding), we offer the following suggestions:

(1) Always include adequate sample sizes in the experimental design. Low sample sizes are unlikely to capture the individual variation often associated with microbiome surveys. Furthermore, without an accurate description of the distribution of data, robust statistical testing may not be possible. Multiplexing with dual indexes and custom library preparations (Kozich et al., 2013) now allow for the inclusion of hundreds of samples per run. Therefore, the cost of sequencing is now less of a barrier to adequate sample sizes.
(2) Consider the limitations of the gene/region of interest. The hypervariable regions of the ribosomal small subunit RNA (SSU rRNA) gene can differ in their ability to detect specific taxa (Kim et al., 2011) and can impact the richness and diversity inferred from a community, including those isolated from shrimp (García-López et al., 2020).
(3) Avoid restricting taxonomic analyses to the phylum level. Phyla are high-level, diverse assemblages of taxa and the differential abundance of a phylum is often too ambiguous to infer specific mechanistic action or interaction with the host and/or other taxa in the microbiome. This is particularly true of Proteobacteria, which often dominate the gut of aquatic invertebrates.
(4) Avoid using marker genes to infer functional potential of aquatic invertebrate gut microbiomes. The lack of annotated genomes from marine microbes creates an analytical bias that may significantly impact gene inference and the assessment of differential abundance of functional gene profiles associated with aquatic invertebrate gut microbiomes (Sun, Jones and Fodor, 2020).
(5) Consider the use of exact sequence variants as opposed to operational taxonomic units (OTUs). There is often valid reason to cluster sequences according to percentage identity, such as accounting for error and taxonomically uninformative variation (e.g. intragenomic polymorphism of multi-copy genes). However, clustering overlooks the high sequencing accuracy possible with modern-day sequencing technologies, and can also obscure meaningful, biological variation (Callahan et al., 2016). Analysis of ESVs allows the generation of sequence clusters that are not dependent on the dataset itself and are therefore comparable across other datasets.
(6) Do not refer to amplicon sequencing data as 'metagenomics'. This is particularly misleading in titles and abstracts of publications. Metagenomics refers to shotgun (not amplicon) sequencing of all DNA in a sample, (sub)sampling genomes of eukaryotes, viruses, and prokaryotes. Amplicon or marker-gene sequencing, by definition and design, targets a very specific region of those genomes and, more often, a very specific region of a single gene (e.g. hypervariable regions of the SSU rRNA gene).

6. Conclusions

The gut microbiomes of penaeid shrimp are becoming increasingly well characterised in comparison to other aquatic invertebrates. There are, however, still substantial gaps in the literature across all the penaeid species, and from the range of farming systems utilised in their culture. In support of the 'pathobiome' concept (Bass et al., 2019), pathogenesis may not be directly linked to the relative abundance of a particular taxon but rather the change in interactions between multiple taxa and the host (Chen et al., 2017; Zhou et al., 2019; Huang, et al., 2020; Dai et al., 2020). However, we currently lack enough data to make generalisations about the gut microbiome of different shrimp species in regard to growth conditions and health status. We propose that a concerted global effort to increase our understanding of microbial complexity in these systems is needed. Inferences made from small datasets may not be representative of a true change or general patterns in terms of differential compositions in relation to disease, and provide little to go on for the development of positive interventions. The contexts in which different microbiome states arise (shrimp species, development stage, culture conditions, treatments, pond ecology, etc.) are very varied and their own influences on shrimp microbiomes are largely unknown. What is 'normal' in a wide range of situations needs to be known before abnormal conditions, for example associated with or predisposing to disease, can be reliably identified. Furthermore, the ability for the global scientific community to access raw sequencing data and experimental information (metadata) needs to improve in order to undertake *meta*-analyses and generalise across studies. This information is vital as demand for aquatic-based protein increases and shrimp aquaculture becomes more intensive. Better characterisation of the microbiota across the entire length of the gut, and across growth and development cycles will likely facilitate the improvement of shrimp probiotics to aid in improving growth and reducing the susceptibility towards disease, which will ultimately maximise the sustainable production of these key species.

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