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Acute hepatopancreatic necrosis disease in penaeid shrimp

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Abstract

Asian countries are the major producers of cultured Penaeid shrimps such as *Penaeus monodon* and *Penaeus* (*Litopenaeus*) *vannamei*. In recent years, shrimp production has declined due to the emergence of a bacterial disease called acute hepatopancreatic necrosis disease (AHPND). This disease is mainly caused by *Vibrio parahaemolyticus*, but other *Vibrio* species are also known to cause AHPND in shrimps. Here, in addition to reviewing recent developments in the field of AHPND diagnosis, host responses to AHPND, the role of microbiota during AHPND infection and the current treatment options for AHPND, we also describe a model of AHPND pathogenesis.

Key words: acute hepatopancreatic necrosis disease, diagnosis, microbiota, pathogenesis, shrimp.

Introduction

Asian countries are major producers of cultured shrimps, with *Litopenaeus vannamei* and *Penaeus monodon* being two of the most commercially important species (FAO, 2017). However, shrimp production continues to be impaired by the emergence of various bacterial, viral and fungal diseases (Lightner 1999; Caroline & Aguinaldo 2012). Most recently, a bacterial disease known as AHPND (acute hepatopancreatic necrosis disease) has become a major threat to the shrimp industry in many Asian countries (Tran *et al.* 2013). AHPND-causing bacteria initially colonize the stomach of infected shrimp (Tran *et al.* 2013; Lai *et al.* 2015). A binary toxin produced by these bacteria subsequently reaches the hepatopancreas, probably at least to some extent via the gastric sieve (Prachumwat *et al.* 2019). In the hepatopancreas, this toxin induces sloughing of the tubule epithelial cells (Tran *et al.* 2013; Fig. 1) and causes the hepatopancreas to turn pale (Fig. 2). The first outbreak of AHPND occurred in 2009 in China (Nunan *et al.* 2014). Since then, outbreaks of the disease have been reported in Mexico, Malaysia, Thailand, the Philippines, Vietnam (Shinn *et al.* 2018), Bangladesh (Eshik *et al.* 2017) and the USA (Dhar *et al.* 2019) and shrimp production in these countries has been drastically reduced (Shinn *et al.* 2018).

In 2013, the causative agent of AHPND was identified as *Vibrio parahaemolyticus* (Tran *et al.* 2013). Subsequently, the disease was shown to be caused only by strains of this ubiquitous, opportunistic, marine pathogen that carry a virulent pVA1 plasmid with binary toxin genes similar to the *Photorhabdus* insect-related (Pir) toxin, PirA and PirB (Lee *et al.* 2015). In addition to these toxin genes, the plasmid also encodes conjugative transfer genes and transposons, suggesting the possibility of plasmid mobilization into other strains or species (Lee *et al.* 2015). In fact, after it was first identified in *V. parahaemolyticus*, the pVA1 plasmid was found in various other species that were also shown to cause AHPND, including *Vibrio owensii*, *Vibrio campbelli* and *Vibrio harveyi* (Kondo *et al.* 2015; Liu *et al.* 2015). PCR-based amplification of the pVA1 plasmid and PirAB toxin regions are presently used as a reliable diagnostic or detection tool for AHPND (Lee *et al.* 2015; Liu *et al.* 2017; Cruz-Flores *et al.* 2019). Recently, to control the spread of AHPND, research has focused on developing easy-to-use on-site diagnostic kits that could help in fast decision-making.

In response to AHPND, the shrimp immune system expresses antimicrobial peptides (AMPs) such as penaeidins and crustins, which provide some protection against AHPND-causing *V. parahaemolyticus* (Maralit *et al.* 2018). Several immune-related factors with antibacterial effects have been identified in *L. vannamei* (Junprung *et al.* 2017; Boonchuen *et al.* 2018; Tinwongger *et al.* 2019), and injecting recombinant proteins of these factors is an effective preventive treatment against AHPND (Junprung *et al.* 2017;

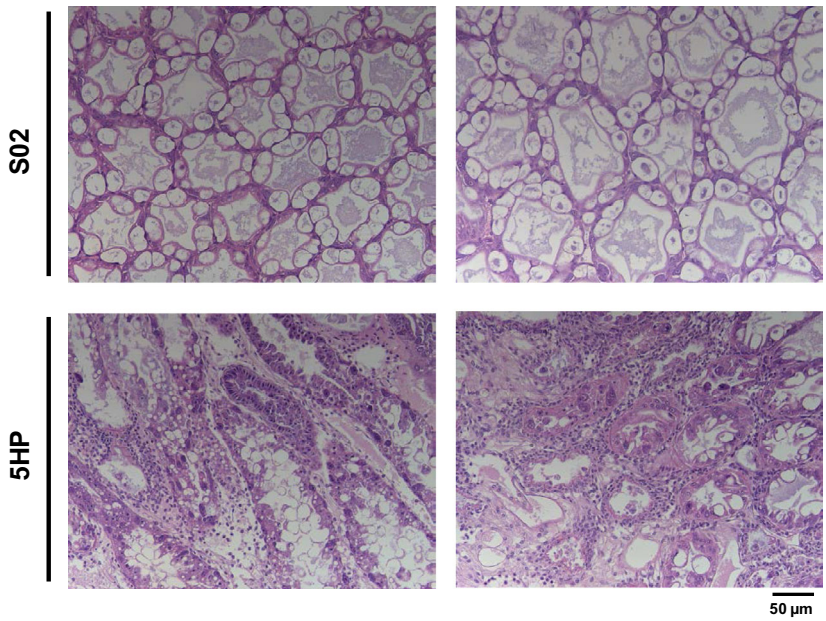

Figure 1 Histology of acute hepatopancreatic necrosis disease (AHPND)-infected shrimp hepatopancreas. Haematoxylin- and eosin-stained hepatopancreas from shrimps infected with non-AHPND-causing (S02) and AHPND-causing (5HP) *Vibrio parahaemolyticus*. The S02-infected group shows normal tubules in hepatopancreas, while the 5HP-infected hepatopancreas shows the characteristic signs of AHPND, with sloughed epithelial cells, necrosis and infiltration of hemocytes. Scale bar: 50 µm.

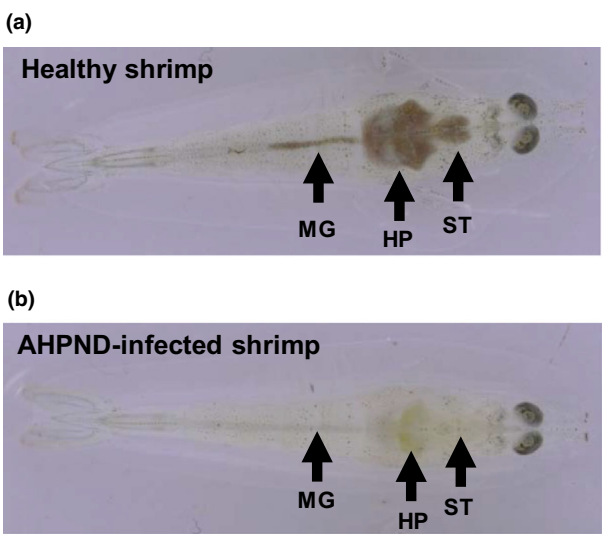

Figure 2 Gross clinical signs of acute hepatopancreatic necrosis disease (AHPND) infection in *Litopenaeus vannamei*. The normally brown midgut (MG), hepatopancreas (HP) and stomach (ST) that as seen in (a) healthy shrimp all turn pale in (b) AHPND-infected shrimp.

Visetnan *et al.* 2017). Some plant extracts and phages that can inhibit the growth of AHPND-causing *V. parahaemolyticus* have also been successfully used as a preventive treatment for AHPND in *L. vannamei* (Jha *et al.* 2016; Jun *et al.* 2018).

The host animal's gut microbiota can also influence the effects of this disease. During AHPND, the bacterial communities in the shrimp stomach and hepatopancreas become dysbiotic (Chen *et al.* 2018). Conversely, enrichment of certain species of bacteria confers protection against AHPND-causing *V. parahaemolyticus* (Yu *et al.* 2018; Yao *et al.* 2018). Beneficial bacteria isolated from healthy shrimps can also be used as probiotics for improving shrimp health and boosting their immune system (Chomwong *et al.* 2018; Pinoargote & Ravishankar 2018a).

In this review, we summarize the research findings and developments in the field of AHPND with respect to disease diagnosis, host responses in shrimp, the involvement of shrimp microbiota, current preventive treatments and various factors contributing to AHPND pathogenesis.

The AHPND-associated plasmid and PirAB toxins

Although several *V. parahaemolyticus* strains have been identified as the causative agents of AHPND (Table 1), not all strains are capable of causing the disease (Tran *et al.* 2013). In Lee *et al.* (2015) sequenced the genome and plasmids of various strains of *V. parahaemolyticus* isolated from AHPND-infected shrimps and grow-out pond, and found that AHPND was caused only by strains of *V. parahaemolyticus* that possessed an extrachromosomal plasmid encoding the binary toxin PirA and PirB. This virulence plasmid had a size of ~69 kbp and was designated pVA1.

Table 1 Summary of bacterial strains carrying an acute hepatopancreatic necrosis disease plasmid

Bacterial pathogen	Bacterial strains/isolates	AHPND plasmid	PirAB toxin	Pathogenicity challenge	Reference
Vibrio parahaemolyticus	3HP, 5HP, M1-1, China, A/3, D/4. PD2	pVA1	Present	*Penaeus vannamei*	Lee *et al.* (2015)
V. parahaemolyticus	THV-1, THV-16, 5HP	pVA	Present	*Litopenaeus vannamei*	Lai *et al.* (2015)
V. parahaemolyticus	13-028/A2 13-028/A3	pVPA3	Present	–	Han *et al.* (2015)
V. parahaemolyticus	D2, D4, D6, E1, E2	pVA	Present	*L. vannamei* *Marsupenaeus japonicus*	Tinwongger *et al.* (2016)
V. parahaemolyticus	VPE61	pVPE61a	Present	–	Theethakaew *et al.* (2017)
V. parahaemolyticus	VP2HP	pVP2HP	Absent	–	Theethakaew *et al.* (2017)
V. parahaemolyticus	XN87	Mutated pVA	Lack of PirAIntact PirB	*P. vannamei*	Phiwsaiya *et al.* (2017)
Vibrio owensii	SH14	pVH	Unstable PirAB	*L. vannamei*	Liu *et al.* (2018)
V. owensii	SH14	pVH	Present	*L. vannamei*	Xiao *et al.* (2017)
V. parahaemolyticus	*Vp*2S01	pVPGX1	Present	*L. vannamei*	Dong *et al.* (2017)
Vibrio campbelli	*Vc*3S01	pVCGX1	Present	*L. vannamei*	Dong *et al.* (2017)
V. campbelli	VH-639, VH-1526, VH-Surat	pVA	Present	*L. vannamei*	Wangman *et al.* (2018)
Vibrio punensis	BA55	pV_{AHPND}	Present	*P. vannamei*	Restrepo *et al.* (2018)

Figure 3 shows the gene organization and location of *pirAB* in the pVA1 plasmid. The pVA1 plasmid includes conjugative transfer and plasmid mobilization genes that make the plasmid self-transmissible. The plasmid also carries a *pndA* post-segregational killing (PSK) system that ensures that the plasmid is always inherited (Lee *et al.* 2015). Damage is caused to the host when the PirA toxin and PirB toxin, which show structural similarities to the *Bacillus* Cry insecticidal proteins, are secreted into the extracellular environment (Lai *et al.* 2015; Lin *et al.* 2017). There has been one report in which the purified recombinant PirB toxin alone was found to cause AHPND-like symptoms when injected into shrimps by reverse gavage (Lee *et al.* 2015), but in this study, the quantity of toxin was approximately 25 000× greater than would be found in 1 μg of lethal crude protein extracted from a virulent strain of bacteria (Prachumwat, *et al.* 2019). It has further been shown that the virulence of AHPND-causing *V. parahaemolyticus* depends on the amount of PirAB toxin released and not on the gene copy number of the plasmid (Tinwogger *et al.* 2016). The cellular damage that this toxin can cause also explains how *V. parahaemolyticus*, which initially colonizes only the shrimp stomach, is able to reach the hepatopancreas (Lai *et al.* 2015; Han *et al.* 2015).

Further study of the pVA1 plasmid and PirAB toxin showed that when non-AHPND strains of *V. parahaemolyticus* were transformed with the plasmid containing PirAB, they become able to cause AHPND-like clinical signs and 100% mortality in shrimps, while a mutant strain of AHPND-causing *V. parahaemolyticus* that lacked the *pirAB* genes failed to cause AHPND (Tinwogger *et al.* 2016). Meanwhile, Theethakaew *et al.* (2017) isolated both an AHPND-causing strain and a non-AHPND-causing strain of *V. parahaemolyticus* from the same infected shrimp; the AHPND-causing strain (VPE61) harboured a virulence plasmid of expected size (69 kbp), while the non-AHPND-causing *V. parahaemolyticus* strain (VP2HP) had a 183 kb plasmid. The 183 kb plasmid included all of the genes found in the 69 kb plasmid except that it lacked the entire 3.4 kb PirA/PirB transposon element (Theethakaew *et al.* 2017).

In another study (Phiwsaiya *et al.* 2017), a virulent *V. parahaemolyticus* isolate, XN87, from a shrimp grow-out pond in Vietnam was found to carry a mutated pVA1 plasmid with a mutant *pirA* gene and a frame-shifted *pirB* gene that failed to produce PirB toxin in the culture media. Immersion challenge of shrimps with this isolate caused 50% mortality but did not show any AHPND-related clinical signs. These findings suggest that *V. parahaemolyticus* can still be virulent and cause shrimp death that can be attributed to the umbrella term 'early mortality syndrome' (EMS) even when the PirAB toxin is absent, and there are no AHPND-related symptoms (Phiwsaiya *et al.* 2017).

In recent years, thanks to advances in sequencing technology, the presence of a pVA-like plasmid with *pirAB* toxin genes has been reported in other species of *Vibrios* such as *V. campbelli* (Wangman *et al.* 2018), *V. harveyi* (Xiao *et al.* 2017), *V. owensii* (Liu *et al.* 2018) and *V. punensis* (Restrepo *et al.* 2018). Two strains of *V. owensii* that were identified as the causative agents of AHPND in China and Vietnam, respectively, were both found to contain plasmids encoding homologues of the PirAB toxin that are present in *V. parahaemolyticus* (Liu *et al.* 2018). A comprehensive genomic study (Xiao *et al.* 2017) of plasmids

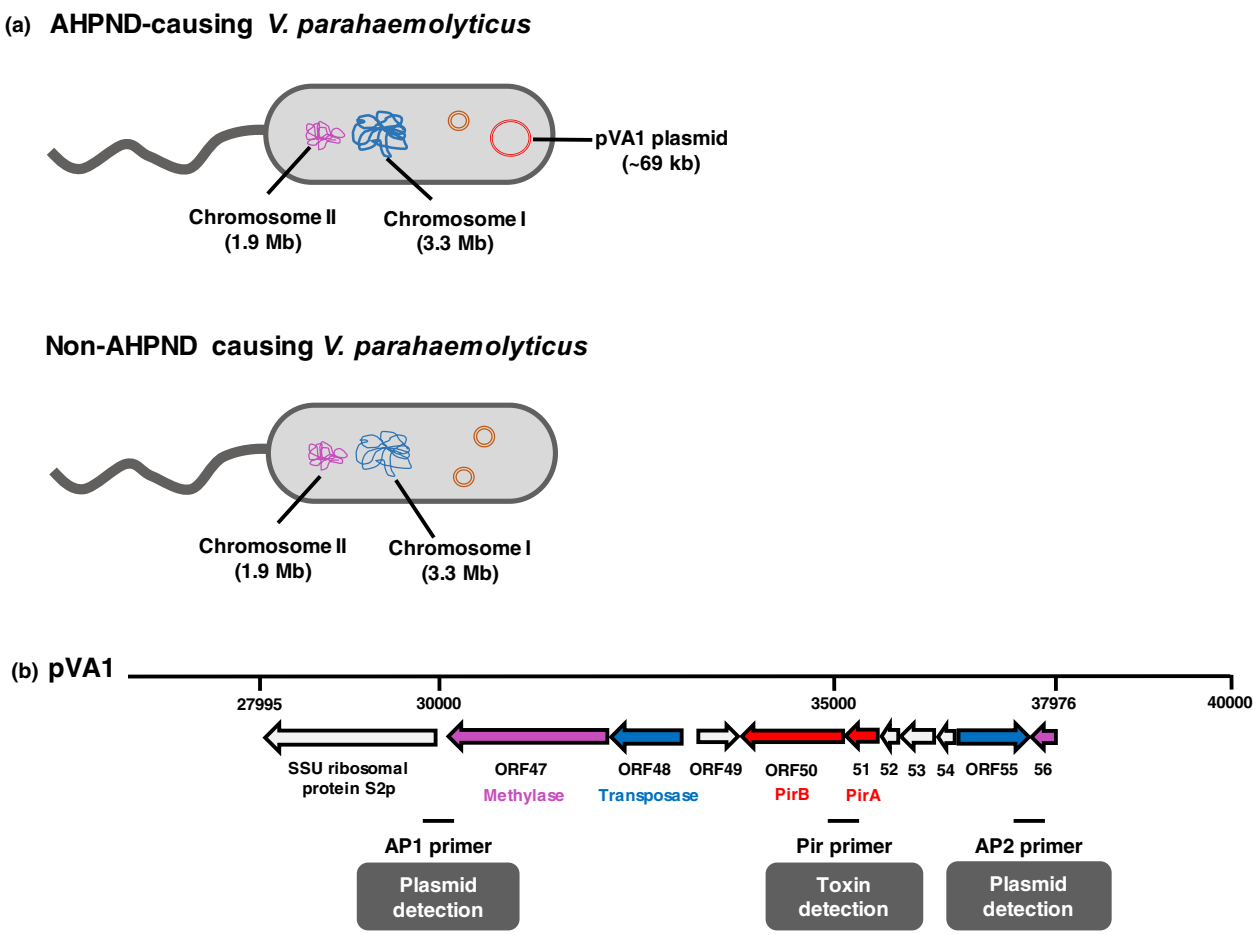

Figure 3 Acute hepatopancreatic necrosis disease (AHPND)-causing plasmid in *Vibrio parahaemolyticus*. (a) The difference between the AHPND-causing and non-AHPND-causing strains of *V. parahaemolyticus* is the presence of a unique 69 kb pVA1 plasmid. (b) The locations of the methylase (purple arrows), transposase (blue arrows) and PirAB toxin genes in the pVA1 plasmid. Regions amplified by the specific primers AP1, AP2 and AP3 on the pVA1 plasmid are also shown. This figure is adapted and modified from Lee et al. (2015).

from different species of *Vibrios* identified a pVH plasmid that was 99% identical to the pVA1 plasmid. The *pirAB* genes in the pVH plasmid were flanked by identical transposon elements known as the *pirAB*-Tn903 composite transposon, and Xiao *et al.* (2017) demonstrated that this transposon was responsible for the insertion of the *pirAB* genes into the ancestral plasmid of pVH. Phylogenetic analysis showed that the *pirB* gene in the pVH plasmid was closely related to the *pirB* genes found in the plasmids in *V. campbelli*, *V. harveyi* and *V. owensii* (Xiao *et al.* 2017).

In a separate study, *V. campbelli* was also shown to cause AHPND in shrimps. Strains of *V. parahaemolyticus* and *V. campbelli* isolated from the same AHPND-infected pond showed similar pathogenicity, as well as the presence of the pVA plasmid and binary toxin genes *pirAB*, and provided an early example of how the virulence plasmid might be acquired by horizontal gene transfer (HGT; Dong *et al.* 2017). At first, the dissemination of virulent plasmids by HGT was observed only within the same *Vibrio* clade (*V. parahaemolyticus*, *V. harveyi*, *V. campbelli* and *V. owensii*), but recently a new strain isolated from AHPND-infected shrimps was found to be positive for the AHPND-associated plasmid encoding the PirAB toxin even though this strain has not yet been experimentally shown to be capable of causing AHPND (Restrepo *et al.* 2018). This strain, *V. punensis*, belongs to the *Orientalis* clade, which usually consists of non-pathogenic *Vibrio* species that are probiotic in nature. Restrepo *et al.* (2018) proposed that the selection pressure in the AHPND-infected shrimp stomach could have facilitated the dissemination of the virulent plasmid into *V. punensis* by HGT. A recent study demonstrated the conjugative transfer of a pVA1-type plasmid from AHPND-causing *V. parahaemolyticus* to a non-pathogenic *V. campbellii* and further showed that challenging shrimps with this transconjugant produced histopathological lesions and the clinical signs of AHPND (Dong *et al.* 2019).

Since HGT enables commensal bacteria to acquire virulent genes and become pathogenic in nature, AHPND can no longer be attributed only to *V. parahaemolyticus*, and as summarized in Table 1, recent evidence increasingly shows that other *Vibrio* species are now capable of causing the disease. We also note that diagnostic methods that use the pVA plasmid and PirAB toxin as markers will, correctly, return a negative AHPND diagnosis for vibrios that lack PirAB toxin even though some of these strains can still cause mortality in shrimps (Phiwsaiya *et al.* 2017). This increasing complexity leads to edge cases such as *V. parahaemolyticus* strain VP2HP (Theethakaew *et al.* 2017) and *V. owensii* strain SH14 (Liu *et al.* 2018), which may or may not properly be counted as 'AHPND causing', and suggests that current diagnostic methods and treatment strategies will need to be revisited.

Diagnosis of AHPND in shrimps

Early detection of *V. parahaemolyticus* either in the shrimp themselves or in the culture habitat can prevent an AHPND outbreak. Since the virulent pVA plasmid that harbours the binary PirAB toxin was first identified and characterized a decade ago, both of these toxin genes have been used as important markers for AHPND diagnosis. Specific primers or probes designed for detecting the plasmid as well as the PirAB toxin are commonly used (Tinwongger *et al.* 2014; Lee *et al.* 2015; Dangtip *et al.* 2015; Sirikharin *et al.* 2015). Most of the commercially available AHPND diagnostic kits are PCR-based and use either conventional PCR (Tinwongger *et al.* 2014; Lee *et al.* 2015; Lai *et al.* 2015; Sirikharin *et al.* 2015), nested PCR (Dangtip *et al.* 2015) or real-time PCR techniques (Cruz-Flores *et al.* 2019), but these kits are time-consuming and require expensive instruments. For diagnostic laboratories, researchers focused recently on developing AHPND detection systems that are less time-consuming with high-throughput and increased sensitivity and specificity. For example, a multiplex real-time PCR (SYBR and Taqman chemistries) using primers targeting *pirA*, *pirB*, shrimp 18S and bacterial 16S genes has been developed for easy, high-throughput detection of AHPND (Cruz-Flores *et al.* 2019). However, since PCR-based kits and thermal cyclers are not affordable by most shrimp farmers, alternative visual detection methods using fluorescence-based assays such as the recombinase polymerase (RPA) assay have been adopted for on-site diagnosis in shrimp farms. RPA is both rapid and sensitive, capable of detecting as few as 2 copies of Pir-toxin-like genes in *V. owensii* (Liu *et al.* 2017). In 2016, Arunrut *et al.* developed another sensitive, visually unaided detection method using loop-mediated isothermal amplification (LAMP) and an ssDNA-labelled nanogold probe (AuNP) to detect *pirA* genes in shrimp and pond sediments in less than one hour. In the same year, another research team developed a LAMP-based detection system that used a water bath instead of an expensive thermocycler; this system detected *pirAB*-like genes in AHPND-causing strains of *V. parahaemolyticus* with greater accuracy and reliability than conventional PCR methods (Koiwai *et al.* 2016). The same group subsequently developed a more rapid and accurate method for diagnosing four shrimp diseases including AHPND using a PCR-DNA chromatography method in which the multiplex PCR products are visualized using a single-strand-tag-hybridized chromatographic printed array strip (Koiwai *et al.* 2018). These reliable on-site diagnostic kits facilitate rapid and timely diagnosis and potentially allow the farmer to control the disease using appropriate treatments. The different AHPND detection methods are summarized in Table 2.

Host responses during AHPND infection

Shrimps exhibit an innate immune response against bacterial infection. This response includes cytokines, melanization, the prophenoloxidase (proPO) system, pattern recognition proteins, antioxidants, antimicrobial peptides and lysosomal enzymes, with the antimicrobial peptides in particular being studied extensively in AHPND pathogenesis. Transcriptomics studies of AHPND-infected shrimp hemocytes and stomach have yielded a list of differentially expressed immune genes on various immune pathways, including penaeidins, crustins, serpins, lectins and antilipopolysaccharide factors (Soonthornchai *et al.* 2016; Maralit *et al.* 2018). In shrimps, a short antimicrobial polypeptide known as antilipopolysaccharide factor (ALF) acts against bacteria, fungi and virus. In *L. vannamei*, the isoform *Lv*ALF AV-R showed binding affinity towards lipopolysaccharides and peptidoglycans and also showed increased expression in hepatopancreas during AHPND (Tinwongger et.al. 2019). In another study, molecular modelling and docking analysis of *Lv*ALF and PirAB toxin showed that *Lv*ALF interacts with PirB toxin through its LPS-binding sites and that knockdown of *Lv*ALF followed by PirAB toxin challenge caused increased mortality in *L. vannamei* (Maralit *et al.* 2018). The single-WAP domain-containing protein (SWD) is a type III crustin antimicrobial peptide that acts as a proteinase inhibitor for subtilisin in *L. vannamei* and also was upregulated in hemocytes of AHPND-infected shrimps (Visetnan *et al.* 2017).

The antimicrobial responses in shrimps are activated by cytokines such as the heat shock proteins (HSP) in hemocytes. Heat shock proteins or chaperonins are involved in temperature-related stress responses (Aguirre-Guzman *et al.* 2009). Junprung *et al.* (2017) found that non-lethal heat shock to *P. vannamei* induced the expression of heat

Table 2 Summary of acute hepatopancreatic necrosis disease diagnostic methods

Bacterial pathogen	Bacterial strain/isolate	Detection method	Specific primers	AHPND-infected shrimp tested	Shrimp tissues tested	References
Vibrio parahaemolyticus	5HP, CN	Nested PCR	AP4	*Litopenaeus vannamei*	Stomach	Dangtip *et al.* (2015)
V. parahaemolyticus	5HP, CN	Conventional PCR	AP3	*Penaeus vannamei*	Stomach Hepatopancreas Faeces	Sirikharin *et al.* (2015)
V. parahaemolyticus	ThV-1, ThV-16, 5HP, M1-1, M2-36	Conventional PCR	AP1, AP2, Pir	*L. vannamei*	Stomach Hepatopancreas	Lai *et al.* (2015)
V. parahaemolyticus	13-028 A3	Multiplex PCR	PirA, PirB Shrimp 18s Bacterial 16sRNA	*P. vannamei*	Hepatopancreas	Cruz-Flores *et al.* (2019)
Vibrio campbelli	D 52B, D3 16-137, D 16-192	Multiplex PCR	PirA, PirB Shrimp 18s Bacterial 16sRNA	*P. vannamei*	Hepatopancreas	Cruz-Flores *et al.* (2019)
Vibrio shiloi	–	Multiplex PCR	PirA, PirB Shrimp 18s Bacterial 16sRNA	*P. vannamei*	Hepatopancreas	Cruz-Flores *et al.* (2019)
Vibrio owensii	SH14	Recombinase polymerase amplification (RPA)	PirA, PirB	–	Hepatopancreas	Liu *et al.* (2017)
V. parahaemolyticus	3HP, 5HP, CHN	Loop-mediated isothermal Amplification (LAMP)	PirA	*L. vannamei* *Penaeus monodon*	Stomach	Arunrut *et al.* (2016)
V. parahaemolyticus	E2, D6, N7, N10, FP11, FP14	Conventional PCR	TUMSAT-Vp1 TUMSAT-Vp2 TUMSAT-Vp3	–	Bacteria	Tinwongger *et al.* (2014)
V. parahaemolyticus	E2, D6, N7, N10, FP11, FP14	LAMP	PirA-likePirB-like	–	Bacteria	Koiwai *et al.* 2016
V. parahaemolyticus	D6	PCR-DNA chromatography	PirA-like	*L. vannamei* *P. monodon*	–	Koiwai *et al.* (2018)

shock proteins HSP70 and HSP90. These HSPs regulated the immune genes proPO and crustin and caused AHPND tolerance in shrimps, while knockdown of these HSPs led to increased mortality (Junprung *et al.* 2017).

Haemocyanin is a protein involved in the storage and transport of oxygen in shrimp haemolymph. However, a recent report suggests that it also shows an antibacterial immune response in *L. vannamei* specifically the agglutination of *V. parahaemolyticus* (Boonchuen *et al.* 2018). A protein–protein interaction assay in the same study further suggested that haemocyanin binds to recombinant PirA and neutralizes the toxin.

The penlectin5 protein, which belongs to the class of fibrinogen-related proteins, was also induced during AHPND infection (Angthong, *et al.* 2017). Silencing the penlectin5 gene using dsRNA prior to challenge with *V. parahaemolyticus* (3HP strain) led to increased mortality (Angthong *et al.* 2017). AHPND-causing *V. parahaemolyticus* has also been shown to activate the Rho-signalling pathway in *L. vannamei*, and this is thought to disrupt the cell adhesion molecules in stomach epithelial cells and facilitate the migration of toxin and bacteria from the stomach to hepatopancreas during AHPND infection (Ng *et al.* 2018). Another study (Kumar *et al.* 2019) found that bile acid stimulated the release of Pir AB toxin, while a reduction in the levels of bile acids in *L. vannamei* stomach resulted in a decrease in AHPND-induced mortality rates.

In recent years, the role of microRNAs (small non-coding RNAs) in disease progression has been widely researched. Although the direct response of shrimp hepatopancreatic tubule epithelial cells has not yet been studied, a comparative transcriptomic analysis of shrimp (*L. vannamei*) hemocytes infected with AHPND and non-AHPND bacteria was used to identify 12 microRNA candidates that were dysregulated during AHPND infection (Zheng *et al.* 2018). These microRNAs were involved in the immune system, metabolism and apoptosis pathways.

Microbiota and microbiome

'Microbiota' is the term used to refer to associations of commensal and symbiotic bacteria that live on or in a host, while 'microbiome' is used to refer to the collective genome (or metagenome) associated with a particular microbiota. Gut microbiome research is an emerging field in shrimp aquaculture. Recent research has focused on the influence of environmental stress, diet and AHPND in modulating the diversity of the gut microbiota. A meta-analysis of gut microbiota in healthy and diseased shrimps showed that in healthy shrimps, the *Gammaproteobacteria*, *Alphaproteobacteria* and *Bacteroides* were the dominant classes in the shrimp stomach. During AHPND, one of the orders of the *Gammaproteobacteria*, the *Vibrionales*, becomes enriched in the gut, which in turn leads to changes in relative abundance in the bacterial community that can serve as gut bacterial signatures for AHPND diagnosis (Yu *et al.* 2018; Yao *et al.* 2018). These signatures are seen at all developmental stages and are robust in the presence of changes in environmental factors and diet that can also influence the composition of the gut microbiota. For example, while changes in the environment and diet can affect the abundance of *Firmicutes*, *Bacteroides* and *Actinobacter*, they have no effect on the abundance of *Proteobacteria* in *L. vannamei*. Similarly, although the composition and co-existence of beneficial and pathogenic bacteria can be modulated by dietary components and environmental parameters, during AHPND, opportunistic *Vibrios* will always become enriched in the shrimp gut compared to the beneficial bacteria (Li *et al.* 2018). A comparative analysis (Chen *et al.* 2018) of the microbiota in both shrimps' guts and the water in the grow-out pond during an AHPND outbreak showed a gradual shift both in the microbial diversity (Fig. 4) and in the species-to-species connectivity. Together these two trends culminated in dysbiosis of the gut bacteria of the infected shrimp. At the same time, the microbial composition of the grow-out pond was largely unchanged, and it remained distinct from the shrimp microbiota (Chen *et al.* 2018). In another study, a metagenomics analysis of the microbiome in the hepatopancreas and intestine of AHPND-infected *L. vannamei* showed significantly enriched metabolic pathways. Specifically, in the infected shrimp, the nucleotide metabolism pathway was enriched in hepatopancreas, while amino acid metabolism, lipid and carbohydrate metabolism were enriched in the intestine. By contrast, in the

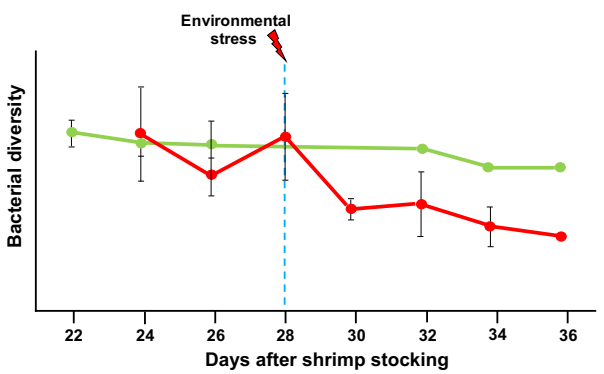

Figure 4 Changes in the diversity of the gut bacteria during acute hepatopancreatic necrosis disease (AHPND). Time series of the diversity of the gut microbiota from 22 days after stocking through to an AHPND outbreak triggered by environmental stress. This figure is adapted and modified from Chen et al. (2018). [—●—] Healthy shrimp; [—●—] AHPND (+) shrimp.

healthy shrimps, signal transduction was enhanced (Cornejo-Granados *et al.* 2017). Although much work still remains to be done, a more complete understanding of the microbial dynamics of shrimps and their culture habitat during AHPND is likely to be crucial for developing strategies to reduce disease outbreaks, restore shrimp health and reduce the use of antibiotics in shrimp aquaculture.

Treatments for AHPND-infected shrimps

In recent years, in addition to improvements in diagnosis, there has been substantial progress towards effective AHPND treatments. In general, bacterial diseases are treated with antibiotics; however, in shrimp aquaculture, there is a danger that the use of antibiotics might lead to drug resistance in bacteria that threaten not only shrimp but also humans. Hence, plant-derived compounds, phages, nanoparticles and recombinant immune-related proteins with antibacterial effects are to be preferred in the treatment of AHPND infection.

When used as feed supplements in the shrimp diet, plant extracts with antibacterial properties have been shown to confer protection against AHPND-causing bacteria. For example, *P. vannamei* that were given shrimp feed supplemented with a mixture of essential oils from 10 different plants showed no gross clinical signs of AHPND 10 days after infection with *V. parahaemolyticus* (Jha *et al.* 2016). Phage therapy is widely used as another alternative for antibiotics in treating bacterial diseases. In *P. vannamei*, the phage pVP-1 was tested for its prophylactic and therapeutic effects against AHPND-causing *V. parahaemolyticus*. The results showed that prophylactic phage treatment was more effective in reducing the mortality of shrimps after *V. parahaemolyticus* challenge (Jun *et al.* 2018). In addition to the antibacterial agents, immunostimulants are also used in the treatment of AHPND: *L. vannamei* treated with recombinant PirA-like toxin showed decreased mortality after *V. parahaemolyticus* challenge (Campa-Córdova *et al.* 2017). In 2019, Tello-Olea *et al.* administered feed supplemented with gold nanoparticles to *L. vannamei* prior to challenge with *V. parahaemolyticus* and found that after challenge, the gold nanoparticles led to increased expression of the immune-related genes *TLR3* (Toll-like receptor) and *proPO* (prophenoloxidase) as well as an increase in the survival rate by 80%.

In *L. vannamei*, most of the proteins that are elicited as an immune response to the AHPND infection show antibacterial activity against *V. parahaemolyticus*. Recombinant immune-related proteins also protect shrimps from AHPND challenge. For example, LvHSP70 recombinant protein is a molecular chaperone involved in innate and adaptive immunity in shrimps (Junprung *et al.* 2019). Shrimps injected with recombinant LvHSP70 and then challenged by *V. parahaemolyticus* showed an increased survival and enhanced resistance to *V. parahaemolyticus* due to the induction of genes involved in activating the prophenoloxidase (proPO) system, the NF-κB signal transduction pathway and antimicrobial peptides (AMPs; Junprung *et al.* 2019). An antimicrobial peptide known as single-WAP domain-containing (SWD) protein with antimicrobial and antiproteinase activity was identified in *L. vannamei*. Shrimps immersed in a mixture of recombinant SWD protein and *V. parahaemolyticus* showed increased survival (Visetnan *et al.* 2017). Likewise, in *L. vannamei*, haemocyanin is a protein involved in non-specific immune responses that is upregulated during AHPND infection. When haemocyanin was isolated and purified from shrimp haemolymph and then injected into AHPND-challenged shrimps along with purified toxin from *V. parahaemolyticus*, PirA toxin was neutralized and mortality was decreased (Boonchuen *et al.* 2018). In another study, when whole egg powders containing either anti-PirA-like IgY or anti-PirB-like IgY were prepared from hens immunized with recombinant PirA-like or PirB-like toxin and used to supplement the basal diets of AHPND-challenged shrimp, survival rates were improved by 86% and 14%, respectively (Nakamura *et al.* 2019). When different doses (15, 30, and 60 ppm) of 5-aminolevulinic acid (5-ALA) were used as a shrimp feed supplement, the survival rates of *L. vannamei* challenged with AHPND-causing *V. parahaemolyticus* were also improved by 95%, 67% and 33% respectively (Pedrosa-Gerasmio *et al.* 2018, 2019). A related study further reported that dietary 5-ALA increased the expression of immune-related genes, increased ATP production and enhanced aerobic energy metabolism (Pedrosa-Gerasmio *et al.* 2018, 2019).

While it is generally considered desirable to maintain a healthy balance of bacteria and algae in the shrimp gut and the culture environment, studies of the microbial community and diversity in the microbiota of shrimp guts and culture ponds have enabled identification of beneficial bacteria that can be used as either probiotics or immunostimulants to improve shrimp health. Pinoargote *et al.* (2018b) isolated pure cultures of *Lactobacillus casei* (lactic acid bacteria), *Rhodopseudomonas palustris* (photosynthetic bacteria) and *Saccharomyces cerevisiae* (yeast) from shrimp gut microbiotas and used these cultures as probiotics. After shrimp were treated with these probiotic cultures and challenged with *V. parahaemolyticus* the microbial composition of shrimp gut and water samples was investigated using NGS, and it was found that the probiotics mitigated the effects of *V. parahaemolyticus*, improved survival and restored microbial diversity in the shrimp gut. Many of the previously cited

Table 3 Summary of treatments for acute hepatopancreatic necrosis disease infection

Shrimp	Effect	Treatment	Mode	Dosage	Challenge	Bacterial pathogen	Bacterial strain	Survival rate (%)	Immune responses	References
Penaeus vannamei	Antibacterial	Blended natural essential oils	Feed	–	Immersion	*Vibrio parahaemolyticus*	VP A/3	53.3	–	Jha *et al.* (2016)
Litopenaeus vannamei	Antibacterial	Recombinant PirA	Immersion	50 mg L^{-1}	Immersion	*V. parahaemolyticus*	No.16	50	–	Campa-Córdova *et al.* (2017)
P. vannamei	Antibacterial	Bacteriophage (pVp-1)	Immersion	1.5×10^6 PFU mL^{-1}	Immersion	*V. parahaemolyticus*	13-028/A3	50	–	Jun *et al.* (2018)
P. vannamei	Antibacterial	Bacteriophage (pVp-1)	Feed	1.5×10^8 PFU mL^{-1}	Immersion	*V. parahaemolyticus*	13-028/A3	75	–	Jun *et al.* (2018)
L. vannamei	Antibacterial	Lorica	Feed	–	Immersion	*V. parahaemolyticus*	–	50	–	Owen *et al.* (2018)
L. vannamei	Probiotic	*Lactobacillus casei*	Feed	9 log10 CFU mL^{-1}	Oral	*V. parahaemolyticus*	13-028/A3	11.67	–	Pinoargote *et al.* (2018b)
L. vannamei	Probiotic	*L. casei* + *Rhodopseudomonas palustris*	Feed	9 log10 CFU mL^{-1}	Oral	*V. parahaemolyticus*	13-028/A3	26.7	–	Pinoargote *et al.* (2018b)
L. vannamei	Probiotic	*L. casei* + *R. palustris* + *Saccharomyces cerevisiae*	Feed	8 log10 CFU mL^{-1}	Oral	*V. parahaemolyticus*	13-028/A3	36.7	–	Pinoargote *et al.* (2018b)
L. vannamei	Probiotic	*Lactobacillus plantarum*	Feed	10^9 CFU g^{-1} feed	Immersion	*V. parahaemolyticus*	XN9	78	–	Nguyen *et al.* (2018)
L. vannamei	Probiotic	*L. plantarum* + *Lactococcus lactics*	Feed	2×10^8 CFU g^{-1} 4×10^8 CFU g^{-1} feed	Immersion	*V. parahaemolyticus*	–	63.3	↑proPO	Chomwong *et al.* (2018)
L. vannamei	Probiotic	*Bacillus licheniformis* *Bacillus flexus*	Feed	2×10^9 CFU g^{-1}	Immersion	*V. harveyi*	GDH11385	80	↑PO ↑lysozyme	Cai *et al.* (2019)
L. vannamei	Probiotic	*Lactobacillus pentosus* *Lactobacillus fermentum* *Bacillus subtilis* *Saccharomyces cerevisiae*	Feed	10^5 CFU g^{-1}	Intramuscular	*V. alginolyticus*	–	50	↑PO	Wang *et al.* (2019)
L. vannamei	Immunostimulant	Gold nanoparticle	Feed	2 μg g^{-1}	Immersion	*V. parahaemolyticus*	IPNGS16	80	↑TLR3 ↑proPO	Tello-Olea *et al.* (2019)
L. vannamei	Immunostimulant	*Dunaliella* sp.	Feed	5% body weight	Immersion	*V. parahaemolyticus*	–	30	↑proPO	Medina Félix *et al.* (2017)
L. vannamei	Immunostimulant	Anti-PirA-IgY	Feed	20% basal diet	Immersion	*V. parahaemolyticus*	D6	86	–	Nakamura *et al.* (2019)
L. vannamei	Immunostimulant	Anti-PirB-IgY	Feed	20% basal diet	Immersion	*V. parahaemolyticus*	D6	14	–	Nakamura *et al.* (2019)
L. vannamei	Immunostimulant	5-ALA	Feed	15 ppm	Immersion	*V. parahaemolyticus*	D6	67	↑NOS ↑catalase	Pedrosa-Gerasmio *et al.* (2018)
L. vannamei	Immunostimulant	5-ALA	Feed	30 ppm	Immersion	*V. parahaemolyticus*	D6	33	↑catalase	Pedrosa-Gerasmio *et al.* (2019)
L. vannamei	Immunostimulant	5-ALA	Feed	60 ppm	Immersion	*V. parahaemolyticus*	D6	50	↑proPO	Pedrosa-Gerasmio *et al.* (2019)

microbiota studies have shown that *Lactobacillus* is abundant in healthy shrimps (Yu *et al.* 2018; Yao *et al.* 2018; Chen *et al.* 2018). When isolated from healthy shrimp hepatopancreas and then used as a probiotic against AHPND, *Lactobacillus* reduced mortality by 28% in AHPND-infected shrimps (Nguyen *et al.* 2018). Similarly, two lactic acid bacterial strains, *Lactobacillus plantarum* and *Lactococcus lactis*, isolated from shrimp stomach have been used as probiotic shrimp feed supplements. These bacterial strains were able to adhere, to and reside in, the shrimp gut, and they were also effective in increasing the proPO expression in haemolymph and resistance to *V. parahaemolyticus* (Chomwong *et al.* 2018). Two other bacterial strains, *Bacillus licheniformis* (LS-1) and *B. flexus* (LD-1), were recently identified as probiotics based on screening that selected for following criteria: tolerance to bile salts, to gastrointestinal stress and to haemolytic activities as well as exhibiting an antibacterial capacity against *Vibrio* pathogens (Cai *et al.* 2019). The efficacy of these two probiotic bacterial strains was then confirmed using biochemical assays and animal experiments in which *L. vannamei* were fed with either one of these probiotics or else with both of them together. These treatments resulted in improved shrimp growth, immunity and disease resistance (Cai *et al.* 2019). Another study showed that feed supplemented with multiple strains of probiotic bacteria (*L. pentosus*, *L. fermentum*, *Bacillus subtilis* and *S. cerevisiae*) were effective in increasing growth, immunity and resistance against *V. alginolyticus* in *L. vannamei* (Wang *et al.* 2019). In shrimp aquaculture, microalgae are also used as immunostimulants. Shrimps fed with *Dunalelia* sp., a microalga rich in carotene, and then challenged with *V. parahaemolyticus* showed an increased survival rate (Medina Félix *et al.* 2017). Recently, Owen *et al.* (2018) reported that a commercial functional diet called Lorica (Skretting, Stavanger, Norway) improved the survival of AHPND-infected *L. vannamei* by ~50%. It was also reported that using ozone nanobubbles to disinfect artificial sea water can kill up to 100% of AHPND-causing *V. parahaemolyticus*

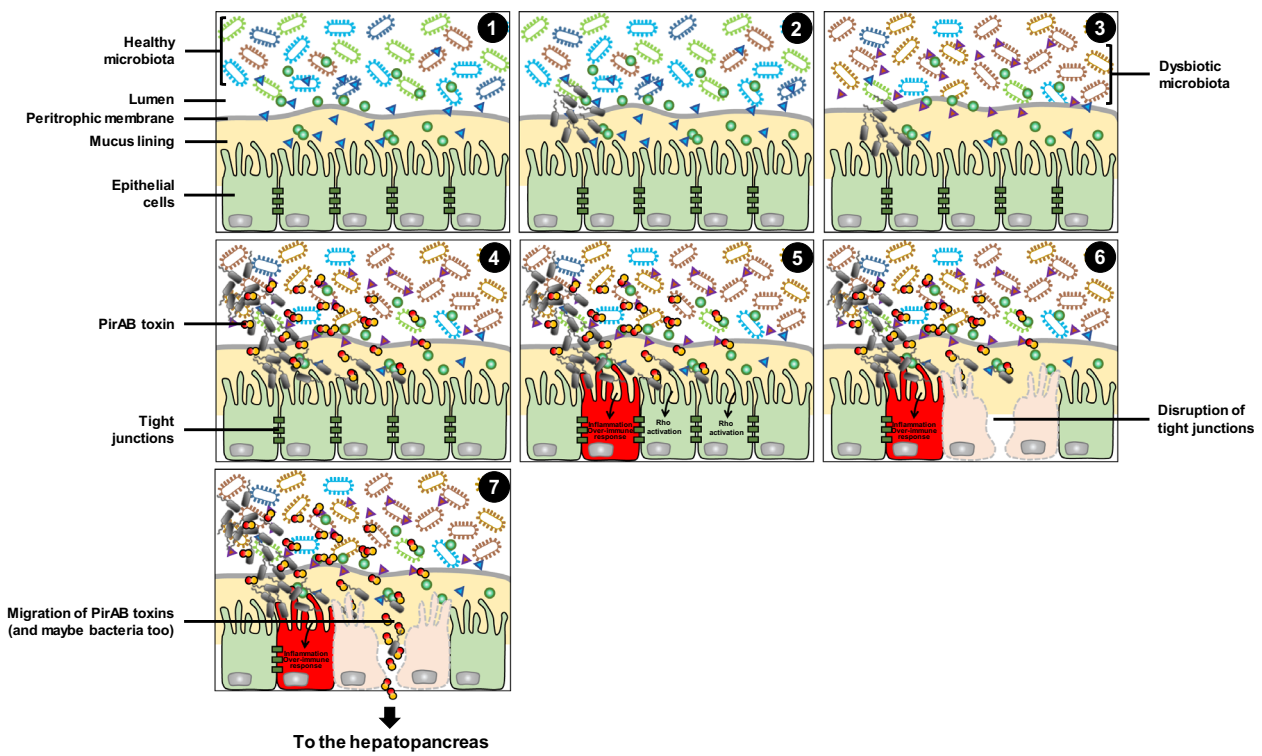

Figure 5 A model of acute hepatopancreatic necrosis disease (AHPND) pathogenesis. (i) Under normal, healthy conditions, the shrimp stomach is composed of epithelial cells, a chitinous layer/mucus lining, peritrophic membrane and a lumen that contains a benign community of gut microbiota. The gut microbiota and shrimp cells release metabolites (circles and triangles) which help in the normal functioning of the digestive processes. (ii) AHPND-causing *Vibrio parahaemolyticus* enters the shrimp stomach through an oral route. (iii) The pathogenic, AHPND-causing bacteria release their own metabolites into the lumen, causing the previously healthy gut microbiota to become dysbiotic. (iv) After replication and colonization, the *V. parahaemolyticus* release PirAB^{VP} toxins into the lumen. (v) The PirABVP toxins trigger an immune response and activation of Rho pathway. (vi) Activation of Rho-signalling pathway disrupts the tight junctions between the stomach epithelial cells, allowing the formation of intercellular gaps. (vii) The PirAB^{VP} toxins and *V. parahaemolyticus* bacteria migrate to the hepatopancreas through the intracellular gaps.

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(Imaizumi *et al.* 2018). A summary of the currently known AHPND treatments is listed in Table 3.

Pathogenesis of AHPND

Despite the progress in AHPND-related research, we do not yet have a detailed understanding of how *V. parahaemolyticus* colonizes the shrimp stomach nor at present (*pace* Lai *et al.* 2015) is there any experimental evidence to show definitely that the AHPND-causing bacteria themselves subsequently migrate to the hepatopancreas. However, Ng *et al.* (2018) found that during AHPND infection, there is a loss of integrity of the brush border of the shrimp anterior midgut and that Rho activation plays a critical role in the disintegration of the epithelial cells in the shrimp stomach. Here, we further propose that these processes might allow not only the toxin but also the AHPND-causing bacteria themselves to reach the hepatopancreas. Below, we elaborate on this proposed aetiology to construct a more comprehensive model of AHPND pathogenesis that also incorporates dysbiotic microbiota.

In this proposed model (Fig. 5), the shrimp stomach is composed of epithelial cells, a chitinous layer/mucus lining bounded by the peritrophic membrane and the stomach lumen itself, which normally contains a high proportion of beneficial bacteria and various metabolites released by both bacteria and shrimp cells. The entry of AHPND-causing *V. parahaemolyticus* into this environment leads to dysbiosis either else because of direct competition for survival or else because of the release of harmful metabolites into the stomach lumen. These dysbiotic conditions enable the *V. parahaemolyticus* to further replicate, colonize and release PirAB toxin, which in turn causes inflammation and an increased immune response in the shrimp stomach cells. At the same time, by an unknown mechanism, the *V. parahaemolyticus* also activates the Rho pathway. This causes disruption of the cell junctions and disintegrates the epithelial lining to produce intercellular gaps that allow the PirAB toxin and bacteria to reach the hepatopancreas.

While the details of this model await further experimental testing and verification, we also note that we presently lack a full understanding of the cross-talk between the AHPND-causing bacteria, the gut microbiome, the host's stress responses and even immune regulation in the shrimp stomach. On the other hand, if the entire microbial population could be accurately characterized, we could then explore ways to enhance the healthy gut microbiome by manipulating the composition of the bacteria with probiotics and perhaps other natural compounds. Ultimately, a detailed understanding of these mechanisms might allow us to use innovative and specific microbiome-mediated strategies to prevent or at least mitigate the effect of shrimp diseases.

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