

# A potential prokaryotic and microsporidian pathobiome that may cause shrimp white feces syndrome (WFS)

Anuphap Prachumwat<sup>1,2#</sup>, Natthinee Munkongwongsiri<sup>1#</sup>, Wiraya Eamsaard<sup>1,2</sup>,  
Kanokwan Lertsiri<sup>1</sup>, Timothy W. Flegel<sup>2,3</sup>, Grant D. Stentiford<sup>4,5</sup>,  
Kallaya Sritunyalucksana<sup>1,2\*</sup>

<sup>1</sup>Aquatic Animal Health Research Team, Integrative Aquaculture Biotechnology Research Group, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Yothi office, Rama VI Rd., Bangkok, Thailand 10400

<sup>2</sup>Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, Thailand 10400

<sup>3</sup>National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Klong Luang, Pathumthani, 12120, Thailand

<sup>4</sup>International Centre of Excellence for Aquatic Animal Health, Centre for Environment Fisheries and Aquaculture Science (Cefas), Weymouth Laboratory, Weymouth, Dorset DT4 8UB, UK

<sup>5</sup>Centre for Sustainable Aquaculture Futures, University of Exeter, College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QD, UK

#These authors equally contributed to this work.

\*Corresponding author, E-mail: [kallaya@biotec.or.th](mailto:kallaya@biotec.or.th)

## Highlights

- White feces syndrome (WFS) shrimp often harbor the microsporidian *Enterocytozoon hepatopenaei* (EHP)
- The hepatopancreas (HP) and midgut of EHP-WFS shrimp had more EHP copies and spores than EHP-non-WFS shrimp
- *Vibrio* spp., *Propionigenium* sp. and EHP dominated in HP microbiomes of EHP-WFS shrimp
- *Propionigenium* copy numbers were uniquely high in the HP of EHP-WFS shrimp
- EHP-WFS shrimp also showed intestinal microbiomes of reduced diversity but more heterogeneity

## 33 Abstract

34 White feces syndrome (WFS) in shrimp cultivation ponds is characterized by the occurrence of  
35 shrimp with abnormal, white intestines (midguts) combined with large floating mats of white,  
36 shrimp fecal strings. The etiology for WFS is complex, similar to diarrhea in humans. EHP-WFS  
37 is a type of WFS characterized by massive quantities of spores from the microsporidian parasite  
38 *Enterocytozoon hepatopenaei* (EHP) together with mixed, unidentified bacteria in the shrimp  
39 hepatopancreas, midgut and fecal strings. However, WFS does not always develop in shrimp with  
40 severe EHP infections in controlled laboratory challenges. Further, in EHP-WFS outbreak ponds,  
41 some shrimp show white midguts (WG) while others in the same pond show grossly normal  
42 midguts (NG). We hypothesized that comparison of the microbial flora between WG and NG from  
43 the same EHP-WFS pond would reveal probable combinations of microbes significantly  
44 associated with EHP-WFS. To test this hypothesis, we selected a pond exhibiting a severe EHP-  
45 WFS outbreak in cultivated *Penaeus vannamei* and used a combination of microscopic and  
46 microbial profiling analyses to compare WG and NG samples. By histology, EHP plasmodia and  
47 spores were confirmed in the hepatopancreas (HP) and midgut of WG and NG shrimp, but  
48 pathological severity and spore quantity was higher in the WG shrimp. In addition, intestinal  
49 microbiomes in WG shrimp were less diverse and had higher abundance of bacteria from the  
50 genera *Vibrio* and *Propionigenium*. *Propionigenium* quantity in the HP of WG shrimp was  
51 significantly higher ( $P = 1.08e-5$ ) than in NG shrimp (4,506 vs. 3 copies /100 ng DNA,  
52 respectively). These findings supported our hypothesis by revealing two candidate bacterial genera  
53 that should be tested in combination with EHP as a potential eukaryote-prokaryote pathobiome  
54 that causes EHP-WFS in *P. vannamei*.

55

56 Keywords: White feces syndrome (WFS), *Penaeus vannamei*, *Enterocytozoon hepatopenaei*  
57 (EHP), EHP-WFS, *Propionigenium*, *Vibrio*

58

## 59 1. Introduction

60 Shrimp cultivation ponds exhibiting white feces syndrome (WFS) are characterized by the  
61 occurrence of shrimp with abnormal, white intestines (midguts) combined with floating mats of  
62 white, shrimp fecal strings. The contents of the midguts and fecal strings vary among WFS  
63 outbreak ponds, but also frequently contain a mixed bacterial component. These two features

64 indicate that WFS has a complex etiology similar to that outlined for other syndromic conditions  
65 in shrimp (Kooloth Valappil et al., 2021) and for animal and plant diseases more generally (Bass  
66 et al., 2019).

67  
68 One type of WFS is characterized by the massive transformation and sloughing of microvilli from  
69 epithelial cells of tubules of the shrimp hepatopancreas (HP). These sloughed microvilli aggregate  
70 in the tubule lumens as vermiform bodies called aggregated, transformed microvilli (ATM) that  
71 superficially resemble gregarines (Sriurairatana et al., 2014). They accumulate in masses at both  
72 the HP center and the midgut and are excreted as white fecal strings that float because of high fat  
73 content. The causal mechanism for ATM formation is still unknown. This type of WFS is of  
74 relatively infrequent occurrence because ATM, although frequently produced, do not often  
75 accumulate in sufficient quantity to cause WFS. Even when they do, the WFS is not associated  
76 with severe mortality or other serious production problems (Sanguanrut et al., 2018). In our  
77 experience, white midguts may also be caused by heavy gregarine infections, severe *Vibrio*  
78 infections and hemocytic enteritis caused by ingested blue-green algae (Anjaini et al., 2018;  
79 Somboon et al., 2012), but they are not usually associated with accumulation of floating fecal mats.  
80 Thus, reports of WFS that are not accompanied by at least microscopic confirmation cannot be  
81 ascribed to any particular causative agent.

82  
83 The type of WFS examined in this study is characterized by the presence in the shrimp midgut and  
84 in white fecal strings of sloughed hepatopancreatic cells, tissue debris and massive quantities of  
85 spores from the microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) (Tourtip et al.,  
86 2009). Shrimp exhibit white to yellow-golden intestines, loose exoskeletons, reduced feeding and  
87 retarded growth, high size variation, reduced average daily growth, elevated feed conversion ratios  
88 and sometimes mortality. This type of WFS (here referred to as EHP-WFS) was first reported from  
89 Vietnam (Ha et al., 2010), but it was soon realized that EHP is not always associated with WFS  
90 and that shrimp can recover from WFS but remain infected with EHP (Flegel, 2012;  
91 Tangprasittipap et al., 2013).

92  
93 EHP-WFS is currently being reported from China (Shen et al., 2019; Wang et al., 2020), Southeast  
94 Asia (Caro et al., 2020; Desrina et al., 2020; Flegel, 2012; Ha et al., 2010; Sajiri et al., 2021; Tang

95 et al., 2016) and South Asia (Rajendran et al., 2016). It typically occurs after 40 days of culture.  
96 In Thailand, EHP-WFS occurrence has significantly increased across all aquaculture regions in  
97 recent years, and it economically threatens Thai shrimp production due to combined losses from  
98 retarded growth and sometimes mortality.

99  
100 Histopathology in the HP of EHP-WFS shrimp can be distinguished from that of usual EHP  
101 infections by the massive, simultaneous production and release of spores by cell lysis, together  
102 with sloughing of whole cells containing spores and sometimes unidentified bacterial cells.

103 Altogether, this results in a loss of integrity of the HP tubule epithelium and may be  
104 accompanied by some shrimp mortality. The spores, sloughed cells and debris from lysed cells  
105 accumulate in the midgut making it and the fecal strings white. This process does not normally  
106 occur even with severe EHP infections in the laboratory where cells with pre-spore plasmodia  
107 greatly outnumber cells that produce spores (Chaijarasphong et al., 2020; Flegel, 2012). In  
108 addition, the cells that do lyse to release spores normally do so in a dispersed manner over time  
109 that allows for cell renewal and leaves the HP structure more-or-less intact. This allows for long-  
110 term infections that result in no external signs of disease but may cause retarded growth.

111  
112 Since WFS is not always associated with EHP infections and cannot be reproduced in the  
113 laboratory in controlled challenge tests, it is possible that EHP may be a component cause of EHP-  
114 WFS, with EHP a necessary but insufficient solo cause of WFS. We previously hypothesized  
115 (Chaijarasphong et al., 2020) that WFS might be induced in shrimp with severe EHP infections  
116 via some unknown causative signal that induced simultaneous production of spores by all or most  
117 of the EHP plasmodia. This opened questions regarding the environmental factors and/or  
118 pathobiomes that might lead to EHP-WFS. It has previously been reported that EHP spores in  
119 white shrimp midguts, in white fecal strings and in severely infected HP tissue are frequently  
120 accompanied by bacterial cells of varied morphology (Tangprasittipap et al., 2013; Thitamadee et  
121 al., 2016). Thus, it is possible that the missing component-cause(s) of EHP-WFS may be bacterial  
122 in nature.

123  
124 To investigate the possibility that the cause of EHP-WFS is a pathobiome that includes a eukaryote  
125 and prokaryote bacteria, we took advantage of the fact that during EHP-WFS outbreaks some of

126 the shrimp in the pond show white midguts (WG) while others show grossly normal midguts (NG).  
127 We hypothesized that comparison of the microbial flora between WG and NG shrimp from the  
128 same EHP-WFS pond would reveal probable combinations of microbes significantly associated  
129 with EHP-WFS. To test this hypothesis, we used a combination of histopathological analysis and  
130 high-throughput 16S rRNA amplicon sequencing analysis of bacterial microbiomes to compare  
131 the HP and guts of WG and NG shrimp. Our comparative analyses showed distinct characteristics  
132 that separated WG and NG shrimp and revealed a significant association between EHP-WFS and  
133 dominant bacterial taxa of the genera *Vibrio* and *Propionigenium*.

134

## 135 **2. Material and methods**

### 136 ***2.1 Shrimp sample collection***

137 A *P. vannamei* shrimp cultivation pond exhibiting a WFS outbreak was chosen because of the high  
138 burden of EHP accompanied by some shrimp mortality. The pond was located in Chanthaburi  
139 Province, Thailand (see Table S1). It was completely polyethylene-lined and was at 27 days of  
140 culture with shrimp average weight 8.70 g and average daily growth of 0.32 g/day. Samples were  
141 collected on the 5<sup>th</sup> day after the WFS outbreak began. Shrimp with white midguts (WG) and  
142 shrimp with grossly normal (digestive tracks) guts (NG) were arbitrarily selected and subjected to  
143 comparative histopathological, molecular and microbiome analyses. Altogether, 15 WG and 15  
144 NG shrimp were collected, 10 each for microbiome and molecular analyses and 5 each for  
145 histopathological examination. Samples were collected under the approved protocol No. BT  
146 07/2561 from BIOTEC Institutional Animal Care and Use Committee (IACUC).

147

### 148 ***2.2 Microbiome and molecular analyses***

149 Each shrimp was dissected to remove the gastrointestinal tract for separate collection of the  
150 stomach, hepatopancreas and midgut (intestine) in 1.5 ml tubes containing 500 µl of lysis buffer  
151 (50 mM Tris pH 9, 0.1M EDTA pH 8, 50 mM NaCl, 2% SDS, 100 µg/ml proteinase K) for DNA  
152 extraction using a QIAamp® DNA Mini Kit (Qiagen). DNA samples were used for bacterial  
153 profiling with high-throughput 16S rRNA amplicon sequencing and quantitative polymerase chain  
154 reactions.

155

156

### 157 **2.3 Histopathological analysis**

158 Shrimp specimens were prepared for histological examination by standard methods (Bell,  
159 Lightner, 1988). Briefly they were fixed in Davidson's AFA fixative for 18-24 hours before  
160 transfer to 70% ethanol before tissue processing, embedding in paraffin, sectioning (4  $\mu$ m thick)  
161 and staining with hematoxylin and eosin (H&E). Slides were examined using a Leica DM 750  
162 equipped with a Leica ICC50 W digital camera.

163

### 164 **2.4 High-throughput 16S rRNA amplicon sequencing**

165 DNA samples were sent for quality control, Illumina library preparation and sequencing at  
166 Macrogen, Inc. (South Korea). Amplicons from the V3-V4 variable region of bacterial 16S rRNA  
167 were obtained using forward (5'-CCTACGGGNGGCWGCAG-3') and reverse (5'-  
168 GACTACHVGGGTATCTAATCC-3') primers (Herlemann et al., 2011) and used for sequencing  
169 library preparation with a Herculase II Fusion DNA Polymerase Nextera XT Index Kit V2. Library  
170 concentration and size distribution were quantified with TapeStation D1000 before sequencing  
171 with the Illumina MiSeq platform using the 2x300 paired end format.

172

### 173 **2.5 Analysis of microbiomes**

174 The raw sequencing reads were trimmed to remove primer sequences by Cutadapt  
175 (<https://cutadapt.readthedocs.io/>) and later processed using QIIME2 (version 2019.7.0) (Bolyen et  
176 al., 2019) with dada2 denoise-paired (Callahan et al., 2016) with truncated lengths of 280 and 235  
177 base pairs for forward and reverse reads, respectively, to produce a set of amplicon sequence  
178 variants (ASVs). Taxonomic classification of ASVs was performed with USearch against an RDP  
179 database (Edgar, 2010). ASVs were imported into R and filtered for ASVs found in  $\geq 2$  samples  
180 and of either  $\geq 1\%$  or  $\geq 0.1\%$  abundance for further analyses. The analyses of  $\geq 1\%$  abundance ASVs  
181 are presented in the main text, whereas those of the  $\geq 0.1\%$  abundance ASVs are given in the  
182 Supplementary Information. Filtered ASV sets were processed with either a compositional data  
183 (CoDa) analysis approach (Gloor et al., 2017) that examines the ratios between ASVs or a standard  
184 count data analysis. For the CoDa approach, zero count ASVs were replaced using the  
185 zCompositions R package (Palarea-Albaladejo, Martín-Fernández, 2015), transformed with the  
186 centered log ratio transform after which a singular value decomposition (SVD) was applied for  
187 principal-component analysis (PCA) plots; differential abundance tests for ASVs were performed

188 with the ALDEx2 v1.6.0 Bioconductor package using significantly abundant ASVs of an expected  
189 effect size difference of  $\geq 1$  (Fernandes et al., 2014). For standard count data analysis, we used  
190 phyloseq (McMurdie, Holmes, 2013) and microbiome  
191 (<http://microbiome.github.com/microbiome>) packages for alpha diversity index calculation and  
192 non-metric multidimensional scaling (NMDS) with a Bray-Curtis dissimilarity distance and  
193 EdgeR (McCarthy et al., 2012) or DESeq2 (Love et al., 2014) packages for differential abundance  
194 tests (FDR < 0.05). Additional graphics were plotted with vegan  
195 (<https://github.com/vegandevs/vegan/>), ggplot2 (<https://ggplot2.tidyverse.org>) and ggpubr  
196 (<https://rpkgs.datanovia.com/ggpubr/>) packages.

197

## 198 ***2.6 Molecular quantification with quantitative polymerase chain reactions***

199 Quantitative polymerase chain reactions (qPCR) were used to quantify copy numbers of EHP and  
200 selected *Propionigenium* taxa per 100ng of total DNA extracted. Each qPCR reaction was  
201 performed in a total volume of 20  $\mu$ L, consisting of 10  $\mu$ L 2X KAPA SYBR FAST qPCR Master  
202 Mix (KAPA Biosystems, USA), 0.2  $\mu$ M of forward primer, 0.4  $\mu$ L Low ROX, 100 ng of template  
203 DNA and a volume of water to the final volume 20  $\mu$ L. Primers for EHP were described in  
204 Jaroenlak et al. (2016) and in Kanitchinda et al. (2020), whereas those for *Propionigenium* taxa  
205 were designed in this study - PG16S-F (5'-TGGACAATGGACCAAAAGTCTG-3') and PG16S-  
206 R (5'-TTCAGCGTCAGTATTCATCCAG-3'). DNA templates for standard curve construction  
207 were derived from purified target fragments with the same sets of corresponding primers in  
208 different estimated copy numbers of ten-fold dilutions from  $10^8$  to  $10^2$  copies/1uL. Amplifications  
209 for qPCR measurement were carried out using a 7500 Fast Real-time PCR System (Applied  
210 Biosystems, USA) with the following conditions: for EHP, 2 min at 94 °C, followed by 40 cycles  
211 of 30 s at 94 °C, 30 s at 64 °C, and 30 s at 72 °C; and for *Propionigenium* taxa, 3 min at 95 °C,  
212 followed by 40 cycles of 15 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. No template control and  
213 DNA samples of HP, midguts and standard curves were obtained in triplicate reactions. Melting  
214 curve and standard curve analyses evaluated specificity of the reactions to obtain estimated copy  
215 numbers of samples with an automatic software-assigned baseline and a manually-set threshold at  
216 0.145 using the ABI PRISM® 7500 Sequence Detection System software (v2.3).

217

218

## 219 **3. Results**

### 220 ***3.1 WFS pond clinical signs and histopathology of shrimp gastrointestinal tracts***

221 WG shrimp had whitish gastrointestinal tracts including the stomach, HP and entire intestine. They  
222 also exhibited loose and soft shells. In contrast, NG shrimp appeared grossly normal (Fig. S1).  
223 Histopathological examination of WG and NG shrimp revealed both shared and different  
224 abnormalities. Shared abnormalities included atrophied cells and EHP spores within the HP tubule  
225 epithelial cells and in epithelial cells of the midgut region located within the HP (Figs. 1 and 2).  
226 Focal lesions comprising shrimp hemocytes encapsulating of aggregated EHP spores were also  
227 observed in both groups (Fig. 1A). Specific characteristics observed in WG shrimp were 1) a  
228 higher prevalence of EHP plasmodia and spores within the HP and midgut epithelial cells (Figs. 1  
229 and 2), and 2) a higher burden of free EHP spores, sloughed HP cells and rod-shaped bacterial  
230 cells in the midgut lumen (Fig. 2A and inset).

231

### 232 ***3.2 Comparison of intestinal microbiomes between WG and NG shrimp***

233 Raw read pairs (5,974,443) were filtered to produce 2,254 amplicon sequence variants (ASVs)  
234 with QIIME2 DADA2 de-noise. These ASVs were filtered for only those found in  $\geq 2$  samples and  
235 of either  $\geq 1\%$  or  $\geq 0.1\%$  abundance. Initial examination of the two filtered datasets by principal-  
236 component analysis (PCA) and non-metric multi-dimensional scaling (NMDS) revealed that WG  
237 and NG samples had different bacterial profiles, except for one WG sample (F8) that closely  
238 clustered with NG samples (Supplementary Figs. S2, S3, S4 and S5; and Materials and Methods).  
239 PCA on the centered log-transformed data of the samples and the associated loadings for the  $\geq 1\%$   
240 abundance ASV dataset (Fig. 3) revealed that intestinal bacterial communities between WG and  
241 NG shrimp differed markedly, except for the one WG shrimp sample (F8) that was more similar  
242 to the NG group. For the subsequent analyses, comparisons were made between the two groups:  
243 WG group of 12 sequenced library samples and NG group of 15 sequenced library samples  
244 (Supplementary Table S2), although similar trends were obtained when the F8 sample was  
245 included (data not shown).

246

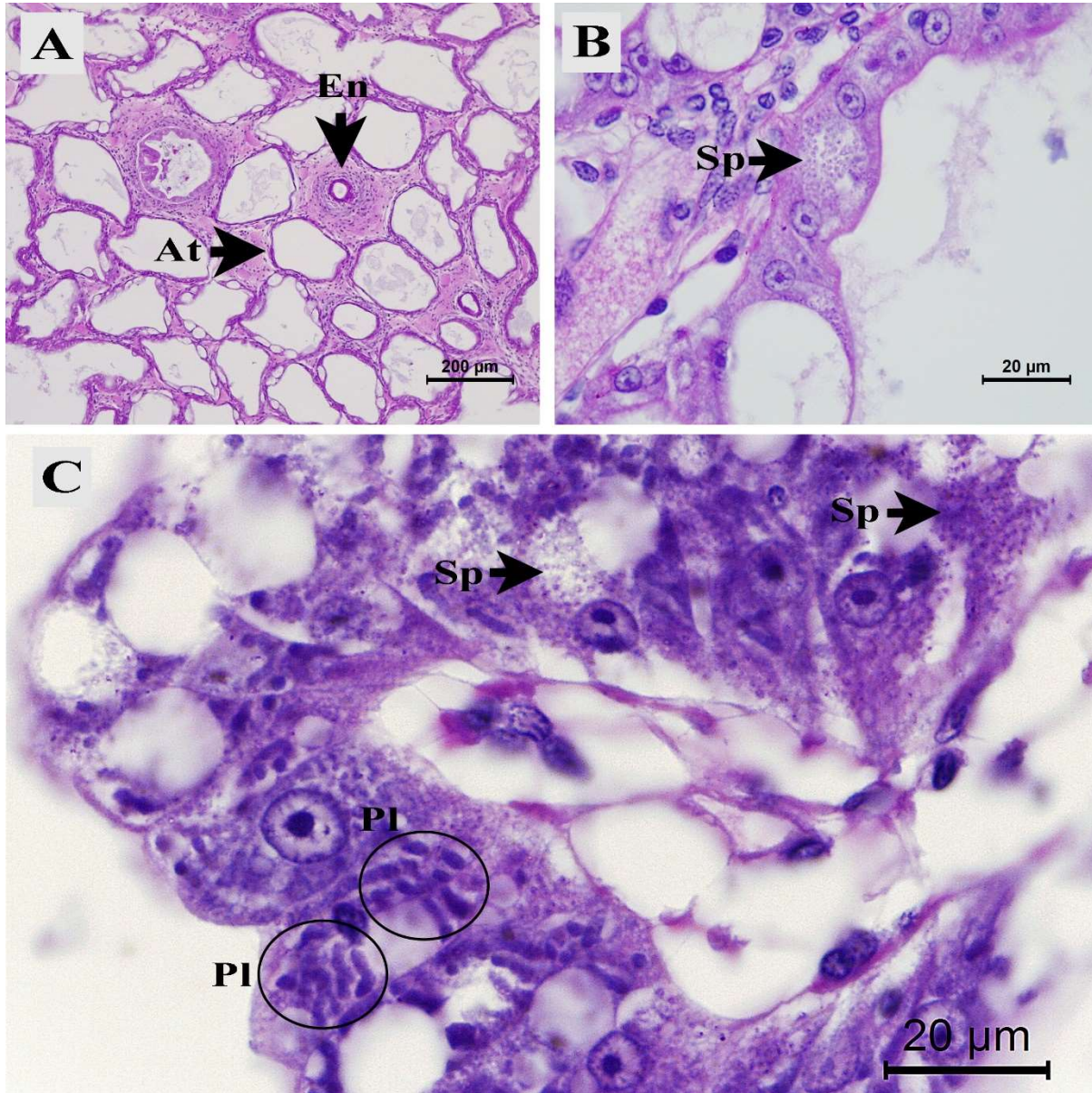
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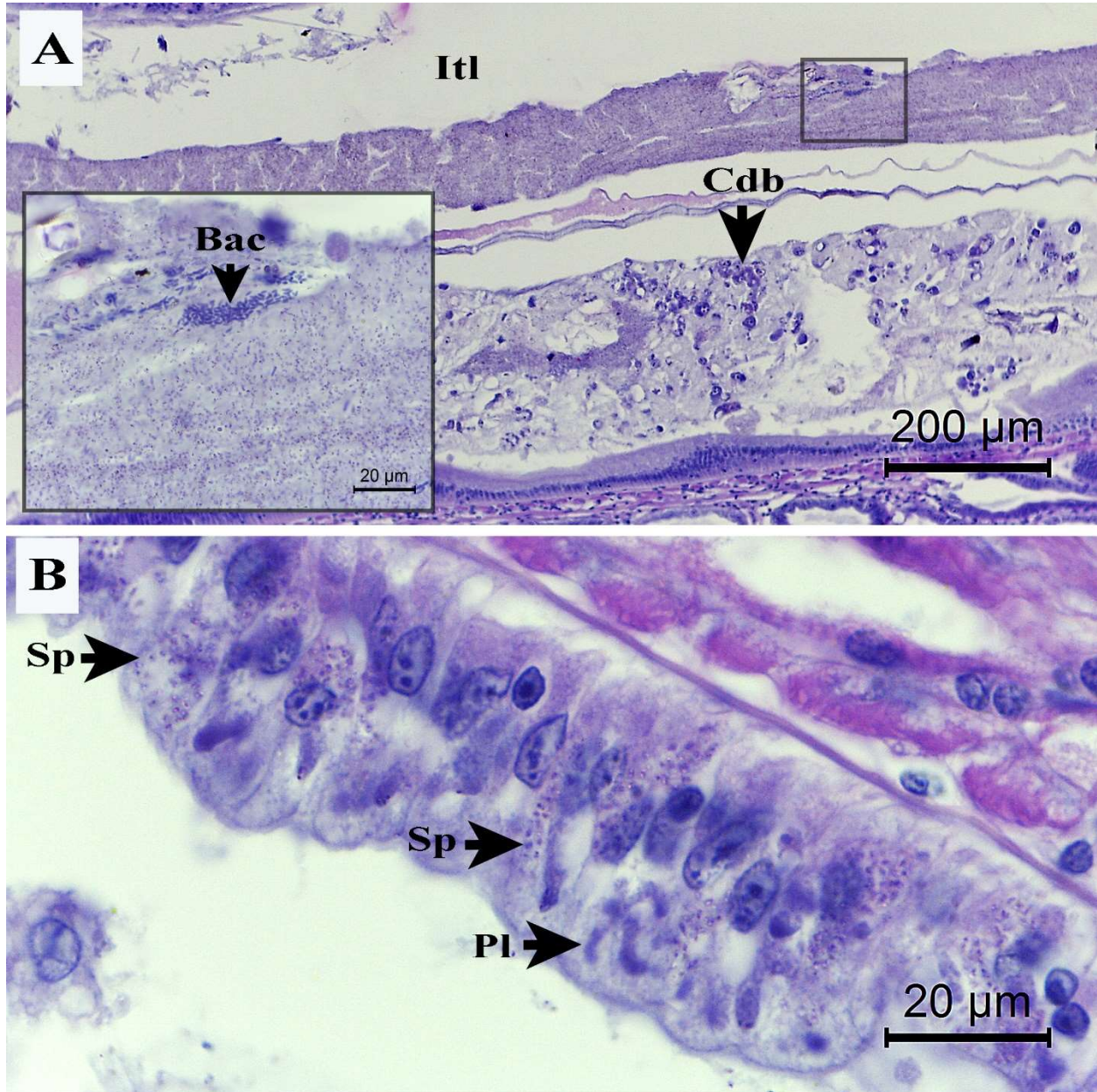


250 **Figure 1.** Photomicrographs of histological characteristics of white midgut (WG) and normal  
251 midgut (NG) shrimp hepatopancreatic tissues. Similar characteristics of WG and NG shrimp were  
252 (A) atrophied cells (At) of the hepatopancreatic epithelial tubule and hemocytic encapsulation  
253 (En), (B) EHP spores (Sp) in hepatopancreatic epithelial cells and (C) high prevalence of  
254 plasmodia (Pl) and spores (Sp) in hepatopancreatic tubule epithelia.



255

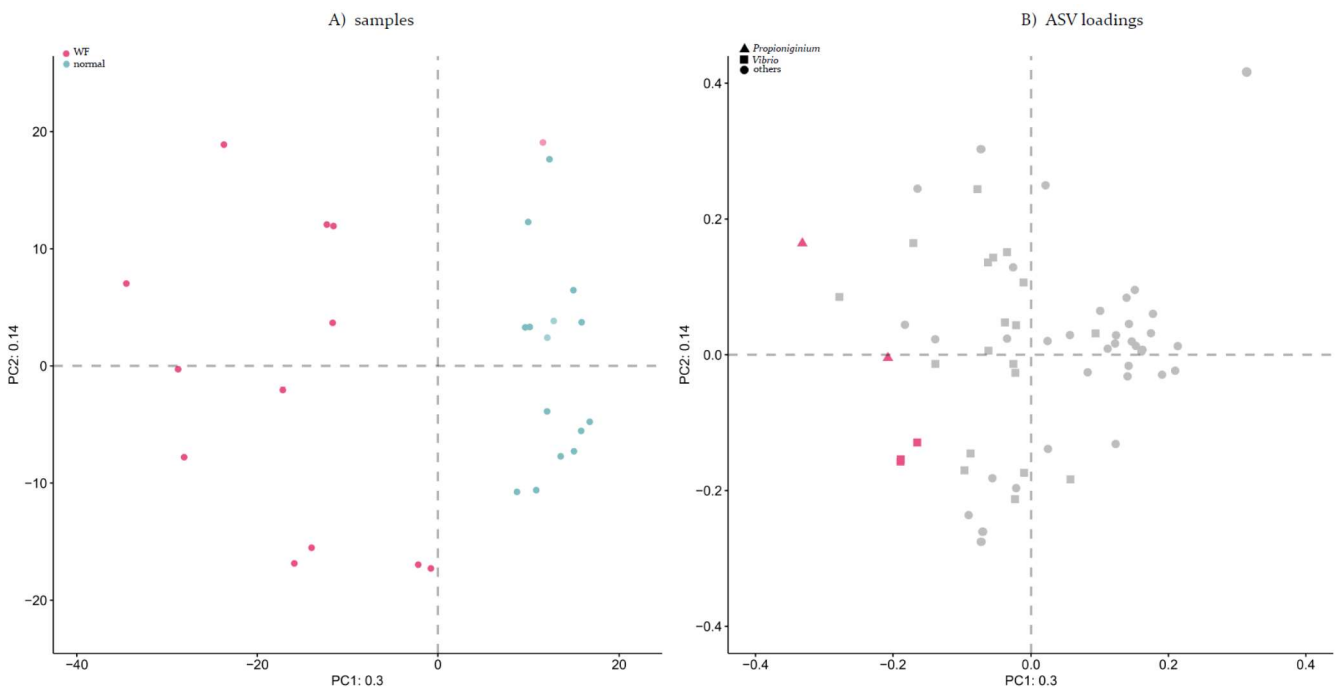
256 **Figure 2.** Photomicrographs of the midgut of white feces shrimp. **(A and inset)** Midgut lumen  
257 (Itl) containing HP epithelial cell debris (Cdb), colonies of rod-shaped bacteria (Bac) and masses  
258 of *Enterocytozoon hepatopenaei* (EHP) spores. **(B)** Spores (Sp) and plasmodia (Pl) of EHP-  
259 infected epithelial cells of the midgut. Note that the midgut epithelium is relatively normal and  
260 intact, despite the presence of EHP stages in some cells.



261

262

263 **Figure 3.** Compositional PCA plot of samples (A) and ASV loadings (B) for the  $\geq 1\%$  abundance  
264 ASV dataset (Materials and Methods). In panel A, each point is a sample [colored for WG (red)  
265 and NG (blue) shrimp groups; Table S1] and the distance between points is proportional to the  
266 multivariate difference between samples. Panel B shows the loadings for panel A in the same  
267 coordinate space, which represents the contributions of the ASVs to the separation of the samples.  
268 In this plot, each point is an ASV (shaped by taxonomic genus and colored by its assigned  
269 significantly higher abundance ASVs in the WG group (red) and the distance and direction from  
270 the origin to the point representing an ASV is proportional to the standard deviation of that ASV  
271 in the data set. The distance between one ASV and another is inversely proportional to their  
272 compositional association: points that are close together may have concordant relative abundances  
273 across all samples. The ability to directly interpret the plot is limited by the proportion of variance  
274 explained (30% on the first component and 14% on the second component).  
275  
276

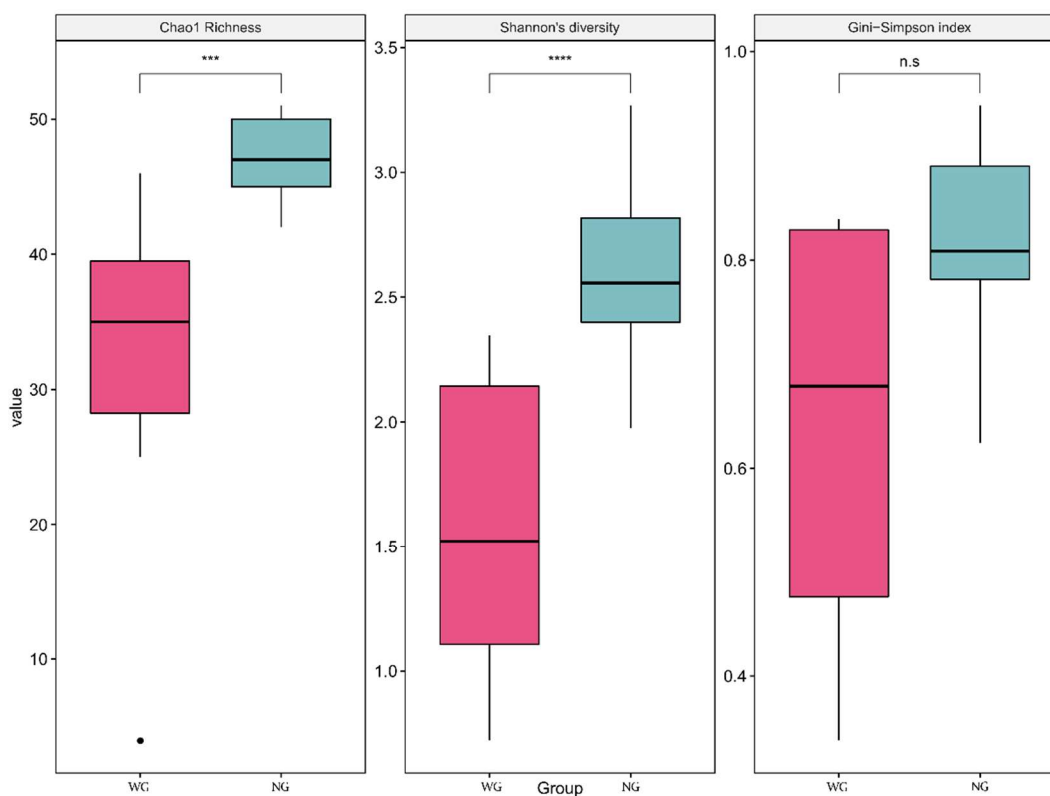


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278

279 A significantly lower alpha-diversity was observed in WG than in NG samples (Chao1's Richness  
280 index,  $P = 2.5e-5$ ; Shannon's diversity index,  $P = 3.8e-7$ ; Gini-Simpson index,  $P = 3e-3$ ; Fig. 4).  
281 On average, distances among WG samples (on PCA and NMDS) were larger than those of the NG  
282 samples (Fig. 3 and Supplementary Figs. S2, S3, S4 and S5), suggesting more variation in bacterial  
283 communities between individual shrimp in the WG group than those in the NG group, i.e. the WG  
284 group was more heterogeneous.

285  
286 **Figure 4.** Comparison of alpha-diversity between the WG shrimp group (n = 12, red) and the NG  
287 shrimp group (n = 15, green) with Chao1's Richness index, Shannon's diversity index, and Gini-  
288 Simpson index. Significant differences are given by asterisks (n.s.,  $P \geq 0.05$  ; \*\*\*,  $0.0001 \leq P <$   
289  $0.001$ ; \*\*\*\*,  $P < 0.0001$ ).

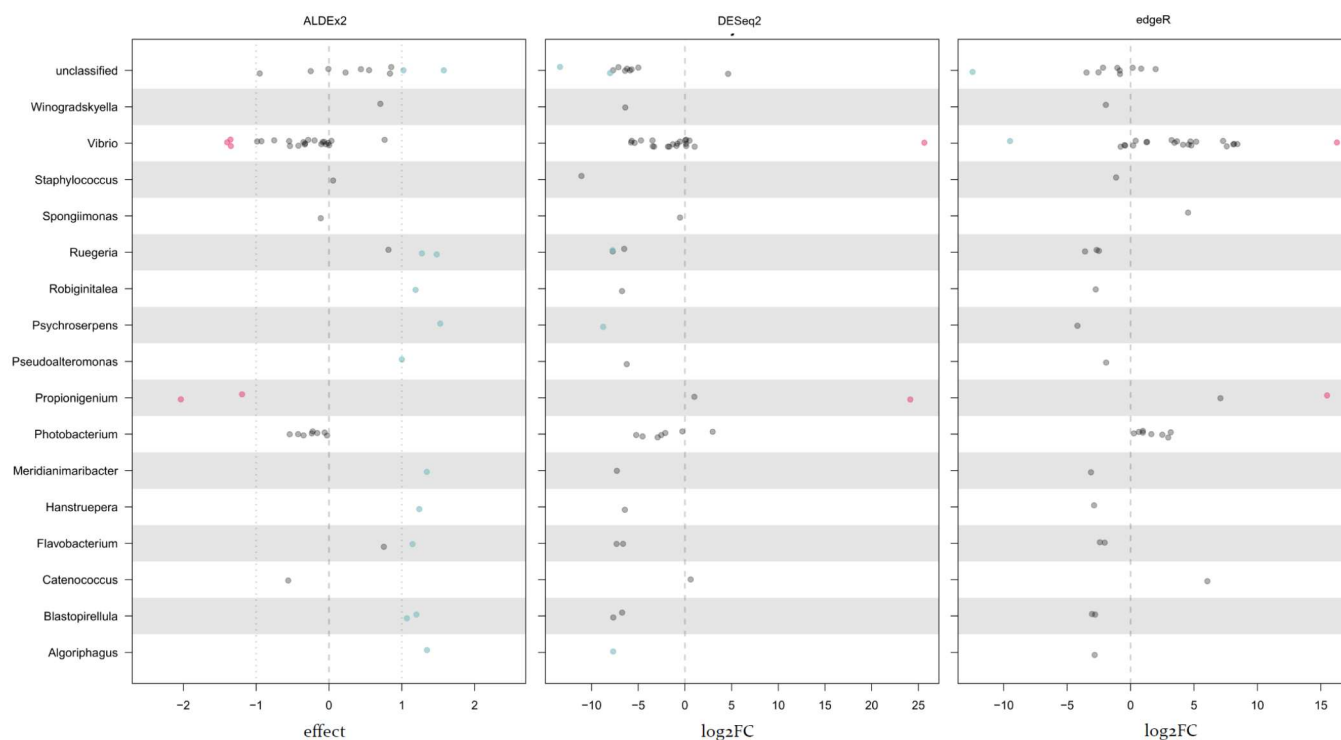


290

291

292 To determine bacterial taxa associated with EHP-WFS shrimp, we analyzed for significantly over-  
293 represented ASVs in WG samples (see Materials and Methods). ASVs of the genera *Vibrio* and  
294 *Propionigenium* were found with significantly higher-fold changes across WG shrimp samples  
295 than NG samples (Figs. 3 and 5; see Materials and Methods). The average fold changes of over-  
296 represented abundances in WG over NG samples for genus *Vibrio* ASVs were 42.2 - 50.7 (3  
297 ASVs), 5.2e7 (1 ASV), and 7.9e4 (1 ASV), from Aldex2, DESeq2 and EdgeR, respectively, while  
298 those for genus *Propionigenium* were 130 - 2004 (2 ASVs), 1.8e7 (1 ASV), 4.6e4 (1 ASV) from  
299 Aldex2, DESeq2 and EdgeR, respectively (Fig. 5). Similar ASVs relating to these *Vibrio* and  
300 *Propionigenium* taxa and some additional genera were also obtained with the other ASV datasets  
301 (see Materials and Methods and Supplementary Table S3).

302  
303 **Figure 5.** Differential relative abundance of ASVs binned by genus determined by (A) ALDEx2,  
304 (B) DESeq2 and (C) EdgeR. Points are colored as red or blue if they are significantly abundant in  
305 the WG or NG shrimp groups, respectively (Materials and Methods).



306  
307  
308 We focused on the significantly WG-over-represented ASVs of *Vibrio* and *Propionigenium* for  
309 further investigation relating to their significance in EHP-WFS. The sequences of significantly

310 over-represented *Propionigenium* ASVs in WG samples all matched with *P. maris* with high  
311 identity (99.75% to those of *P. maris* or identical to those of uncultured and identified  
312 *Propionigenium* sp., which are likely strains of *P. maris*) such that specific primer sequences could  
313 be designed to compare abundance of *Propionigenium* in the shrimp gut and HP. However,  
314 significantly WG-over-represented *Vibrio* ASV sequences all matched records for multiple  
315 members of the *Vibrio harveyi* clade due to the short 16S rRNA region targeted. So also did the  
316 non-WG-over-represented *Vibrio* ASVs. Thus, it was not possible to make a species specific  
317 primer pair for comparative quantification of WG-over-represented *Vibrio*.

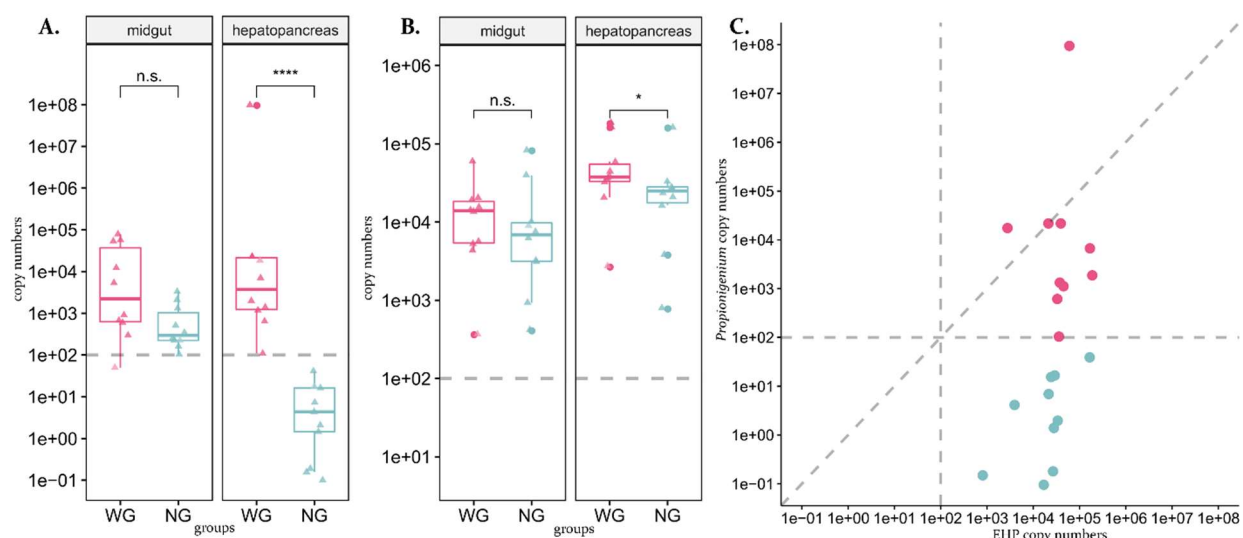
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### 319 **3.3 High *Propionigenium* spp. levels in the hepatopancreas of WG but not NG shrimp**

320 To investigate different levels of EHP and *Propionigenium* between WG and NG shrimp, we  
321 carried out qPCR on the HP and midguts of all 10 WG and 10 NG shrimp. Copy number of both  
322 EHP and *Propionigenium* were significantly different between WG and NG shrimp in the HP but  
323 not in the midguts (Fig. 6 and Supplementary Table S2). Within the HP, there was a significantly  
324 higher copy number for *Propionigenium* in WG shrimp than in NG shrimp (medians of  
325 4,506/100ng vs. 3/100ng DNA for WG and NG, respectively;  $P = 1.08e-5$ , Mann–Whitney U test;  
326 Fig. 6A). Specifically, all HP samples of NG shrimp had lower *Propionigenium* levels ( $< 50$   
327 copies) than the lowest standard copy number used (100 copies), implying that *Propionigenium*  
328 might be present at a low abundance or absent in the HP of NG shrimp. But all 10 HP samples  
329 from WG shrimp had  $> 100$  copies of *Propionigenium*, suggesting its presence at high abundance  
330 in the HP of WG shrimp. For EHP within the HP, WG shrimp samples had a significantly higher  
331 copy numbers than did NG shrimp (medians of 37,573 and 24,966, respectively;  $P = 0.0432$ ,  
332 Mann–Whitney U test; Fig. 6B), although both WG and NG shrimp had HP samples with similar  
333 maximum EHP levels ( $1.6e5 - 1.8e5$  copies). Within midgut samples, both EHP and  
334 *Propionigenium* levels tended to be higher in WG than in NG (*Propionigenium* medians of 3,148  
335 and 300 for WG and NG, respectively;  $P = 0.0524$ , Mann–Whitney U test; Fig. 6A; EHP medians  
336 of 13,928 and 6,900 for WG and NG, respectively;  $P = 0.4359$ , Mann–Whitney U test; Fig. 6B).  
337 Note that, contrary to its levels in the HP, *Propionigenium* levels in all midguts of NG shrimp were  
338  $> 100$  copies, where 100 copy number was the lowest standard copy number used in this  
339 experiment (Fig. 6A). When *Propionigenium* copy numbers were plotted against EHP copy  
340 numbers in shrimp HP samples (Fig. 6C), higher co-occurrence of *Propionigenium* and EHP was

341 observed in WG shrimp while *Propionigenium* copies in the HP of NG shrimp were undetectable  
342 (< 50 copies/100ng DNA) by our qPCR assays (Fig. 6A and 6C).

343  
344 **Figure 6.** Copy numbers of *Propionigenium* (A) and EHP (B) in the 100 ng DNA samples of  
345 hepatopancreas and midguts between the WG and NG groups, and scatterplot (C) of the copy  
346 numbers of *Propionigenium* against those of EHP in the hepatopancreas samples. The points are  
347 colored red and blue for WG and NG groups, respectively. The estimated copy numbers were  
348 obtained by qPCR reactions described in the text.



349

350

#### 351 4. Discussion

352 We have revealed the co-occurrence of EHP and distinctive bacterial communities which appear  
353 to contribute as a prokaryotic-eukaryotic pathobiome to cause the clinical manifestation of WFS  
354 in penaeid shrimp. Shrimp exhibiting these co-occurring microbial consortia in the HP displayed  
355 white gut (WG) characteristic of WFS, while others collected from the same shrimp pond but with  
356 normally colored guts (NG) lacked this pathobiome in the HP. With respect to gut histopathology,  
357 we confirmed earlier reports that WG shrimp exhibited more severe HP lesions characterized by  
358 higher numbers of spores and more tissue destruction (e.g., lysed, atrophied and sloughed cells)  
359 than did the NG shrimp (Figs. 1 and 2). We also proved that WG shrimp had significantly higher  
360 burdens of EHP than NG shrimp by qPCR counts. This was despite having similar maximum copy  
361 numbers for EHP by qPCR. In addition, high numbers of epithelial cells containing EHP plasmodia

362 and/or spores were observed in the region of the midgut within the HP only in WG shrimp. Our  
363 examinations also confirmed earlier reports of rod-shaped bacterial cells being present together  
364 with the EHP spores.

365

366 In some of the WG and NG specimens, HP tissues showed lesions with hemocytic aggregation  
367 and encapsulation (Fig. 1A), but because bacteria were also present in many of the specimens,  
368 there was uncertainty as to whether these responses were induced by EHP or bacteria or both.  
369 Intracellular parasites usually do not elicit immune responses. For example, the microsporidian  
370 *Agmasoma penaei* in *P. monodon* muscle tissue rarely does (Flegel et al., 1992). Perhaps an  
371 inflammatory response can be initiated by tissue damage or cell lysis leading to release of  
372 intracellular parasite antigens. During microsporidiosis some insects such as lepidoptera and  
373 orthoptera display signs of cellular immunity by increased number of hemocytes, phagocytosis,  
374 encapsulation, nodule formation and melanization in infected tissues (Hoch et al., 2004; IaL et al.,  
375 2004; IuIa et al., 2000; Tokarev et al., 2007). However, some microsporidia species may escape  
376 or suppress host immunity for their advantage (Antúnez et al., 2009). Our histopathological  
377 examination also revealed a higher accumulation of rod-shape bacterial cells in the midgut lumen  
378 in WG than in NG shrimp, suggesting possible involvement of bacteria in conjunction with EHP  
379 in causing EHP-WFS.

380

381 Our high-throughput 16S rRNA amplicon sequencing analysis revealed that bacteria of the genera  
382 *Vibrio* and *Propionigenium* were significantly associated with WG shrimp (Figs. 3 and 5). It was  
383 subsequently confirmed that *Propionigenium* levels in HP and intestine samples of WG were  
384 higher than those in NG shrimp by qPCR (Fig. 6 and Supplementary Table S2). Similar  
385 comparisons could not be done with the dominant *Vibrio* species, the sequences of which were all  
386 related to the *Vibrio harveyi* clade (Darshanee Ruwandepika et al., 2012; Ke et al., 2017;  
387 Urbanczyk et al., 2013). This was because the primers used for generic amplification of 16S rRNA  
388 yielded amplicons too short and too similarity to allow identification of individual *Vibrio* species  
389 within the clade. In this respect, we cannot discount the role of *Vibrio* taxa in the pathobiome of  
390 clinical EHP-WFS. . These associations between bacteria of the genera *Propionigenium* and *Vibrio*  
391 to EHP-WFS were observed and supported by both high-throughput 16S rRNA amplicon profiling  
392 and qPCR analyses.



393 Increased abundances of opportunistic *Vibrio* spp. measured by traditional plate counts have been  
394 reported in WFS ponds of both *P. monodon* and *P. vannamei* in many Asian countries.  
395 Specifically, reported *Vibrio* isolates from WFS shrimp gastrointestinal tracts and rearing water  
396 have been *V. harveyi*, *V. alginolyticus*, *V. parahaeolyticus*, *V. anguillarum*, *V. fluvialis*, *V. mimicus*,  
397 *V. vulnificus*, *V. damsela*, and *V. cholera* (Huang et al., 2020; Somboon et al., 2012; Supono et  
398 al., 2019; Wang et al., 2020). Some isolates have shown virulence in subsequent experimental  
399 bioassays by causing shrimp mortality but without WFS clinical signs (Wang et al., 2020). Using  
400 culture-independent approaches for high-throughput targeted amplicon or metagenomic shotgun  
401 sequencing, recent WFS studies have examined whether WFS intestinal microbial community  
402 assemblies differ from those of healthy shrimp. With WFS *P. vannamei* ponds in China and  
403 Indonesia, recent WFS microbiome studies reveals markedly different structures of WFS intestinal  
404 microbiomes that shift to gut “dysbiosis” with less diversity but more heterogenous bacterial  
405 composition than in healthy shrimp (Alfiansah et al., 2020; Hou et al., 2018; Huang et al., 2020;  
406 Wang et al., 2020). Shrimp gut dysbiosis has been observed in some EHP-WFS studies (Wang et  
407 al., 2020), but the other studies (Alfiansah et al., 2020; Hou et al., 2018; Huang et al., 2020) did  
408 not investigate EHP presence in their studied shrimp ponds. Importantly, key bacterial candidates  
409 associated with WFS were obtained by statistical analyses showing significantly more abundant  
410 bacterial taxa in WFS than in normal shrimp. These included taxa affiliated with *Vibrio*,  
411 *Candidatus* Bacilloplasma, *Aeromonas*, *Phascolarctobacterium*, *Ruminococcus*,  
412 *Rhodobacteraceae*, *Alteromonas*, *Marinomonas*, *Photobacterium*, *Pseudoalteromonas* and  
413 *Flavobacteraceae* (Alfiansah et al., 2020; Hou et al., 2018; Huang et al., 2020; Wang et al., 2020).  
414 Our microbiome analyses (Figs. 3 and 4) supported characteristics of lower bacterial diversities in  
415 WG samples and shifting of intestinal microbiome compositions to intestinal dysbiosis in WG  
416 shrimp. Our work added a bacterium from the genus *Propionigenium* to a list of WFS associated  
417 bacteria, specifically in EHP-WFS ponds exhibiting abnormal shrimp mortality.

418  
419 The stark difference in absence of *Propionigenium* in the HP of NG shrimp but significant presence  
420 in the HP of WG shrimp in our study (Fig. 6A) was of particular interest. In contrast, its  
421 concentrations in the intestine of NG and WG were similar (Fig. 6A). It is possible that that  
422 progression of NG into WG shrimp might be associated to movement of *Propionigenium* (perhaps  
423 together with *Vibrio*) from the intestine to the HP.

424 The HP of healthy shrimp is usually devoid of bacteria, and presence of bacteria in the HP signifies  
425 poor health status (e.g., Vibriosis (Lightner, 1996)). The genus *Propionigenium* has not previously  
426 been associated with shrimp disease. The genus so far comprises two strictly anaerobic bacterial  
427 species (*P. maris* and *P. modestum*) that are capable of decarboxylating succinate to propionate  
428 for growth (Schink, 2006). They are found in marine habitats, typically in sediments. They are  
429 Gram negative, coccoid to ovoid or short rod-like cells with rounded ends (Schink, 2006). Of the  
430 two currently known species, our short 16S rRNA amplicon sequence showed the highest  
431 similarity to *P. maris*. Anoxic and metabolic conditions in shrimp intestines and HP might promote  
432 growth of *Propionigenium* during WFS progress.

433  
434 Recently, succinic acid was one of metabolites found to be positively associated with WFS and  
435 with abundances of potential pathogenic bacteria such as *Vibrio*. Succinic acid is also a carbon  
436 source for *Propionigenium* that yields propionic acid. In addition, succinate supplemented feed in  
437 healthy shrimp can induce intestinal bacterial profile changes similar to those in WFS shrimp  
438 (Huang et al., 2020). Suggested avenues of further work include 1) tests on the possibility that  
439 propionic acid may induce spore formation and HP damage in *P. vannamei*, 2) work on the  
440 isolation and cultivation of *Propionigenium* from WFS shrimp for bioassays with EHP-infected  
441 shrimp and for species identification, and 3) epidemiological work to determine the risks factors  
442 (including the presence or absence of *Propionigenium* and *Vibrio* species) associated with WFS  
443 outbreaks.

#### 444 445 **Acknowledgements**

446 We would like to thank P. Wechprasit and W. Pattarayingsakul for their help in laboratory, D.  
447 Bass for helpful discussion on microbiome analysis, BIOTEC's Biostatistics & Informatics  
448 Laboratory, K. Anekthanakul and Sai T. Y. A. for their programing and computational support.  
449 This work was financially supported by the Newton Institutional Links 2017, the Newton prize's  
450 Chairman's award, the International Collaborative Award (ICA\R1\180038) from the Royal  
451 Society (to GDS, Cefas/UK and KS, BIOTEC/Thailand). We also thank RDI management for  
452 National Strategic and Network Division (P19-51879), the National Science and Technology  
453 Development Agency (NSTDA).

454

455 **Declaration of Competing Interest**

456 The authors declare that they have no conflicts of interest.

457

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