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1 **Survival and immune response of white shrimp *Litopenaeus***
2 ***vannamei* following single and concurrent infections with WSSV**
3 **and *Vibrio parahaemolyticus***

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23 ABSTRACT

24 The survival and immune responses of *Litopenaeus vannamei* were evaluated during
25 white spot syndrome virus (WSSV) or *Vibrio parahaemolyticus* single and concurrent
26 infections. The mortality, WSSV load, activities of 4 immune enzymes: acid
27 phosphatase (ACP), alkaline phosphatase (AKP), peroxidase (POD) and superoxide
28 dismutase (SOD), and the transcription of Evolutionarily Conserved Signaling
29 Intermediate in Toll pathways of *L.vannamei* (LvECSIT) were quantified at 0, 3, 6, 12,
30 24, 48, 72 and 96 h post-infection (pi). The results showed: (i) the cumulative
31 mortality of the co-infection group (WSSV and *V. Parahaemolyticus* 83 %) was
32 significantly lower than the WSSV infection group (97%) ($P < 0.05$) at 96 hpi; (ii)
33 copies of WSSV in the co-infection group were significantly lower than that of the
34 single infection group from 24 to 96 hpi ($P < 0.05$); (iii) ACP, AKP,POD and SOD
35 activity in the gills of the co-infection group was higher than that of the WSSV group
36 at 12, 48 and 96 hpi ($P < 0.05$).The expression of LvECSIT mRNA in the co-infection
37 group was significantly higher than in the WSSV infection group from 12 to 72 hpi (P
38 < 0.05).The results indicate that proliferation of WSSV is inhibited by
39 *V.parahaemolyticus* infection. In addition, infection with WSSV alone causes a
40 significant reduction in some immune responses of shrimp than co-infection with
41 WSSV and *V.parahaemolyticus* occurs at 26 °C. Third, LvECSIT, an essential
42 member of TLR signaling pathway might play a crucial role in shrimp defense against
43 WSSV – *Vibrio* co- infection.

44 Keywords: *Litopenaeus vannamei*, Immune response, White spot syndrome virus
45 (WSSV), *Vibrio parahaemolyticus*, Co-infection

46 1. Introduction

47 Shrimp aquaculture has developed very fast in China over the last two decades,
48 but the production of shrimp has been seriously affected by white spot syndrome virus
49 (WSSV) and *Vibrio* spp. [1]. WSSV - *Vibrio* co-infection is the normal manner of
50 shrimp disease breakouts and shrimp infected with the virus are more susceptible to
51 *Vibrio* spp.[2].It has been reported previously that *Vibrio alginolyticus* was isolated
52 from shrimp during a breakout of white spot syndrome virus [3].Another study
53 showed that during a WSSV and *Vibrio anguillarum* co-infection test in shrimp,
54 WSSV increased more rapidly under co-infection conditions than in the single
55 infection[4]. Similarly, the transcription of immune-related genes was suppressed in
56 the co-infection groups, and the shrimp would suffer higher mortality in multiple
57 infections [5]. Unlike the above observations, an outbreak of WSSV was postponed
58 after co-infection with WSSV and *Vibrio harveyi* in *Penaeus vannamei* [6]. These
59 studies about the WSSV - *Vibrio* co-infections in shrimp seem to be conflicting and
60 the pathogenesis involved is unclear.

61 Although the defense mechanism of shrimp to WSSV - *Vibrio* co-infections
62 remains unknown, it has been reported that bacterial infection could reduce the copies
63 of virus in some arthropods [7-8].*Drosophila melanogaster* infected with *Wolbachia*
64 appeared to inhibit the proliferation of Drosophila C virus[7].Furthermore, *Wolbachia*
65 induces reactive oxygen species (ROS)-dependent activation of the Toll/Toll-like
66 receptor (TLR)-mediated signaling pathway to control dengue virus in the mosquito
67 *Aedes aegypti*. Some Toll pathway-related genes (*Spn27A*, *SPZ1*, *CECD*, and *DEFC*)

68 were up-regulated in *Aedes aegypti* after co-infection with *Wolbachia* and dengue
69 virus [8]. Such virus suppression mechanisms may exist in shrimp, which warrants
70 further exploration.

71 In shrimp, the innate immune system is the first line of defense against
72 pathogenic infections [9]. When pathogens invade shrimp, they stimulate a series of
73 immune responses including lymphatic hemocyte agglutination, melanisation,
74 hemocyte phagocytosis, formation of cysts [10-12] and humoral immune factors (a
75 variety of enzymes have been identified). It was reported that ACP, AKP, POD and
76 SOD were susceptible to WSSV and *Vibrio* infections, and they could be used as
77 indicators of immune response to these pathogens [13-15].

78 Under the stimulus of pathogens, various humoral and cellular immune
79 responses of shrimp are activated through signaling pathways, among which
80 Toll/Toll-like receptor (TLR)-mediated signaling pathway are the best known and can
81 be activated by pathogenic related molecular patterns (PAMPs) [16]. After PAMP
82 recognition, TLRs can either directly or indirectly trigger downstream signaling
83 cascades, resulting in the regulation of cytokine gene expression [17]. TRAF6 is an
84 important downstream signal ligand of Toll-1 receptor protein and ECSIT is the first
85 gene that has been approved to interact with TRAF 6 [18]. As an important adaptor
86 protein of TLR, ECSIT have been demonstrated to be an immune-response gene since
87 its transcript expression level is up-regulated after *Vibrio anguillarum* [19] or WSSV
88 infection [20].

89 White spot syndrome virus (WSSV) is one of the most detrimental pathogens

90 affecting shrimp [21]. It is a baculovirus with double stranded DNA [22], and the
91 mortality rate of WSSV-infected shrimp can reach 100% in 7-10 days. Recently,
92 researchers found another serious shrimp disease (acute hepatopancreatic necrosis
93 disease AHPNS/early mortality syndrome EMS), which is characterised by empty
94 stomach, severe atrophy of hepatopancreas and soft carapace. *Vibrio*
95 *parahaemolyticus* is one of the causative agents of AHPNS / EMS, and it has caused
96 big economic losses in the shrimp industry in China [23-25]. Nowadays, there is
97 limited information available on molecular immune responses in shrimp under WSSV
98 or *V. parahaemolyticus* single and concurrent infections.

99 In an attempt to provide a theoretical basis for the control of WSSV in *L.*
100 *vannamei*, a number of parameters (mortality, WSSV load, the activities of the several
101 immune enzymes, transcription of LvECSIT) were investigated following single
102 infections and co-infection with WSSV and *V. parahaemolyticus*.

103

104 **2. Materials and methods**

105 2.1. Experimental animals and conditions

106 *L. vannamei* (size 7.66 ± 0.82 cm) were obtained from the East Sea Island
107 Marine Biological Research Center in Guangdong Ocean University. Before the
108 experiment, 20 shrimp were randomly selected to ensure that they were free of WSSV
109 and *V. parahaemolyticus*, according to Sun *et al.*[14]. They were fed with artificial
110 pellet diets twice a day and were kept at 26°C and salinity at 25 ‰. Filtered seawater
111 was sterilized with 1.5 ppm trichloroisocyanuric acid and the residual chlorine was

112 detected to ensure that it was safe for shrimp. About 1/3 of the water was replaced and
113 un-eaten pellet diet was removed by siphoning daily.

114

115 2.2 Preparation of virus and *V. parahaemolyticus* suspension for injection

116 WSSV extracts were prepared from crude extracts of disease shrimp and stored
117 at - 80 °C. Healthy shrimp were injected intramuscularly with 3.3×10^2 copies μL^{-1}
118 virus (in PBS) and mortalities occurred at 48 h post-injection (pi). Following removal
119 of the exoskeletons, WSSV infected shrimp were homogenized in cold PBS (KH_2PO_4
120 0.27g, Na_2HPO_4 0.01g, NaCl 8g, KCl 0.2g, diluted with water to 1 L and adjust pH to
121 7.4) (1 mL g^{-1}). After centrifugation at 12,000 g for 10 min, the crude viral
122 supernatant was filtered using a membrane filter (220 nm).

123 *V. parahaemolyticus* was obtained from the Economic Aquatic Animal Disease
124 Control Laboratory of the Guangdong Ocean University [26]. *V. parahaemolyticus*
125 was cultured in trypticase soy broth (TSB, Huankai Co Ltd., Guangzhou, China) at
126 28 °C for 18 h. The culture medium was centrifuged in an 8 mL tube at 4000 g for 15
127 min. The supernatant was removed and *V. parahaemolyticus* was re-suspended in
128 PBS to 1.22×10^6 CFU mL^{-1} .

129

130 2.3 Experimental design

131 The laboratory challenge test contained 4 treatments in triplicate (n=40 for each
132 sample group, n=10 for mortality group). For *V. parahaemolyticus* treatment, shrimp
133 were intramuscularly injected with 50 μL of *V. parahaemolyticus* (1.22×10^6 CFU

134 mL⁻¹). For WSSV treatment, shrimp were intramuscularly injected with 50 µL of
135 WSSV viral suspension (3.3×10^2 copies µL⁻¹). For co-infection treatment, shrimp
136 were intramuscularly injected with 50 µL of cocktail suspensions containing *V.*
137 *prahaemolyticus* (1.22×10^6 CFU mL⁻¹) and WSSV (3.3×10^2 copies µL⁻¹). The PBS
138 treatment was injected with 50 µL of PBS. Tissues (muscle, gills) of one shrimp per
139 group were sampled individually at PBS 0 h post-infection (pi), and at each time point
140 (3, 6, 12, 24, 48, 72 and 96 hpi) from each group to measure virus load,
141 immune-related enzymes, and immune-related gene LvECSIT expression analysis
142 (Table 1-2). The experiments were repeated three times.

143

144 2.4 Analysis of virus load

145 The muscle of the first abdominal segment (about 0.05 g) was dissected and
146 added to 45 µL 50 mM NaOH and homogenized on ice, mixed and then boiled in
147 water bath for 10 min. Then, 5 µL 1M Tris solution was added, mixed and centrifuged
148 at 12,000 g for 10 min [14]. The supernatant was used as WSSV template for
149 quantitative PCR. The qPCR was carried out in 15 µL volume, and the primer
150 sequences are shown in Table 3. The standard curve was made according to the
151 method of Xin *et al.*[27].

152

153 2.5 Determination of activities of immune-related enzymes in the gills

154 The gills (0.2g) were cut off from the samples stored in liquid nitrogen and
155 homogenized on ice after adding 1.8 mL PBS. The samples were centrifuged at 3000g

156 for 10 min at 4 °C, the precipitate was removed and the supernatant was used for acid
157 phosphatase (ACP), alkaline phosphatase (AKP), peroxidase (POD) and superoxide
158 dismutase (SOD) immune enzyme analysis. Enzymatic activities for ACP, AKP, POD,
159 SOD were determined using kits purchased from Jiancheng Bioengineering Institute
160 (NJJCbio, Nanjing, China), according to the methods described by Sun *et al.* and Liu
161 *et al.* [14,28]. ACP and AKP activities are expressed in King unit (mg protein)⁻¹. POD
162 and SOD activities are expressed in U (mg protein)⁻¹. Each enzymatic assay was
163 performed in triplicate.

164

165 2.6 Immune-related gene LvECSIT expression analysis by real-time PCR

166 Gills from one shrimps were sampled [20] at PBS 0 h post-infection (pi) and at
167 each time point (3, 6, 12, 24, 48, 72 and 96 hpi) from each group. The transcriptional
168 level of LvECSIT was detected with real-time PCR. Primers for LvECSIT (Genbank
169 accession No. is XM_027378031) were shown in Table 3. β -actin was used as internal
170 reference. RNA extraction, cDNA synthesis, real-time PCR for analysis of immune
171 gene expression were as described by Li *et al.* [29].

172

173 2.7 Statistical analysis

174 Statistical analysis was carried out using the software SPSS 21. Results were
175 analyzed using One-way ANOVA and Duncan's multiple comparisons of the means.
176 Differences were considered significant when $P < 0.05$.

177

178 3. Results

179 3.1 Effect of WSSV and *V. parahaemolyticus* infection on shrimp survival

180 Shrimp in each challenge group started to die at 12 hpi. The cumulative mortality
181 reached peak at 96 hpi, and the mortality of WSSV group (97 %) was significantly
182 higher than co-infection group (83 %) and *V. parahaemolyticus* group (34 %) ($P < 0.05$)
183 (Fig.1).

184

185 3.2 Effects of WSSV and *V. parahaemolyticus* infection on the proliferation of WSSV 186 in *L. vannamei*

187 In the experiment, we collected the muscle of shrimp to detect the copies of
188 WSSV by real time PCR. The results illustrated that WSSV could be detected in
189 muscle within 3 h, and the maximum viral load in the WSSV infection group was
190 6.71×10^5 copies μL^{-1} at 72 hpi, significantly higher than that in co-infection group
191 (1.80×10^4 copies μL^{-1}). The viral load in the WSSV infection group was
192 approximately 10 times more than that in co-infection group at 24, 48, 72 and 96 hpi
193 (Fig.2).

194

195 3.3 Effects of WSSV and *V. parahaemolyticus* infection on shrimp gill immune 196 enzyme activity

197 The ACP activity in the gills of shrimp infected with *V. parahaemolyticus* alone
198 and the co-infection groups showed an initial rise and subsequent fall, and reached
199 maximum activity at 24 and 6 hpi respectively. In the *V. parahaemolyticus* group and

200 co-infection group, the maximum ACP activity was significantly higher than the PBS
201 group and WSSV group at 6, 12, 24, 48, 72 and 96 hpi ($P < 0.05$). By the end of the
202 experiment, the ACP activity of WSSV group remained at a low level, and was
203 consistently lower than both the *V. parahaemolyticus* and the co-infection groups.
204 Comparison of the degree of variation of each treatment group showed the following
205 trend: PBS group (0.14) < WSSV group (0.33) < *V. parahaemolyticus* group (0.45) <
206 co-infection group (0.58) (Fig.3A).

207 In the *V. parahaemolyticus* group and co-infection group, the AKP activity
208 decreased after the initial rise, and was higher than the WSSV group and PBS group
209 at all time points, and the maximum AKP activity was recorded at 6 h and 24 hpi
210 respectively. The AKP activity of WSSV group was significantly lower than the
211 co-infection group from 6-96 hpi. The AKP activity of *V. parahaemolyticus* group
212 varied over the course of the experiment whereas the AKP activity of the PBS group
213 was stable. Degree of variation: PBS group (0.18) < WSSV group (0.21) <
214 co-infection group (0.29) < *V. parahaemolyticus* group (0.45) (Fig.3B).

215 The POD activity of the PBS group remained higher than 3 challenge groups
216 until the end of experiment, and the difference was significant at 48 hpi ($P < 0.05$).
217 For the *V. parahaemolyticus* group, co-infection group and WSSV group, the
218 minimum POD activity occurred at 3, 6 and 24 hpi respectively. The POD activity of
219 the co-infection group was higher than the WSSV group at 6, 12, 48 and 96 hpi, and
220 was significantly higher at 6 hpi. Degree of variation: PBS group (0.05) < *V.*
221 *parahaemolyticus* group (0.11) < co-infection group (0.14) < WSSV group (0.15)

222 (Fig.3C).

223 SOD activity of the WSSV and co-infection groups showed the lowest value at
224 96 h pi, which was significantly lower than PBS group ($P < 0.05$). The SOD activity
225 of the co-infection group was significantly higher than the WSSV group at 48 hpi ($P <$
226 0.05). The SOD activity of *V. parahaemolyticus* group was significantly higher than
227 WSSV group at 3, 6, 48 and 96 hpi ($P < 0.05$). SOD activity in each group variation
228 coefficient: PBS group (0.11) $<$ *V. parahaemolyticus* group (0.18) $<$ co-infection
229 group (0.24) $<$ WSSV group (0.32) (Fig.3D).

230

231 3.4 Effects of WSSV, *V. parahaemolyticus*, and WSSV and *V. parahaemolyticus*
232 co-infection on LvECSIT expression in shrimp

233 In the challenge test, the expression of LvECSIT was detected in gill at 0, 3, 6,
234 12, 24, 48, 72 and 96 hpi. The transcription levels of LvECSIT in the PBS group
235 up-regulated from 6 to 48 hpi. WSSV infection group showed a degree of fluctuation
236 and reached maximum expression at 48h. Furthermore, LvECSIT expression
237 up-regulated significantly in WSSV infection group more than co-infection group at
238 3hpi, and was significantly more up-regulated than *V. parahaemolyticus* group at 6
239 hpi. The LvECSIT expression was significantly up-regulated in *V. parahaemolyticus*
240 group or co-infection group when compared with the WSSV infection group from 12
241 to 72 hpi ($P < 0.01$). There was no significant difference between the *V.*
242 *parahaemolyticus* group and co-infection group from 12 to 48 hpi. Each treatment
243 group showed minimum LvECSIT expression at 96 hpi and was all significantly

244 lower than PBS group ($P < 0.05$) (Fig. 4).

245

246 **4. Discussion**

247 In complex aquaculture environments, the outbreak of shrimp disease is
248 accompanied with sharply defined changes of physical factors or secondary infection
249 and co-infection by pathogens [30-32]. Nonetheless, the conclusions about *Vibrio* spp.
250 and WSSV co-infection in shrimp have been conflicting. Previous studies have shown
251 that mortality in co-infections (39%) was significantly higher than in single WSSV
252 infections (25%) and single infections with *Vibrio anguillarum* (25%) [5]. However,
253 other studies have revealed that the outbreak of WSSV was postponed after *P.*
254 *vannamei* co-infection with WSSV and *V. harveyi* [6]. In this study, the mortality of
255 WSSV group (97 %) was significantly higher than the co-infection group (83%) and
256 *V. parahaemolyticus* group (34 %) ($P < 0.05$), which conflicted with the reported in *L.*
257 *vannamei* after co-infection with WSSV and *V. anguillarum* [5], but was similar to
258 previous findings in *P.vannamei* after co-infection with WSSV and *V. harveyi* [6]. The
259 synergistic effect between WSSV and *Vibrio* may be influenced by the species of the
260 *Vibrio* bacteria [6].

261 In this experiment, the WSSV copy number measured in the co-infection group
262 was always lower than in the WSSV group. It might be the key factor of lower
263 mortality in the co-infection group. The proliferation of WSSV result also
264 demonstrated that the WSSV replication was controlled under co-infection conditions.
265 It is possible that WSSV must make use of the metabolites in the host cell to assemble

266 nucleotides and proteins of the virus [33] after infection of the shrimp, but the
267 metabolites were used by *V.parahaemolyticus* or the metabolism of shrimp was
268 slowed down by *V.parahaemolyticus*. This suggests that virus couldn't replicate
269 without the metabolites, hence the WSSV proliferation was inhibited.

270 ACP is a typical lysosomal enzyme and plays a key role in eliminating and
271 hydrolyzing microbes [34]. In *Chlamys farreri* [35], the ACP activity was
272 significantly increased at the early stage of *Vibrio anguillarum* challenge. In this
273 experiment, the ACP activity is most sensitive to *V. parahaemolyticus* infection from
274 3 h after infection and reached the peak at 6 hpi. However, the ACP activity of the
275 WSSV infected group declined at 3 hpi then increased and reached the peak at 12 hpi.
276 The result was consistent with ACP activity in *Penaeus monodon* with WSSV in
277 latent period on reinfection [36], but the time of appearance of the peak varied. The
278 difference in the appearance of the peak might be associated with the dose of infection
279 and environment. Furthermore, ACP activity in the virus infected group was always
280 significantly lower than that of the co-injection group throughout the experimental
281 period. In other words, the *V. parahaemolyticus* infection has, to some extent, affected
282 ACP vitality of the shrimp. The ACP activity of the co-injection group from 3 to 96 h
283 pi was always higher than the WSSV group. The ACP activity of the co-injection
284 group from 6 to 24 hpi was significantly higher than that of the *V. parahaemolyticus*
285 injected group which suggests that co-infection stimulates the immune response in *L.*
286 *vannamei*. In the co-infection group, the ACP activity declined from 48 hpi, but
287 remained significantly higher than the WSSV group. The co-infection may cause

288 disturbance of cell metabolism and immune function, which is consistent with the
289 previous report in *Penaeus (Marsupenaeus) japonicus*[37].

290 AKP is a regulatory enzyme associated with the metabolism and can be seen as
291 an important index in the assessment of the immune status of shrimp [38]. After an
292 initial rise at 3 hpi, the AKP activity of WSSV-injected group decreased significantly
293 at 6 hpi in this experiment which was similar to previous reports [39]. We observed
294 that AKP activity in the gills of the shrimp is more sensitive to *V. parahaemolyticus*
295 infection than WSSV infection; the AKP activity of the co-injection group varied in a
296 similar manner.

297 Reactive oxygenspecies (ROS), including superoxide anion (O_2^-),hydroxyl
298 radical (OH) and hydrogen peroxide (H_2O_2) are an important part of the innate
299 immune defense system that is produced to help eliminate invading
300 microbes[40].Antioxidant enzymes such as peroxidases (POD) and superoxide
301 dismutase (SOD) either convert O_2 to H_2O_2 (SOD), convert H_2O_2 to water and oxygen
302 bycatalase (CAT), or use H_2O_2 to oxidize substrates by various peroxidases [41].POD
303 activity can serve as an immune index to evaluate the immune status of
304 crustacean[42].After infection with WSSV, the POD activity of *Cherax*
305 *quadricarinatus* was shown to decrease significantly [43].In this study, the POD
306 activity in gill decreased initially in all 3 challenge groups at 3 hpi. The minimum
307 activity of the WSSV-injected groups was recorded at 6 hpi and was significantly
308 lower than other groups. The POD activity in the co-infection group was significantly
309 higher than WSSV group at 6 hpi, which may have contributed to enhancing the

310 ability of the co-infection group to resist the infection of WSSV at 6 hpi.

311 SOD is an enzyme that catalyses the rapid two-step dismutation of the toxic
312 superoxide anion to molecular oxygen and hydrogen peroxide through the alternate
313 reduction and oxidation of the active-site metal ion [44]. A previous study indicated
314 that a significant decrease in SOD activity occurred earlier at 3 hpi in white shrimp
315 *L.vannamei* that received *V. alginolyticus* injection, followed by recovery after 96 hpi
316 [45]. In this study, the SOD activity of the *V. parahaemolyticus* -injected group
317 significantly increased at 3 hpi which conflicted with the previous report [45]. A
318 significant decrease in SOD activity occurred in WSSV -injected group at 6 hpi. It
319 was consistent with reports in the shrimp *Penaeus monodon* [46] and *L. vannamei*
320 [47], which showed a decrease of SOD activity after WSSV infection. According to
321 the study in *Fenneropenaeus indicus* [48], the lower activities of SOD may have been
322 due to inactivation of SOD by the oxidative stress generated singlet oxygen. In the
323 present study, the SOD activity of co-infection group and *V. parahaemolyticus* group
324 was significantly higher than that in WSSV group at 48 hpi, which suggests that the
325 shrimp in the co-infection and *V. parahaemolyticus* group could clear the oxyradical
326 more efficiently compared to WSSV group, and avoid the oxidative damage induced
327 by pathogens. Previous studies have shown an increase in activity of antioxidant
328 enzymes in shrimp during bacterial infections, with a decrease observed during viral
329 infection with WSSV [41].

330 As far as we know, viral suppression mechanisms exist in arthropods [7]. Studies
331 had revealed that the proliferation of West Nile and chikungunya virus were

332 suppressed in individuals after infection with *Wolbachia* [50, 51]. In mosquito during
333 co-infection with *Wolbachia* and dengue virus, the TLR signaling pathway was
334 activated by ROS and expressed more immune factors than in the mosquito group
335 infected with virus only [8]. ECSIT is a multifunctional adaptor protein of TLR
336 signaling pathway, and represented a constitutive expression pattern in some tissues
337 [51]. In shrimp, MjECSIT was previously shown to be expressed in hemocyte, gill,
338 hepatopancreatic, stomach, heart, intestinal, testicular, and ovarian tissues, and the
339 expression level in gill was higher than in hemocyte [19]. The mRNA transcript of
340 LvEcsit in gill was also higher than in hemocyte (Data will be showed in another
341 paper), which are considered with the result in MjECSIT [19]. So gill was chosen for
342 the sample tissue in this study. TLR pathway is of major importance during innate
343 immunity. Most genes in TLR pathway are reported to up-regulated in the stress of
344 pathogen. ECSIT, an essential member of this pathway, was found to be significantly
345 up-regulated after *Vibrio anguillarum* challenge in *Crassostrea gigas* [52], and by
346 challenge with microorganisms (*Vibrio alginolyticus*, *Staphylococcus haemolyticus*
347 and *Saccharomyces cerevisiae*) in the Hong Kong oyster *Crassostrea hongkongensis*
348 (ChECSIT) [17]. In this study, the expression of LvECSIT was up-regulated by
349 infection with *V. parahaemolyticus* (Fig.4). The transcription level of LvECSIT in the
350 co-infection group was higher than WSSV group from 12h to 72hpi (Fig.4), which
351 was consistent with the expression pattern of Toll pathway-related genes in *Aedes*
352 *aegypti* [8]. Furthermore, the transcription levels of LvECSIT in the PBS group
353 up-regulated from 6 to 48 hpi, was consistent with MjECSIT at 6 hpi [19], and

354 ChECSIT at 3 and 12 hpi [17].The difference in the kinetics of expression between
355 these studies could be associated with the animal, dose of infection and environment.
356 However, further study is required to elucidate the potential mechanism in shrimp.
357 In summary, this study demonstrated that 1) shrimp in co-infection groups suffered
358 lower mortality than groups with single infection by WSSV only; 2) the amount of
359 WSSV in co-infection group was always lower than that of WSSV single infection
360 group over the course of the trial; 3) ACP and AKP activity in gills of shrimp
361 co-infected with *V. parahaemolyticus* and WSSV was significantly higher than that of
362 WSSV single infection group from 6 to 72 hpi; ACP and AKP enzyme activity can be
363 used as indicators of immune response to these pathogens; POD and SOD activity
364 may not be the best indicators of immune response to WSSV - *Vibrio* infections.4) the
365 transcription level of LvECSIT was up-regulated in *V. parahaemolyticus* infected and
366 multiple infection groups. This study provided information for understanding the
367 effect of WSSV - *Vibrio* infections on survival and immune responses in shrimp.
368 Further study is needed to develop prevention and management strategies to reduce
369 losses caused by multiple pathogens in aquaculture.

370

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375

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553 Table1 Design of experiment for virus load, enzymes, and gene expression analysis.

Treatments	WSSV copies μL^{-1}	<i>V.pra</i> CFU mL^{-1}	No. of shrimp	Sampling Number of shrimp at hours post-injection (hpi)							
				0	3	6	12	24	48	72	96
1 PBS	-	-	40×3	1×3	1×3	1×3	1×3	1×3	1×3	1×3	1×3
2 <i>V. pra</i>	-	1.22×10^6	40×3	0	1×3	1×3	1×3	1×3	1×3	1×3	1×3
3 WSSV	3.3×10^2	-	40×3	0	1×3	1×3	1×3	1×3	1×3	1×3	1×3
4 Co-infection	3.3×10^2	1.22×10^6	40×3	0	1×3	1×3	1×3	1×3	1×3	1×3	1×3

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Table2 Design of experiment for Lethality

Treatments	WSSV copies μL^{-1}	<i>V.pra</i> CFU mL^{-1}	No. of shrimp
1 PBS	-	-	10×3
2 <i>V. pra</i>	-	1.22×10^6	10×3
3 WSSV	3.3×10^2	-	10×3
4Co-infection	3.3×10^2	1.22×10^6	10×3

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Table 3 Sequences of primers used in this study.

Primer name	Primer sequence(5'-3')	references
WSSV-F	AAACCTCCGCATTCCTGTGA	[28]
WSSV-R	TCCGCATCTTCTTCCTTCAT	
LvECSIT-F	ATGATTCTTATGAACGCTT	This study
LvECSIT-R	AATTTGGGCATCCAGTAC	
β -actin-F	GAAGTAGCCGCCCTGGTTGT	This study
β -actin-R	GGATACCTCGCTTGCTCTGG	

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639 Figure 1. Cumulative mortality in shrimp, *L. vannamei* infected by intramuscular
640 injection with *V. parahaemolyticus* only, by white spot syndrome virus (WSSV)
641 only, or concurrently infected with *V. parahaemolyticus* and WSSV (Co-infection) at
642 different time intervals pi (0, 3, 6, 12, 24, 48, 72, and 96 hours). Injection with PBS
643 served as negative control. Groups that don't share a letter are significantly different
644 ($P < 0.05$).

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647 Figure 2. Effect of single injection (*V. parahaemolyticus* or WSSV) and co-infection
648 injection (*V. parahaemolyticus* and WSSV) on the amount of WSSV (copies μL^{-1})
649 estimated in *L. vannamei* muscle at different time intervals pi (3, 6, 12, 24, 48, 72, and
650 96 hours). Values are expressed as mean \pm SD. Groups that don't share a letter are
651 significantly different ($P < 0.05$).

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654 Figure 3. Effect of single injection (*V. parahaemolyticus* or WSSV) and co-infection
655 injection (*V. parahaemolyticus* and WSSV) on the gill ACP(A), AKP(B), POD(C) and
656 SOD(D) activity of *L. vannamei* at different time intervals pi (0, 3, 6, 12, 24, 48, 72,
657 and 96 hours). Groups that don't share a letter are significantly different ($P < 0.05$).

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660 Figure 4. Effect of single injection (*V. parahaemolyticus* or WSSV) and co-infection
661 injection (WSSV and *V. parahaemolyticus*) on the mRNA expression of LvECSIT of
662 *L. vannamei* at different time intervals pi (0, 3, 6, 12, 24, 48, 72, and 96 hours).
663 Groups that don't share a letter are significantly different ($P < 0.05$).

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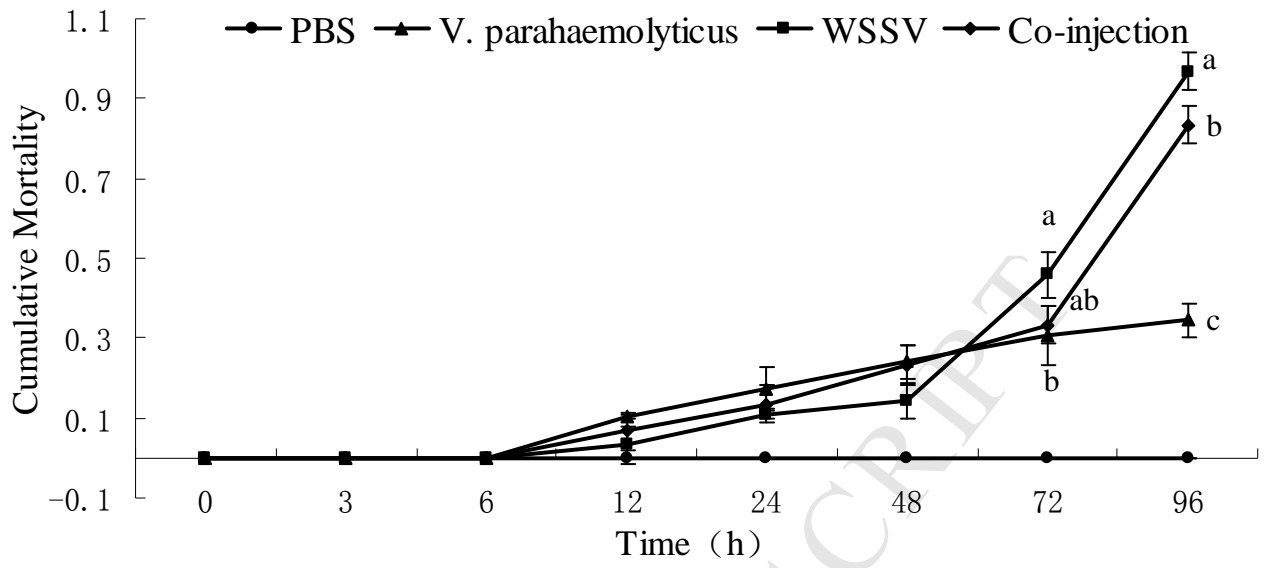


Fig.1

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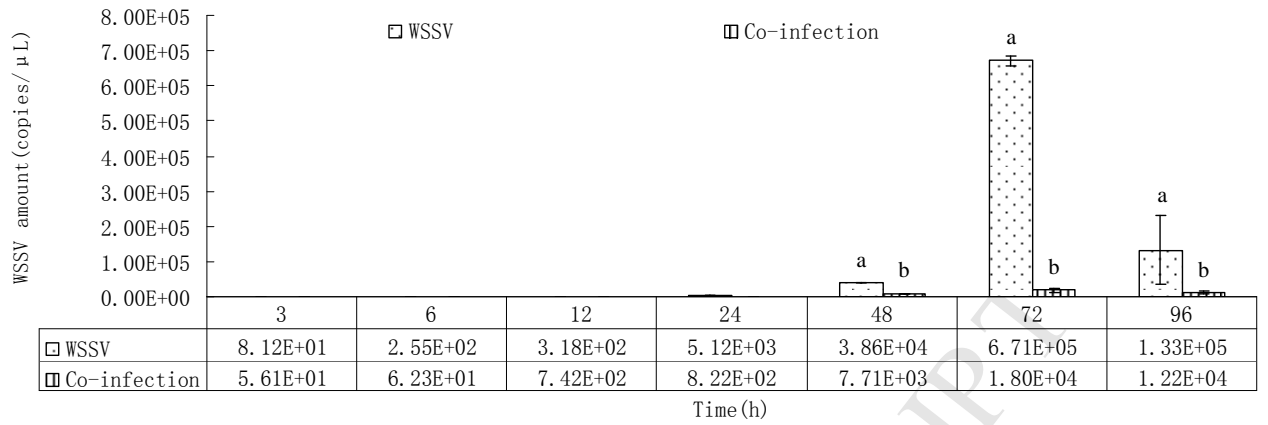


Fig.2

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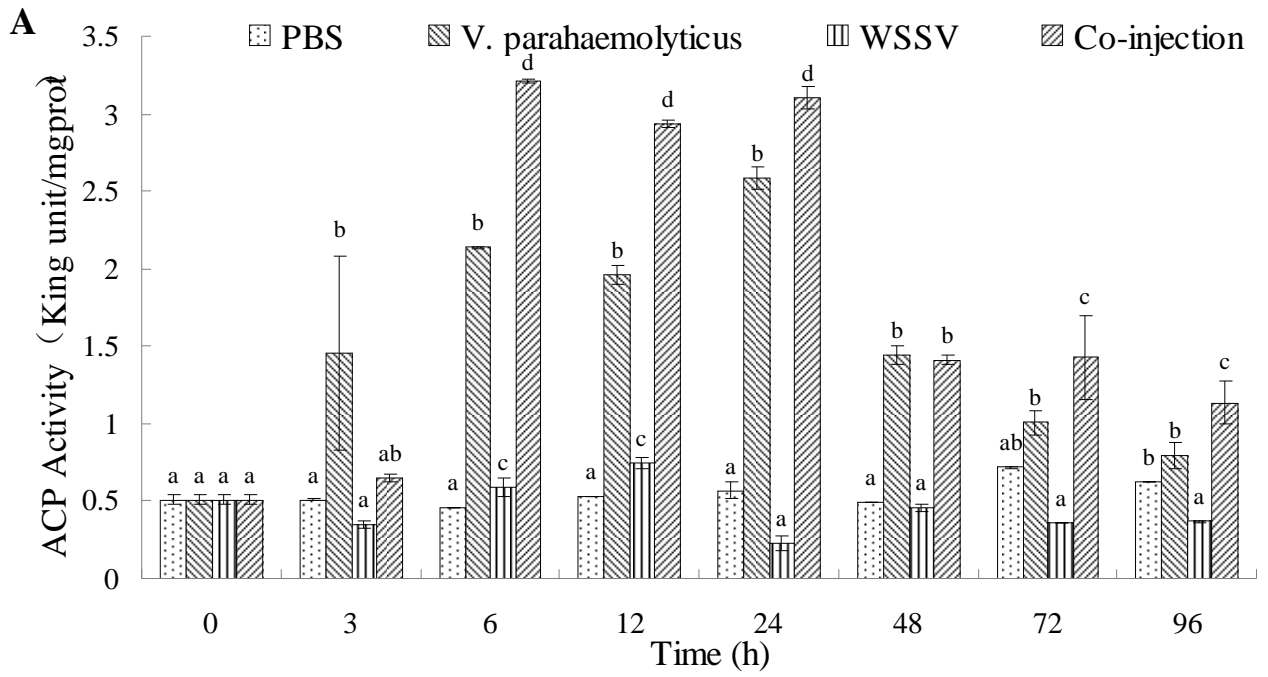
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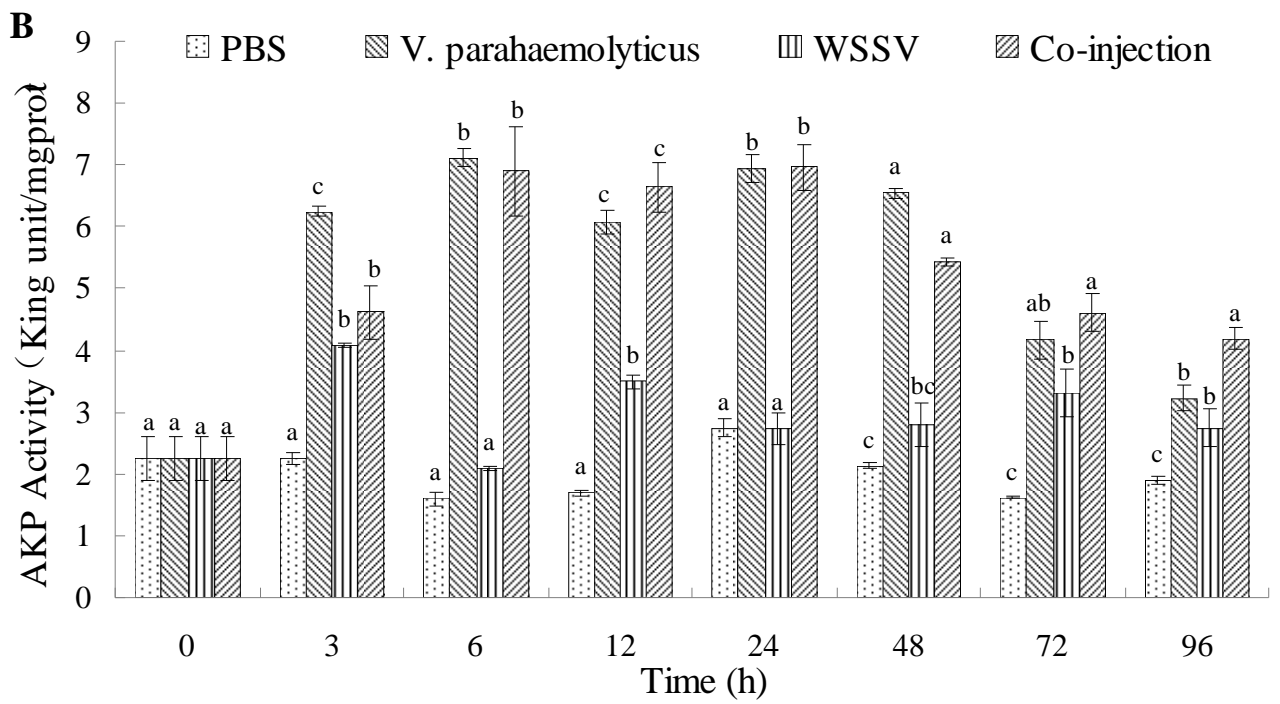
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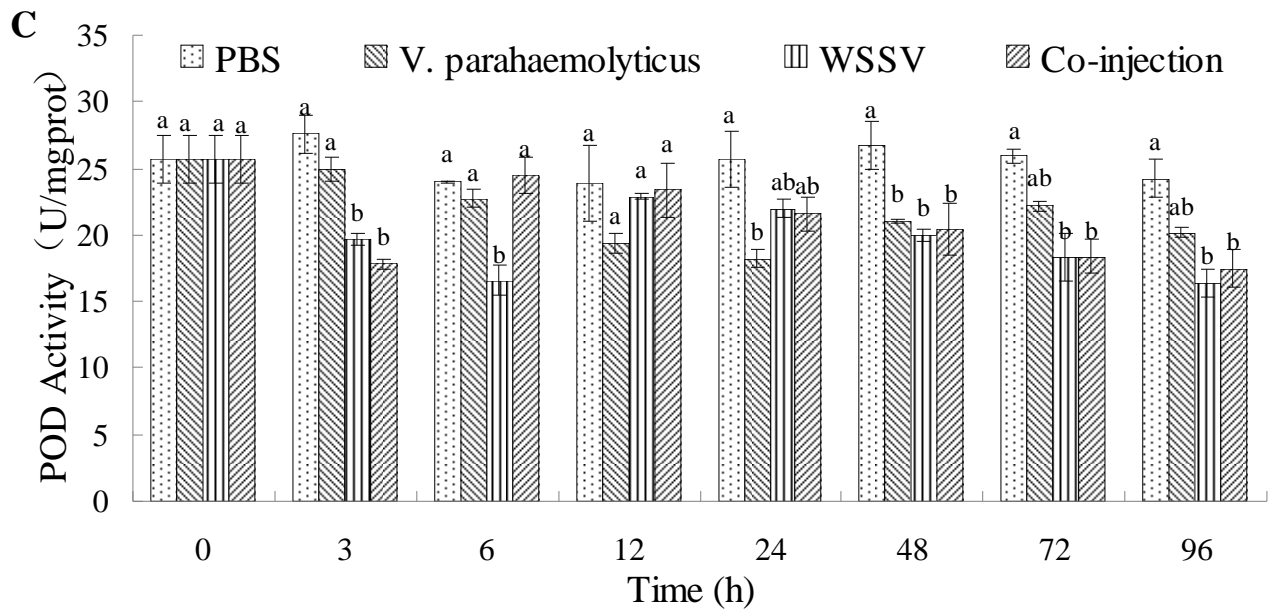
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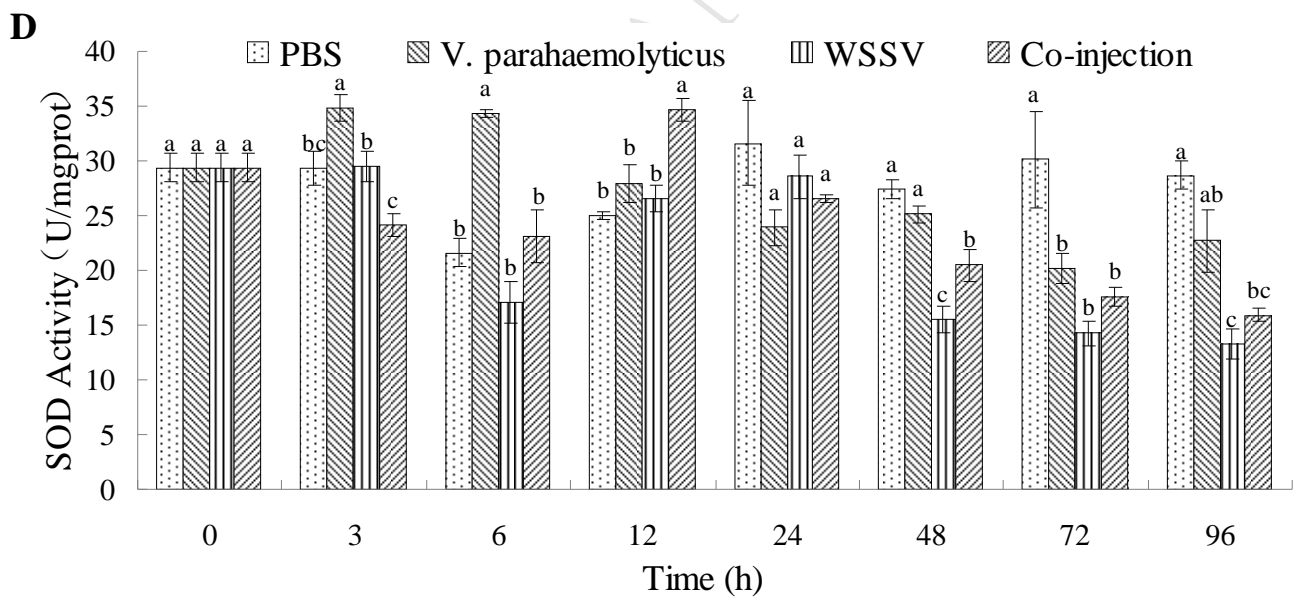
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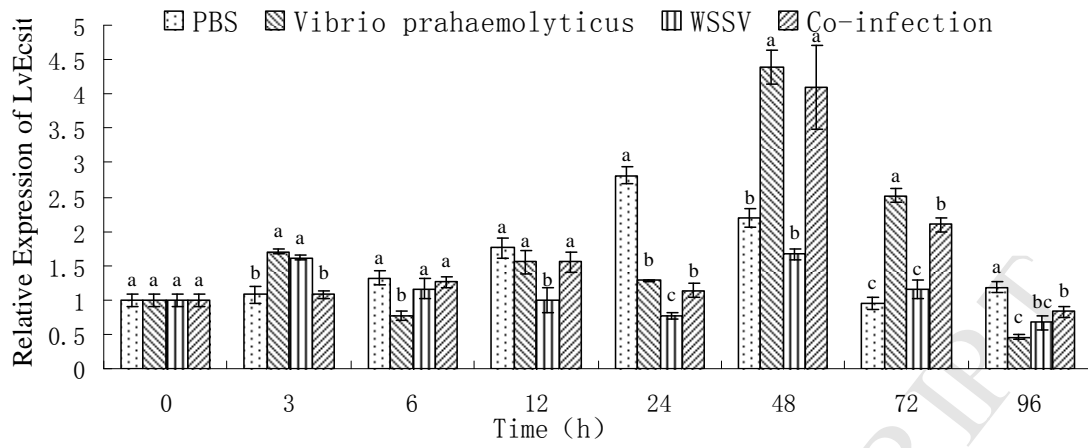
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Fig.3



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Fig.4

Shrimp in co-infection groups suffered lower mortality than WSSV group.

The amount of WSSV in co-infection group was lower than in WSSV group.

ACP and AKP enzyme activity can be used as indicators to co-infection.

The transcription level of LvECSIT was up-regulated in co-infection groups.