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Immunostimulation of shrimp through oral administration of silkworm pupae expressing VP15 against WSSV

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Abstract

White spot syndrome virus (WSSV) is one of the most concerning pathogens in penaeid shrimp and can cause severe loss in shrimp aquaculture worldwide. Among the WSSV structural proteins, VP15, a DNA-binding protein located in the WSSV nucleocapsid, is an antiviral protein candidate to protect kuruma shrimp (*Marsupenaeus japonicus*) from WSSV infection. We identified that the truncated VP15, $\mathrm{VP15}_{(26\text{–}57)}$, is responsible for the protective effect against the WSSV. This study attempts to develop an immunizing agent against WSSV using silkworm pupa as a delivery vector through oral administration. The VP15, $\mathrm{VP15}_{(26\text{–}57)}$, and SR11 peptide derived from $\mathrm{VP15}_{(26\text{–}57)}$ were expressed in silkworm pupae. Oral administration of feed mixed with the powdered pupae that expressed VP15-derived constructs enhanced the survivability of kuruma shrimp with an overall relative percent survival (RPS) higher than 70%. There is no death for the group receiving pupa/$\mathrm{VP15}_{(26\text{–}57)}$, and the RPS is 100%. In addition, we also investigated the relative mRNA expression levels of immune-related genes by qPCR at different time points. Our results indicate that the oral administration of pupa/VP15-derived products could provide a high protective effect against WSSV and be a practical approach for controlling WSSV in aquaculture.

Keywords: White spot syndrome virus, *Marsupenaeus japonicus*, VP15, Oral administration, Silkworm pupa

40 ## 1. Introduction

41 Shrimp farming began centuries ago by Asian people before growing into large-scale 42 commercial shrimp farming in the 1970s. Back in the 1930s, the Japanese started cultivating 43 the kuruma shrimp (*Marsupenaeus japonicus*) for the first time, and later it became one of 44 Japan's most economically important species [1, 2]. The kuruma shrimp is widely distributed 45 from Japan through South-East Asia to The Red Sea/East Africa region [3]. However, kuruma 46 shrimp farming is carried out only in East Asian countries. In Japan, the production of kuruma 47 shrimp is around 1,500–1,600 tons annually [4, 5], but diseases hamper productivity which 48 could cause an economic loss of millions of dollars. Among all the causative agents, white spot 49 syndrome virus (WSSV) is the most threatening pathogenic virus causing the white spot 50 disease (WSD) in shrimps with a mortality rate of 80–100% within one week after the first 51 infection [6, 7].

52 The WSSV belongs to the *Whispovirus* genus, the only genus in the *Nimaviridae* family 53 [8–10]. The virus contains a supercoiled circular double-stranded DNA (dsDNA) of about 290 54 kb harboring at least 180 putative open reading frames [11, 12]. The virion size is 55 approximately 80–100 nm in width and 250–350 nm in length, with a rod-shaped nucleocapsid 56 surrounded by a trilaminar membrane and a tail-like appendage at one end [10, 13, 14]. The 57 WSSV comprises more than 34 structural proteins, including at least six major virion proteins. 58 VP19, VP24, VP26, and VP28 are the most abundant proteins on the envelope, while VP15 59 and VP664 are nucleocapsid-associated proteins [15, 16].

60 The transmission routes of the WSSV can be both vertical and horizontal. Vertical 61 transmission occurs from mothers to offsprings in the hatcheries through the contaminated viral 62 particles during the spawning [17]. However, the horizontal transmission of WSSV in farms is 63 a more concerning subject. Since the transmission happens through the cannibalism of the dead 64 WSSV-infected shrimp or by exposure to the WSSV-contaminated water it is (still) difficult to

65 control [18]. Therefore, vertical transmission of WSSV can be easily controlled compared to
66 the latter by several countermeasures such as using a specific-pathogen-free broodstock, PCR
67 detection for the contaminated egg, disinfection of the eggs, decontamination of the
68 environment [19], and even the polyculture system [20].

69 Previously, the view of vaccination/immunization was limited only to the vertebrate
70 species due to the concept of adaptive immunity that exclusively existed in the vertebrates. In
71 contrast, invertebrate species possess only an innate immunity [21]. Later, a 'quasi-immune
72 response' or an 'acquired resistance' was first described in kuruma shrimp that survived a
73 second WSSV infection after four months of the prior infection [22]. Another study
74 demonstrated that shrimp developed resistance against the virus and produced a viral
75 neutralizing factor three weeks after exposure [23]. These evidences suggest that the immune
76 response in shrimp is triggered with some degree of specificity and can be trained or primed to
77 confer protection against the target pathogen, so-called 'immune priming'. Since then, many
78 shrimp immunizing strategies have been demonstrated, including live (at sublethal level) or
79 inactivated viruses, subunit proteins, and nucleic acid-based approaches [24, 25]. Our recent
80 studies found that the recombinant VP15 could provide substantial protection against WSSV
81 via a prime-and-boost strategy [26]. Later, we identified that the antigenic domain $VP15_{(26–57)}$
82 is responsible for the enhancement in shrimp survivability and narrowed it down to the peptide
83 (SR11) level [27].

84 In this study VP15, $VP15_{(26–57)}$, and the VP15-derived peptide SR11 were expressed in
85 silkworm pupae using the silkworm-bacmid expression system. We demonstrated the
86 protective effect of pupa-expressed VP15-derived proteins against WSSV through an oral
87 administration of the pupa powder-mixed feed. In addition, the generated immune responses
88 in kuruma shrimp were evaluated by quantitative polymerase chain reaction (qPCR) of the
89 genes involved in the innate immune signaling pathways. To our knowledge, this might be the

90 first demonstration in oral administration of silkworm pupa containing a VP15-derived product
91 as an immunizing agent, This could be useful for a field application in shrimp aquaculture (Fig.
92 1).

93

94 2. Materials and Methods

95 *2.1. Generation of a recombinant bacmid encoding GST and GST-fusion proteins/peptide and*
96 *protein expression in silkworm pupae*

97 In our previous studies, the VP15, VP15$_{(26–57)}$, and SR11 encoding genes were cloned into
98 the pGEX-6P-1 GST-fusion vector for protein expression in *Escherichia coli* [27]. The
99 plasmids were used as a template for amplifying the GST-fused VP15, VP15$_{(26–57)}$, and SR11
100 gene cascades using KOD-PLUS-NEO kit (Toyobo, Tokyo, Japan). The amplicons were
101 cloned into the pFastBac-1 (Thermo Fisher Scientific, Tokyo, Japan), screened for the positive
102 clone(s), and confirmed the sequences via DNA sequencing. The positive plasmids were
103 designated as pFastBac/GST-VP15, pFastBac/GST-VP15$_{(26–57)}$, and pFastBac/GST-SR11. The
104 pFastBac/GST was also cloned using the described procedure.

105 The recombinant plasmids were used for the transformation into *E. coli* BmDH10Bac (CP^{-}
106 /Chi^{-}) for recombinant *Bombyx mori* nucleopolyhedrovirus (BmNPV) bacmid generation [28],
107 resulting in rBmNPV/GST, rBmNPV/GST-VP15, rBmNPV/GST-VP15$_{(26–57)}$, and
108 rBmNPV/GST-SR11 bacmids. The bacmids were prepared via alkaline-lysis with sodium
109 dodecyl sulfate (SDS) [29] and were then transfected to silkworm larvae (Ehime Sansyu,
110 Ehime, Japan) [28]. Silkworms were reared with an artificial diet, Silkmate S2 (Nosan, Japan),
111 under a controlled environment (25°C, 65 ± 5% relative humidity). Silkworm larval
112 hemolymph and fat bodies were collected six days after the transfection. Hemolymph was kept

113 as a recombinant BmNPV (rBmNPV) stock in a –80°C freezer for subsequent silkworm
114 larva/pupa infection.

115 Silkworm pupae were purchased from Ehime Sansyu (Ehime, Japan). Upon arrival,
116 silkworm pupae were kept inside the 4ºC refrigerator until needed. The hemolymph stocks
117 containing rBmNPVs were diluted 100-fold and injected into silkworm pupae (approximately
118 50 µl/pupa). The pupae were left inside the chamber for 5–6 days to express the recombinant
119 proteins. Then, pupae were collected and ground to a powder in the presence of liquid nitrogen
120 using a mortar and a pestle. The resulting pupa powder was transferred to a 50 mL centrifuge
121 tube and freeze-dried overnight under a vacuum.

122

123 *2.2.Quantitative western blot analysis*

124 To quantify the recombinant proteins expressed in silkworm pupae, the recombinant GST-
125 VP15, -VP15$_{(26–57)}$, and -SR11 were expressed using *E. coli* Rosetta gami-B (Novagen, Tokyo,
126 Japan) and purified with the glutathione Sepharose 4 Fast Flow (Cytiva, Tokyo, Japan)
127 accordingly to the previous report [27]. The purified proteins were used as a standard for
128 protein quantification. The freeze-dried silkworm pupae powder was resuspended in
129 phosphate-buffered saline (PBS, pH 7.4), mixed with an equal volume of 2 × SDS-loading
130 buffer, and heated at 98ºC for 5 min. The expression of the VP15-related constructs in pupae
131 was analyzed by western blotting with an anti-FLAG antibody (MBL, Tokyo, Japan), and the
132 intensities/area under the curves (AUC) of protein bands were determined using ImageJ and
133 Quantity One (Bio-Rad) software. The expressed recombinant protein amount was quantified
134 by comparing it to the standard calibration of the corresponding protein.

135

136 *2.3. Synthesis of the SR11 peptide*

137 The VP15-derived peptide SR11 was commercially synthesized (GL Biochem Ltd.,
138 Shanghai, China) and was characterized using high-performance liquid chromatography
139 (HPLC) and electrospray ionization mass spectrometry (ESI-MS). HPLC was employed to
140 purify the synthetic peptide using an Inertsil ODS-SP column to achieve a purity of >95%. The
141 purity and molecular masses of the purified SR11 peptide were analyzed on electrospray
142 ionization coupled with liquid chromatography-mass spectrometry (LC-MS/ESI, Agilent-
143 6125B).

144

145 *2.4. Shrimp and WSSV inoculum*

146 The kuruma shrimp (body weight: 0.65±0.14 g) produced at a shrimp farm in Oita
147 prefecture were used in the present study. The shrimp were reared with dechlorinated
148 electrolyzed seawater (21 ± 1°C) in a flow-through system inside double-bottomed tanks with
149 sand beds and fed with a custom-made crumbled diet (i.e., without shrimp meal) at 3% of body
150 weight per day. The shrimp were confirmed to be WSSV-free by qPCR just before using for
151 the following experiments.

152 The WSSV suspension was prepared according to our previous report [19]. Briefly, the
153 muscle of moribund WSD shrimp was homogenized with four-time volumes of PBS and
154 centrifuged at 3000 *g* for 10 min at 4°C. The resulting supernatant was stored as a WSSV
155 inoculum in a –80°C freezer until needed.

156

157 *2.5. Preparation of shrimp diet containing GST and GST-fusion proteins/peptide*

158 To prepare silkworm pupae for oral administration, powdered pupae containing GST
159 and GST-fusion proteins were suspended in PBS at a volume equivalent to 6% of feed weight
160 (w/w) and mixed with custom dried feed (Higashimaru Co., Ltd). These diets were bound with
161 SD Tenpaku No. 1 (Japan Nutrition Co., Ltd.), a 0.5% feed weight binder. The diet containing
162 SR11 peptide was also prepared in the same manner.

163

164 *2.6. Oral administration of shrimp for WSSV challenge study*

165 The kuruma shrimp were divided into six groups (n = 40 per group) and fed on a custom
166 dry diet (Table 1). These rations were provided for 23 d. From 24 d to 30 d, the ration for
167 shrimp was changed to a normal commercial diet. Seven days after the final feeding, shrimp
168 were exposed to WSSV by immersion route (n = 20 – 32 per group) for 2 h in seawater
169 containing 4.6×10^7 copies mL^{-1} of WSSV. The WSSV doses used in challenge studies were
170 adjusted to produce 60% cumulative mortality among negative control shrimp based on the
171 LD_{50} data. In the challenged groups, dead shrimp were removed twice daily and stored at −30°C
172 for qPCR analysis to confirm that WSSV infection was the cause of death. To detect WSSV
173 by qPCR, total DNA was extracted from shrimp using a QIAamp DNA Mini Kit (Qiagen)
174 following the manufacturer’s instructions.

175 Real-time PCR was performed using a total volume of 20 µL, containing 70 ng template
176 DNA and 2 × Probe qPCR Mix (Takara Bio, Shiga, Japan)10 µL. For WSSV quantification,
177 0.25 µM of TaqMan probe [Pr (5′-FAM-AGCCATGAAGAATGCCGTCTATCACACA-
178 BHQ-3′)] and 0.3 µM of each WSSV-specific primers (Table 2) were used for the detection.
179 Thermal cycling consisted of an initial denaturation step at 95°C for 30 s, followed by 40 cycles
180 of 95°C for 5 s and 60°C for 30 s annealing and extension steps on the CFX Connect (Bio-
181 Rad., USA). The quantity of each sample was determined using CFX Operating Software

182 version 4.0. The copy number of the target amplicon in the plasmid was estimated, and 10-fold
183 serial dilutions were made for use as absolute standards for quantification. The viral copy
184 number was normalized on a nanogram genomic DNA basis or a milliliter basis for water. For
185 each new run, at least 2 non-template control were performed as a negative control.

186

187 *2.7. RNA extraction and cDNA synthesis*

188 Total RNA from the gills of *M. japonicus* was extracted using the commercial RNA
189 extraction kit, NucleoSpin RNA (Macherey-Nagel, Germany), following the manufacturer's
190 protocols. First-strand cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix
191 (Toyobo, Japan), according to the manufacturer's protocol. The ND-1000 NanoDrop
192 spectrophotometer (Thermo Fisher Scientific, Japan) was used to determine the amount of
193 nucleic acid in each total RNA sample.

194

195 *2.8. Real-time PCR analysis*

196 Total RNA was extracted from the gills on days 0, 1, 3, and 14 after oral administration of
197 the recombinant protein expressed-pupa powder. The gill tissue was collected from three
198 individuals in each group. Total RNA was reverse transcribed into cDNA, and the cDNA was
199 used as a template for quantitative PCR (qPCR) of *Dorsal*, *Akirin*, *Relish*, *STAT*, and *proPO*.
200 Additionally, the changing patterns of effector molecule mRNA transcripts, including *Anti-*
201 *Lipopolysaccharide Factor* (*Alf-D2*), *Crustin*, *Penaeidin*, and *Lysozyme,* were investigated.
202 The primers are listed in Table 2. The *β-actin* served as an internal control gene. The qPCR
203 was performed with the Thunderbird SYBR qPCR Mix (Toyobo, Japan) and was programmed
204 at 95ºC for 1 min, followed by 40 cycles at 95ºC for 15 s, 55ºC for 30 s, and 72ºC for 1 min.

205 Melting curve analysis from 55ºC to 95ºC was then performed. The qPCR data were analyzed

206 with the $2^{-\Delta\Delta Ct}$ method (Ct, cycle threshold) [30].

207

208 *2.9. Statistical analysis*

209 Statistical analysis of the time-mortality relationship was performed with chi-squared

210 analysis with a significant level of 1% (χ^2 test, $p < 0.01$). The protective effect against WSSV

211 was calculated as survival rate (%) or relative percent survival (RPS) with the following

212 equations [31].

213 $$Survival\ rate\ (\%) = \left(\frac{alive\ shrimp\ number}{initial\ shrimp\ number}\right) \times 100$$

214 $$RPS\ (\%) = \left[1 - \left(\frac{\%\ mortality\ in\ vaccinated\ group}{\%\ mortality\ in\ the\ PBS\ group}\right)\right] \times 100$$

215 The relative expression data are represented as mean ± standard deviation (SD). The

216 differences between a quantified gene in immunized and control groups were analyzed by one-

217 way analysis of variance (ANOVA). p-value of < 0.05 was considered statistically significant.

218

219 **3. *Results***

220 *3.1. Generation of recombinant BmNPV for protein expression in B. mori pupae*

221 The expression of GST-VP15, -VP15$_{(26–57)}$, and -SR11 in silkworm pupae were analyzed

222 by western blot with an anti-Flag antibody (Fig. 2) compared with the purified proteins. The

223 bands corresponding to the recombinant GST-VP15, -VP15$_{(26–57)}$, and -SR11 could be detected

224 at the expected height similar to the purified proteins (Fig. 2A–C). The theoretical sizes of

225 GST-VP15, -VP15$_{(26–57)}$, and -SR11 are expected to be 37, 31.6, and 29.2 kDa respectively.

226

227 *3.2. Quantitative western blot analysis*

228 The quantitative western blot analysis was performed against the corresponding purified
229 product to quantify VP15, $VP15_{(26–57)}$, and SR11 expressed in silkworm pupae. From the
230 analysis, the amounts of GST-VP15, $-VP15_{(26–57)}$, and -SR11 in silkworm pupae were
231 estimated to be 496.8, 142.4, and 535.0 ng/mg powdered pupa, respectively (Fig. 2D and S1).
232 Calculations based on the band intensities/area under the curve from both programs yielded a
233 similar result.

234

235 *3.3. Oral administration of feed containing immunizing agent*

236 After oral administration of pupa/VP15-, pupa/$VP15_{(26–57)}$-, pupa/SR11-, or SR11 peptide-
237 containing feed, shrimp were challenged with WSSV via an immersion route (Fig. 3A) and
238 were observed for 20 d. In the PBS-administered group, death events started at 7 d post-
239 infection (dpi) and rapidly decreased during 7 to 10 dpi. At the end of the observation, the
240 survival rate was 52% (48% of cumulative mortality) in the PBS group. On the other hand, no
241 death was observed in a group of shrimp fed with pupa/$VP15_{(26–57)}$-containing feed (Fig. 3B).
242 Shrimp fed with pupa/GST and pupa/SR11 showed a similar survival rate of 85% (15% of
243 cumulative mortality) and 86.7% (13.3% of cumulative mortality) respectively. However,
244 these were not significantly different from the PBS group (χ^2 test, $p < 0.01$). While, groups of
245 shrimps receiving pupa/VP15 and SR11 showed survival rates of 90.9% (10.1% of cumulative
246 mortality) and 95.5% (4.5% of cumulative mortality) respectively, which were significantly
247 higher (χ^2 test, $p < 0.01$) than the survival rate from the control feed (PBS group).

248 The RPS values are summarized in Table 3. Shrimp that fed the pupa/$VP15_{(26–57)}$-
249 containing feed showed an RPS of 100%, the highest RPS among all the experimental groups

250 and no death was observed in this group. RPSs in groups of shrimps fed with pupa/VP15- and
251 pupa/SR11-containing feed were 81.0% and 72.3% respectively. Interestingly, the group of
252 shrimp receiving SR11 through an oral route showed an RPS of 90.6%. Overall, the oral
253 administration of VP15-derived proteins or peptides could confer protection in kuruma shrimp
254 against WSSV with an RPS of over 70%. The group of shrimp receiving pupa/GST had the
255 lowest RPS value (68.8%).

256

257 *3.4. Quantitative analysis of genes expression by qPCR*

258 Quantitative polymerase chain reaction (qPCR) was used to determine the changes in
259 expression levels of shrimp (*M. japonicus*) *MjDorsal*, *MjAkirin*, *MjRelish*, *MjSTAT*, and
260 *MjproPO* genes from their gills at 1-, 3-, and 14-d post-immunization. The groups receiving
261 pupa/VP15, pupa/VP15$_{(26–57)}$, and pupa/SR11 showed a similar trend of *MjDorsal*, *MjRelish*,
262 and *MjSTAT* mRNA levels which were significantly upregulated at 14 d in comparison to the
263 pupa/GST and PBS (or un-immunized) group (Figs. 4–6). The relative *MjAkirin* mRNA levels
264 showed a similar increasing trend and level in the pupa/GST and pupa/VP15-derived products
265 immunized groups. However, the group receiving pupa/VP15$_{(26–57)}$ showed the highest mRNA
266 level on average (Fig. 4B). The *MjproPO* level in the pupa/SR11 group was highest on day 3
267 and gradually decreased, while the pupa/VP15 and pupa/VP15$_{(26–57)}$ groups displayed the
268 highest relative *MjproPO* mRNA levels on day 14 (Fig. 6B).

269 Interestingly, we also noticed that the (mRNA) expression levels of *MjDorsal*, *MjAkirin*,
270 *MjRelish*, and *MjSTAT* in the pupa/GST group were upregulated on day 14 but at lower levels
271 than the group receiving pupa/VP15-derived products, except for the *MjAkirin* which showed
272 a similar level (Figs. 4–6). The group receiving synthetic SR11 through an oral route showed
273 a similar changing pattern of mRNA levels. *MjDorsal*, *MjAkirin*, *MjRelish*, *MjSTAT*, and

274 *MjproPO* were spiked on day 1 of feeding. However, the mRNA levels gradually decreased as
275 time progressed (Figs. 4–6).

276 Additionally, the relative mRNA expression levels of the effector molecules (*MjAlf-D2,*
277 *MjCrustin, MjLysozyme,* and *MjPenaeidin*) were also investigated (Fig. 7). In pupa/VP15- and
278 pupa/VP15$_{(26–57)}$-immunized groups, the levels of *MjAlf-D2, MjCrustin, MjLysozyme,* and
279 *MjPenaeidin* were significantly upregulated at almost all time points (Fig 7A-D). The group
280 immunized with pupa/SR11 showed elevated levels of *MjCrustin, MjLysozyme,* and
281 *MjPenaeidin* (Fig. 7B–D). The group receiving artificial SR11-mixed feed showed a
282 significant upregulation of *MjAlf-D2, MjLysozyme,* and *MjPenaeidin* at earlier times. Still, the
283 levels on day 14 were higher than the PBS control group on average (Fig. 7A, C, and D). In
284 the pupa/GST group, the *MjCrustin* and *MjLysozyme* were significantly upregulated, and the
285 average levels of *MjPenaeidin* at all time points were higher than the PBS group.

286 The group of shrimps fed with control feed (PBS group) showed a small or no change in
287 the mRNA levels of all investigated genes throughout the observation period (Figs. 4–7).

288

289 ## ***4. Discussion***

290 WSSV is one of the most concerning shrimp pathogenic viruses among the causative agents.
291 Many researchers have used WSSV structural proteins (*e.g.,* VP19, VP24, VP26, and VP28),
292 nucleic acid-based agents, inactivated WSSV particles, etc., to induce protection against the
293 pathogen. The discovery of a quasi-immune response opens the possibility of immunizing
294 agent development for combating WSSV outbreaks in shrimp aquacultures. However, most
295 shrimp immunizing studies are usually based on the intramuscular (IM)-injection method,
296 which is not practical for field application. Later, several research groups attempted to induce

297 anti-WSSV immunity through an oral route using various delivering vehicles, including *E. coli*,
298 *Bacillus subtilis*, yeasts, and silkworm (homogenate) [32].

299 In recent decades, silkworm larva/pupa has been used as a platform for recombinant
300 protein production. The system has been applied for many recombinant protein productions,
301 including virus-like particles, eukaryotic proteins, and pharmaceutically-related proteins (*e.g.*,
302 cell or viral proteins) [33]. Silkworm pupa exhibits several advantages over the larva for protein
303 production. i) The synthesis of viral (BmNPV) proteins is efficient because the metabolic rate
304 is low during the pupa stage; therefore, the host's low protein synthesis is beneficial for proteins
305 requiring high co-/post-translational processing or the production of secretory proteins. ii) Pupa
306 can survive at 4ºC for a long period and require no diet during this stage [33, 34]. Besides,
307 silkworm pupa is an edible protein source and is considered to be one of the future foods.
308 Dried silkworm (*B. mori*) pupae consist of 55% and 32% of total protein and lipid, respectively,
309 along with a high content of essential amino acids such as valine, leucine, lysine, and lipid such
310 as omega-3 and omega-9 [35]. Therefore, silkworm pupae are used to feed farmed animals
311 such as cattle and fish.[36].

312 Recent findings indicated that silkworm pupa is a source of pharmaceutical-valued
313 bioactive compounds, *e.g.*, several publications demonstrated proteins or peptides extracted
314 from silkworm pupae could function as immunomodulatory molecules by enhancing non-
315 specific immune responses in a host [37]. A more recent study reported the finding of
316 silkworm-derived bioactive compounds with an anti-viral activity [38]. Furthermore, the
317 presence of protease inhibitors and biocapsule-like fat in silkworms may increase the stability
318 of recombinant proteins from the harsh environment during oral administration and delivery of
319 immunizing agents [39]. These findings support the potential usage of silkworm pupae as a
320 functional food supplement and even a vehicle for delivering any immunizing agent.

321 In this study, we demonstrated silkworm pupae as a vehicle for delivering the VP15 and
322 its derived products to kuruma shrimp via oral administration. After immunization and the
323 WSSV challenge, we observed a significant improvement in survival rates, particularly in the
324 groups receiving pupa/VP15$_{(26–57)}$- or synthetic SR11-supplemented feed. The overall RPS
325 value was more than 70% in all immunized groups, indicating that the VP15-derived products
326 could substantially protect kuruma shrimp against WSSV. We also noticed that the pupa/GST
327 group had a higher survival rate than the control group. This suggests that the presence of pupa
328 powder may positively affect shrimp survivability by enhancing immunomodulation. Despite
329 the promising results, cultivating silkworm larvae/pupae at a large scale requires space and
330 staffing; hence, it is a limiting factor for a field application. A suitable facility and highly
331 trained personnel are crucial for generating enough recombinant BmNPV for protein
332 expression in a silkworm-based system.

333 We have observed an improvement in the survival rate in the group receiving pupa/GST
334 or BmNPV-infected pupae. Several reports demonstrated the use of silkworm pupae for
335 immunizations in fish and shrimp through an oral administration. The oral administration of
336 grass carp (*Ctenopharyngodon idella*) with BmNPV-infected pupae did not improve the
337 survival rate after being challenged by grass carp reovirus (GCRV). The carps receiving
338 silkworm pupae expressing GCRV proteins showed an elevated survival rate [40]. Similar
339 studies have applied silkworm expressing WSSV structural proteins to immunize crayfish
340 (*Procambarus clarkia*). The groups immunized with silkworm expressing VP28 or VP19
341 improved survival rates after the WSSV challenge. However, the groups receiving either mock-
342 infected silkworm or HyNPV-infected silkworm did not show an elevation in survival rates
343 [41, 42]. However, our result suggested otherwise. We considered that the improvement in the
344 RPS of the pupa/GST group was affected by a prophylactic effect. Several studies reported the
345 prophylactic potency of peptidoglycan from *Bifidobacterium thermophilum* in enhancing

346 resistance to a pathogenic bacterium (*Vibrio panaeicida*) [43] and of several probiotic
347 microorganisms (*Pediococcus pentosaceus*, *Staphylococcus hemolyticus*, *Lactobacillus*
348 *plantarum*, *Lactococcus lactis*, *Bacillus megaterium*, and yeast-like *Candida haemulonii* and
349 *C. sake*) in reducing the virulence of WSSV [44–47]. Another study reported that the oral
350 administration of *E. coli* cells ~~alone~~ could enhance the survival rate of kuruma shrimp (*M.*
351 *japonicus*) after the oral WSSV challenge [19]. In mice experiments, oral administration of
352 silkworm pupae also showed a prophylactic property. Silkworm pupae expressing recombinant
353 urease subunit B (UreB) and heat shock protein A subunit (HspA) of *Helicobacter pylori*
354 showed therapeutic and prophylactic effects against *H. pylori* in mice [48, 49]. Another
355 research used silkworm pupae expressing amyloid-β peptide (Aβ42), a biomarker of
356 Alzheimer's disease, as prophylaxis for preventing Alzheimer's disease in a mice model [50].
357 Therefore, these data support our hypothesis on the prophylactic effect of silkworm pupae in
358 enhancing shrimp resistance to WSSV, and the successful delivery of VP15-derived products
359 further enhanced the immune responses.

360 As an invertebrate species, shrimp lack adaptive immunity, thus, solely relying on innate
361 immunity to fight against the invading pathogen. The innate immune responses of these
362 invertebrates initiate upon the recognition of pathogen-associated molecular patterns (PAMPs)
363 by pattern-recognition receptors or proteins (PRRs or PRPs), which can be generally
364 categorized into cellular defenses and humoral defenses. Cellular defenses include circulating
365 hemocytes for pathogen clearance by phagocytosis, encapsulation, RNA interference, and
366 apoptosis. In contrast, humoral responses involve the production of soluble effector molecules
367 such as antimicrobial peptides (AMPs) through signal transductions of the immune-related
368 pathways and activation of the prophenoloxidase (proPO) system [51]. Nuclear factor kappa B
369 (NF-κB) signaling pathway, Toll and immune deficiency (IMD) pathway, and Janus Kinase
370 (JAK)/STAT signaling pathway are the three major pathways in the regulation of AMP

371 production [52]. To support the immunization efficacy induced by pupa/VP15-derived
372 products, the changes in mRNA expression patterns of immune-related genes in the immunized
373 host were analyzed: *Dorsal* of the Toll pathway, *Relish* of the IMD pathway and its positive
374 regulator *Akirin*, and *STAT* of JAK/STAT pathway, as well as the *proPO* of proPO system (Fig.
375 8).

376 The NF-κB signaling pathways have been well established in shrimp and are known for
377 their importance in humoral immunity against infections. Two major transcriptional factors
378 regulate these pathways i) Dorsal of the Toll signaling pathway and ii) Relish of the IMD
379 signaling pathway. Both pathways can be initiated upon the recognition of PAMPs by PRPs
380 [53, 54]. Toll signaling pathway, Toll4 has been newly identified as the potential PRP for
381 WSSV [55]. Upon the activation of Toll receptors, MyD88 is recruited and forms a complex
382 with Tube and Pelle through interactions of death domains. Pelle has a kinase activity that can
383 phosphorylate the NF-κB inhibitor (IκB) Cactus, resulting in a dissociation of Cactus from the
384 Dorsal [53] (Fig. 8A).

385 In contrast to the Toll signaling pathway, the mechanism of the IMD pathway is still
386 unclear. Although many core components of the pathway are being identified, including IMD,
387 TAB2, TAB1, TAK1, IKKβ, IAP2, and the transcription factor Relish, some pivotal
388 components such as FADD and DREDD homologs are waiting to be discovered from shrimp
389 [53] (Fig. 8B). Recently, a new regulator of the IMD pathway Akirin was found to be a positive
390 regulator for several IMD-Relish-targeted AMPs via direct interaction with the Relish [56, 57].
391 Moreover, the RNAi for silencing Akirin revealed a decrease in the Relish level, and shrimp
392 were prone to the WSSV infection [58]. In general, the activation of the IMD cascade results
393 in the phosphorylation of Relish and the cleavage of the C-terminal IκB-like domain containing
394 six ankyrin repeats (ANKs) [59]. The unmasked Dorsal and the truncated Relish or N-terminal
395 Rel homology domain (RHD) then translocate into the nucleus and activate the production of

AMPs, including anti-lipopolysaccharide factors (ALFs), crustins, penaeidins, and lysozymes [60].

Here, we analyzed the changes in ~~NF-κB pathway-related gene~~ expression levels of NF-κB pathway-related genes (*Dorsal*, *Relish*, and *Akirin*) and NF-κB-targeted AMPs (*Alf-D2*, *Crustin*, *Lysozyme*, and *Penaeidin*) in immunized shrimp by qPCR. The shrimp fed with pupa/VP15-derived products or synthetic SR11 peptide enhanced the *Dorsal, Relish*, and *Akirin* mRNA levels. Interestingly, the groups fed with pupa/GST showed an increase in *Dorsal*, *Relish*, and *Akirin* mRNA levels on day 14, but at relatively lower levels than the groups receiving pupa/VP15-derived products except for the *Akirin,* showing a similar mRNA level to the group receiving pupa/VP15-derived products. We further analyzed the expression of several NF-κB targeted AMPs, including *Alf-D2*, *Crustin*, *Lysozyme*, and *Penaeidin*. These four genes were significantly upregulated in the groups receiving pupa/VP15 and pupa/VP$_{(26–57)}$ following the trends of *MjDorsal* and *MjRelish.* The group receiving pupa/SR11 showed an upregulation of *Crustin*, *Lysozyme*, and *Penaeidin*. The SR11 group showed a different pattern in the upregulations of *Alf-D2*, *Lysozyme*, and *Penaeidin*, which had a high level on day 1, and the transcript levels tended to be stable or slightly increased as time progressed. The shrimp fed on pupa/GST presented an upregulation of *Crustin* and *Lysozyme*. The level of *Penaeidin* in this group was also elevated as it was higher than the PBS control group on average; however, statistically not significant. Thus, these results indicate that pupa/VP15-derived or SR11 products could induce the expression of immune-related genes, which might be one of the key contributions to protective effects against WSSV. In contrast, pupa/GST may generate the minimum immune response, which explains the increase in the survival rate of this group.

The JAK/STAT pathway is an interferon (IFN)-mediated antiviral response in mammals. Later, with increasing evidence, this pathway also plays an antiviral role in invertebrate species and is evolutionarily conserved [61, 62]. The pathway has three main components cytokine-

421 like receptors or domeless at the cell surface, Janus kinases (JAKs), and signal transducers and
422 activators of transcription (STATs) [63]. The JAK/STAT pathway can be activated through the
423 interaction of C-type lectin or Vago to the surface receptor resulting in an upregulation of
424 AMPs expression [62, 64] (Fig. 8C). Vago genes have been identified from shrimp species.
425 They have been involved in anti-WSSV and anti-bacterial responses through JAK/STAT
426 activation resulting in an enhancement in the transcription of the immune effector [64–66]. In
427 fruit fly (*Drosophila melanogaster*) and mosquito (*Aedes aegypti*), Vagos are under the
428 regulation of the IMD pathway [67, 68], hence suggesting potential crosstalk between the IMD
429 pathway and JAK/STAT pathway (Fig. 6C). Our findings showed an upregulation of the *STAT*
430 gene after an oral administration of pupa/VP15-derived products or SR11 peptides. We
431 hypothesized that the NF-κB-controlled Vago could mediate the upregulation of STAT due to
432 the increase in the *Relish* level. Taken together, the JAK/STAT could be the third life-support
433 that enhances shrimp resistance against WSSV in addition to the Toll and IMD pathways.

434 Another important humoral arm is the melanization mediated by the proPO activation
435 cascade. This non-self-recognition system also plays a role in shrimp defense against pathogens,
436 supporting cellular responses through hemocyte attraction, enhancing phagocytosis activities,
437 melanization, and particle encapsulation [69]. The proPO system can be activated upon the
438 recognition of PAMPs on pathogens by PRPs, which induces the serine proteinase cascade that
439 eventually activates the proPO-activating enzymes (PPAEs) (Fig. 8D). The PPAEs, then
440 activate the proPO by proteolytic cleavage of the proPO zymogen, yielding the enzyme
441 phenoloxidase (PO), which leads to the melanization at the site of infections [51]. Studies have
442 suggested that viral infection could hamper PO activity, and the silencing of *proPO* led to
443 increased mortality [70, 71]. Therefore, the proPO system is another critical player in antiviral
444 immunity in shrimp species. Our results indicated that the shrimp fed with either pupa/VP15-
445 derived products or synthetic SR11 peptide showed increased *proPO* mRNA levels. It is

446 possible to mention that the WSSV-VP15 or the antigenic VP15-derived peptide SR11 is
447 sufficient to induce the *proPO* system, which may be another critical factor for survivability in
448 shrimp against the devastating pathogen.

449

450 ## 5. Conclusion

451 We successfully developed an oral immunizing agent using silkworm pupa as a delivery
452 vehicle for VP15-derived products, that can be combined with a commercial feed. The oral
453 administration of pupa/VP15-derived products, particularly the group receiving pupa/VP15$_{(26–}$
454 $_{57)}$, provided substantial protection against WSSV and induced the expression levels of
455 immune-related genes in kuruma shrimp compared to the unimmunized group. Moreover, the
456 unique high biosafety profile of the silkworm makes the system an attractive choice for
457 developing an oral immunization strategy. Therefore, as presented here, oral immunization of
458 shrimp using silkworm pupae as an immunizing agent carrier may provide a new avenue of
459 field-applicable immunization in aquaculture. Further investigations may focus on improving
460 delivery efficacy, optimization, and dependency on the dose of transgenic pupae and feeding
461 duration.

462

463 ## Author Contributions

464 **Jirayu Boonyakida**: Investigation, Methodology, Writing – original draft preparation.
465 **Takafumi Nakanishi**: Resource, Methodology. **Jun Satoh**: Methodology, Investigation.
466 **Yoshiko Shimahara**: Methodology. **Tohru Mekata**: Resource. **Enoch Y. Park**:
467 Conceptualization, Funding acquisition, Writing – revision & editing, Supervision.

468

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477

478 **Compliance with Ethical Standards**

479 Not applicable
480

481 **Conflict of interest**

482 The authors declare that they have no conflicts of interest.
483

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739 **Table 1. Set-up of the oral immunization experiments**

Group	Form	Feeding amount[a]	mg freeze-dried powdered pupa[b]	No. of shrimp/group
PBS (control 1)	-	-	-	40
GST		10.8	10.8	40
GST-VP15		20.41	41.1	40
GST-$VP15_{(26–57)}$	Pupa	3.74	46.4	40
GST-SR11		24.59	45.9	40
SR11	Synthetic peptide	1.28	-	40

740 [a]: μg of recombinant protein per gram of shrimp per day (g^{-1} of shrimp d^{-1})

741 [b]: per day

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763 **Table 2. Primers used in this study**

Primer name	**Sequence (5’- 3’)**
For WSSV detection	
WSSV-1011F	TGGTCCCGTCCTCATCTCAG
WSSV-1079R	GCTGCCTTGCCGGAAATTA
For qRT-PCR	
MjDorsal-FW	AGACTGGGTTTTCTCATCGTAATC
MjDorsal-RV	TAAATGGGATCTGACACTTGTGG
MjRelish-FW	CACCACAGCACACTGTTCC
MjRelish-RV	GGAGACATCACACTGTACTG
MjAkirin-FW	GTGCGAGAAGAGATCCGGAG
MjAkirin-RV	CTTGAAGACGGTGCTGGAGA
MjSTAT-FW	GGTCCCAGTTCTGTAAGGAG
MjSTAT-RV	AGCATCTCTTCAGCCTGGCG
MjproPO-FW	CCAAGTGCCAGAACGAAATG
MjproPO-RV	CGATGAGACGCGAGGAAG
MjPenaeidin-FW	GCTGCACCCACTATAGTCTTT
MjPenaeidin-RV	CTACCATGGTGATGAAACAAA
MjCrustin-FW	CATGGTGGTGGCTTAGGAAA
MjCrustin-RV	GTAGTCGTTGGAGCAGGTTA
MjLysozyme-FW	TCCTAATCTAGTCTGCAGGGA
MjLysozyme-RV	CTAGAATGGGTAGATGGA
MjAlfD2-FW	CGCAGGCTTATGGAGGAC
MjAlfD2-RV	AGGTGACAGTGCCGAGGA
MjActin-FW	CAGCCTTCCTTCCTGGGTATGG
MjActin-RV	GAGGGAGCGAGGGCAGTGATT

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765

766 **Table 3. Mortality and RPS from orally immunized kuruma shrimp**

Group	Form	Number of dead individuals (WSSV detected)	Mortality (%)	RPS (%)	Prevalence of WSSV detection in surviving individuals
PBS (control 1)	-	12/25	48.0	-	0
GST		3/20	15.0	68.8	35.3
GST-VP15		2/22	9.1[a]	81.0	0[a]
GST-VP15$_{(26–57)}$	Pupa	0/17	0[a]	100.0	5.9[b]
GST-SR11		2/15	13.3	72.3	5.9[b]
SR11	Synthetic peptide	1/22	4.5[a]	90.6	19.0

767 [a]: $p < 0.01$

768 [b]: $p < 0.05$

769

770 ## Figure legends

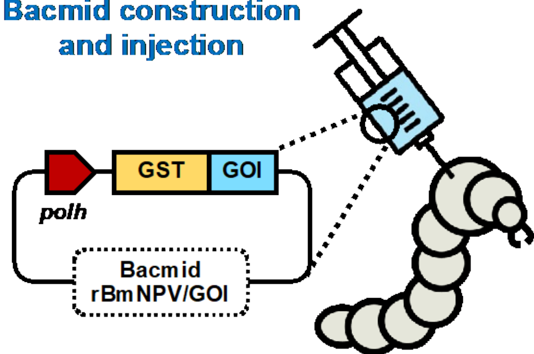
771 **Fig. 1.** Schematic diagram of the recombinant BmNPV expression vector system for
772 development of oral immunization using VP15-derived products in kuruma shrimp. The gene-
773 of-interest (GOI), VP15, VP15$_{(26–57)}$, or SR11, was cloned as a GST-fusion gene, and
774 recombinant BmNPV bacmids were prepared for protein expression in the silkworm. The
775 recombinant BmNPVs coding VP15-derived constructs were injected into the silkworm pupa.
776 The VP15-derived constructs-expressed pupae were ground to a powder form and freeze-dried.
777 The freeze-dried powdered pupae were then applied for oral immunization of kuruma shrimp.
778

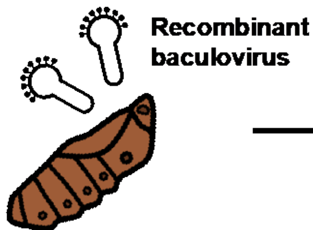
779 **Fig. 2.** Quantitative Western blot analysis of GST-fused VP15 (A), VP15$_{(26–57)}$ (B), and SR11
780 (C) expressed in silkworm pupae using anti-Flag antibodies. Quantification of GST-VP15,
781 GST-VP15$_{(26–57)}$, and GST-SR11 expressed using silkworm pupae compared with the purified
782 correspondent proteins purified GST-fused VP15 (A), VP15$_{(26–57)}$ (B), and SR11 (C) from *E.*
783 *coli*. (D) The amount of the expressed recombinant proteins in silkworm pupae was calculated
784 using a calibration line, as shown in Fig. S1.
785

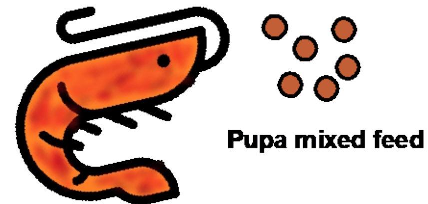
786 **Fig. 3.** Protective effect of the feed containing pupa/GST, pupa/VP15, pupa/VP15$_{(26–57)}$,
787 pupa/SR11, or synthetic SR11 peptide against WSSV through oral administration. (A) Time-
788 schedule of shrimp immunization, WSSV challenge, and observation. (B) The survival rate of
789 immunized kuruma shrimp. The survival rates from all groups were plotted against time (in
790 day unit) after the challenge. PBS served as a negative control representing the un-immunized
791 group.
792

793 **Fig. 4.** The relative mRNA expression of *MjDorsal* representing the Toll pathway analyzed by
794 qPCR of kuruma shrimp fed with pupa/GST, pupa/VP15, pupa/VP15$_{(26–57)}$, pupa/SR11,

795 synthetic SR11 peptide, or the controlled diet after 1 day, 3 days, and 14 days. The asterisks
796 indicate a significant difference ($p < 0.05$) compared to the PBS control group at different time
797 points.
798
799 **Fig. 5.** The relative mRNA expression of *MjRelish* representing the IMD pathway (A) and
800 *MjAkirin*, the positive regulator of IMD pathway (B), was analyzed by qPCR after 1 day, 3
801 days, and 14 days of feeding. The asterisks indicate a significant difference ($p < 0.05$) compared
802 to the PBS control group at different time points.
803
804 **Fig. 6.** The relative mRNA expression of *MjSTAT* representing the JAK/STAT pathway (A)
805 and *MjproPO* of proPO system (B) was analyzed by qPCR after 1 day, 3 days, and 14 days of
806 feeding. The asterisks indicate a significant difference ($p < 0.05$) compared to the PBS control
807 group at different time points.
808
809 **Fig. 7.** The relative mRNA expression of effector molecules; *MjAlf-D2* (A), *MjCrustin* (B),
810 *MjLysozyme* (C), and *MjPenaeidin* (D), analyzed by qPCR after 1 day, 3 days, and 14 days of
811 feeding. The asterisks indicate a significant difference ($p < 0.05$) compared to the PBS control
812 group at different time points.
813
814 **Fig. 8.** Schematic diagram of the shrimp (*M. japonicus*) immune system. The green box
815 represents the up-regulated immune-related gene after oral immunization. A, B, C, and D
816 indicate the Toll pathway, IMD pathway, JAK/STAT pathway, and proPO system,
817 respectively.
818







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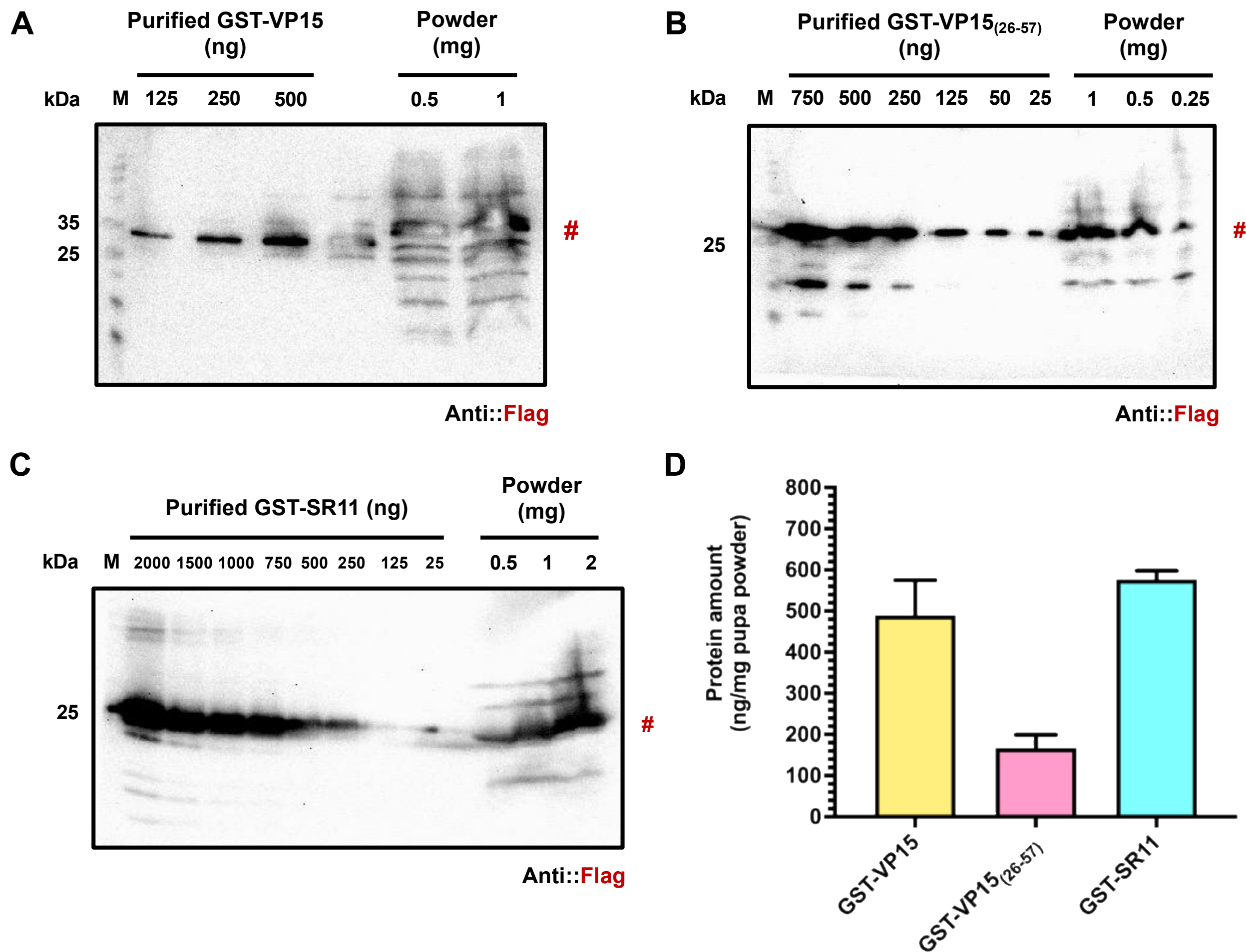

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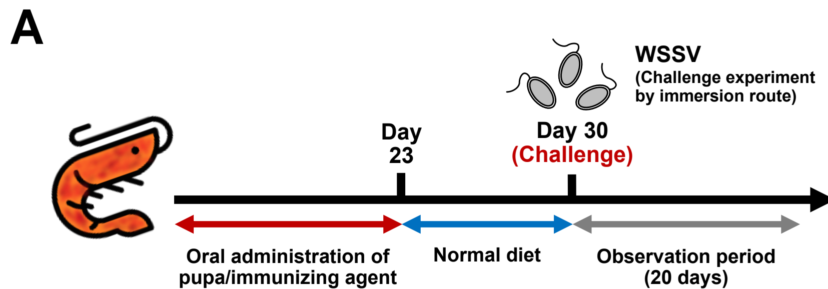

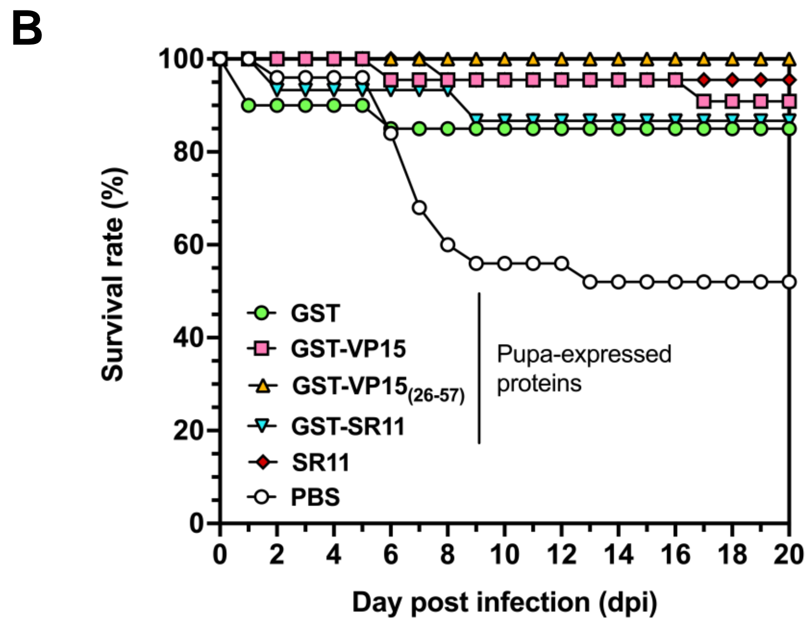

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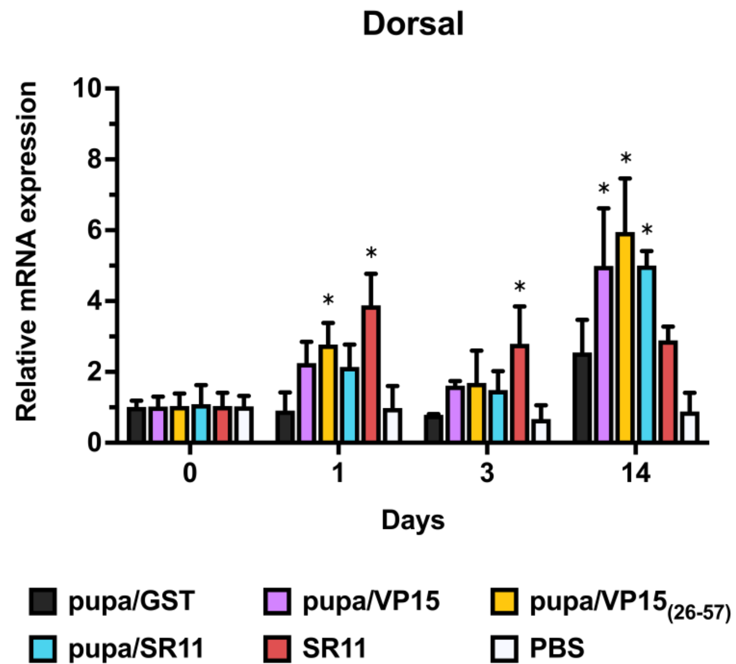

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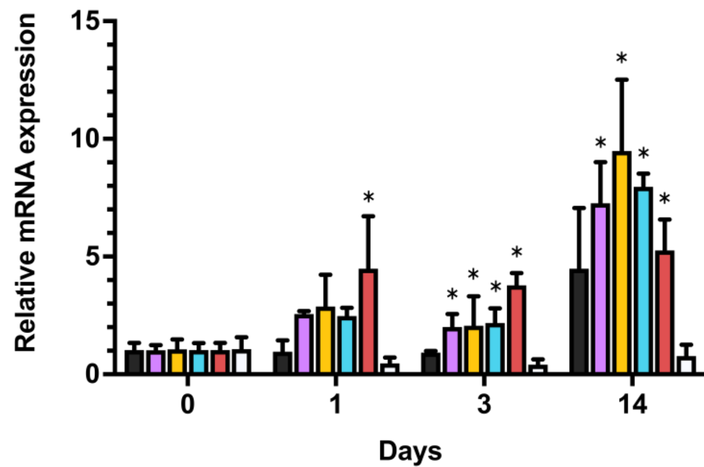

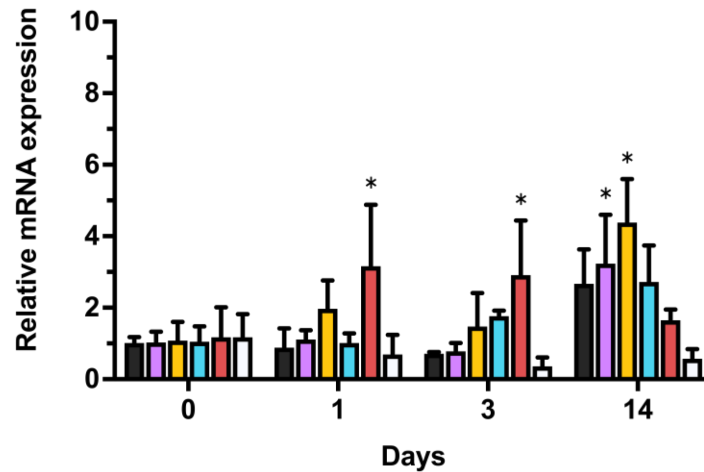

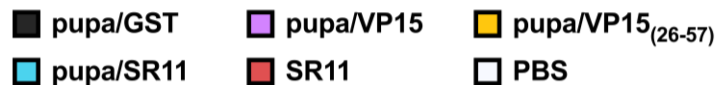

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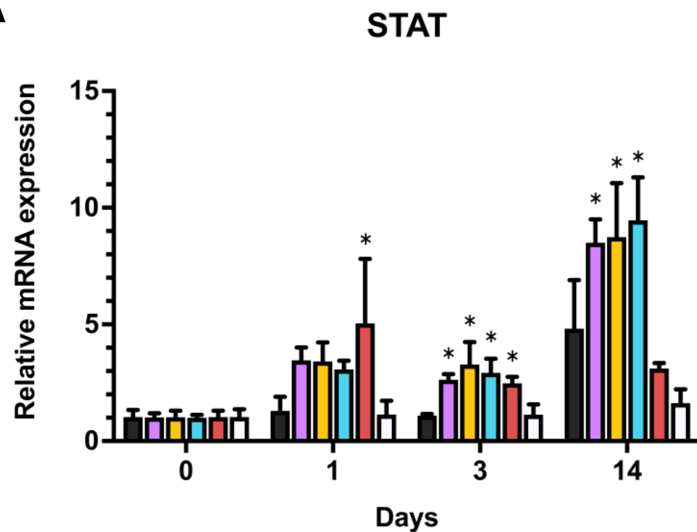
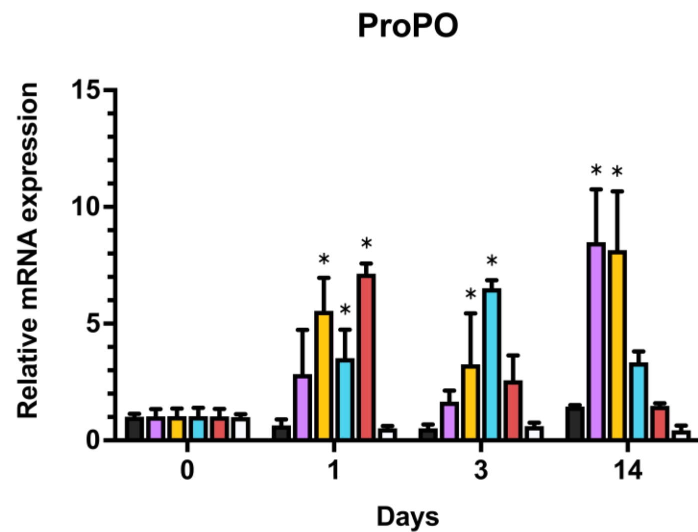




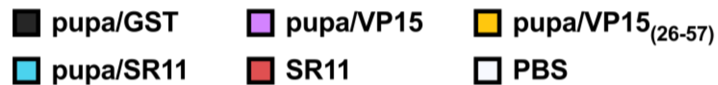
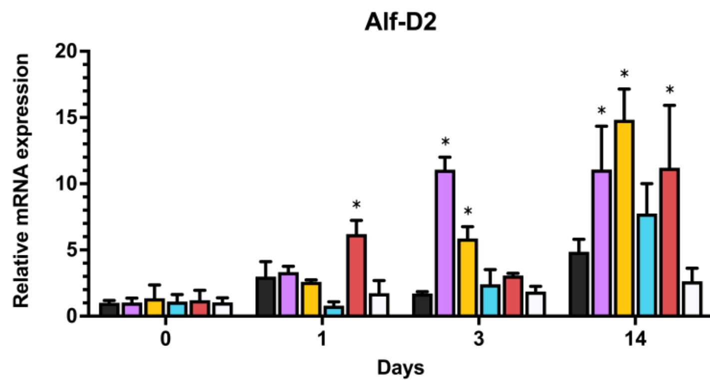


Fig. 6: *Boonyakida* et al.

A



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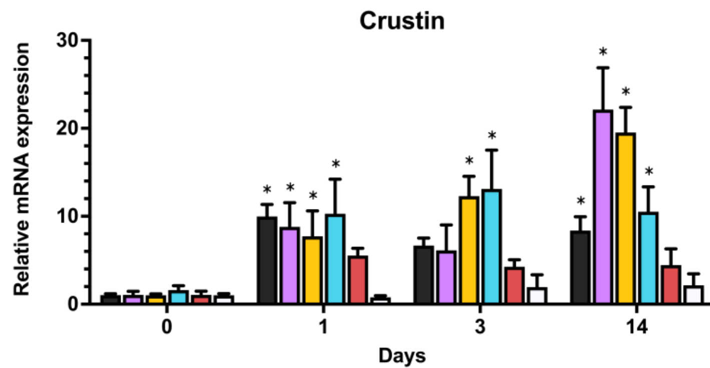

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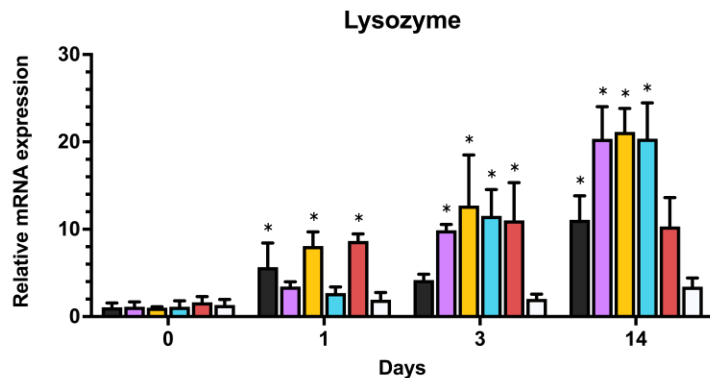

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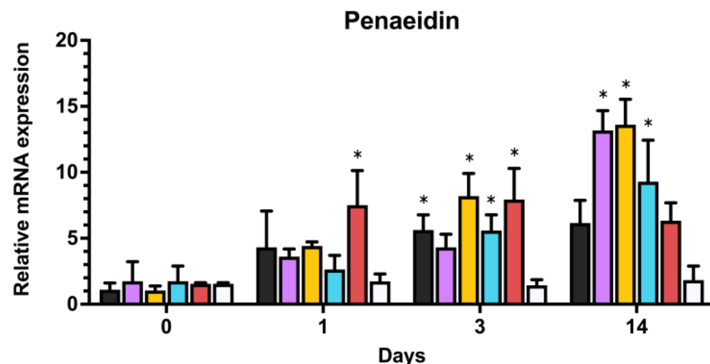

pupa/GST pupa/VP15 pupa/VP15$_{(26-57)}$
pupa/SR11 SR11 PBS

Fig. 7: ***Boonyakida*** **et al.**

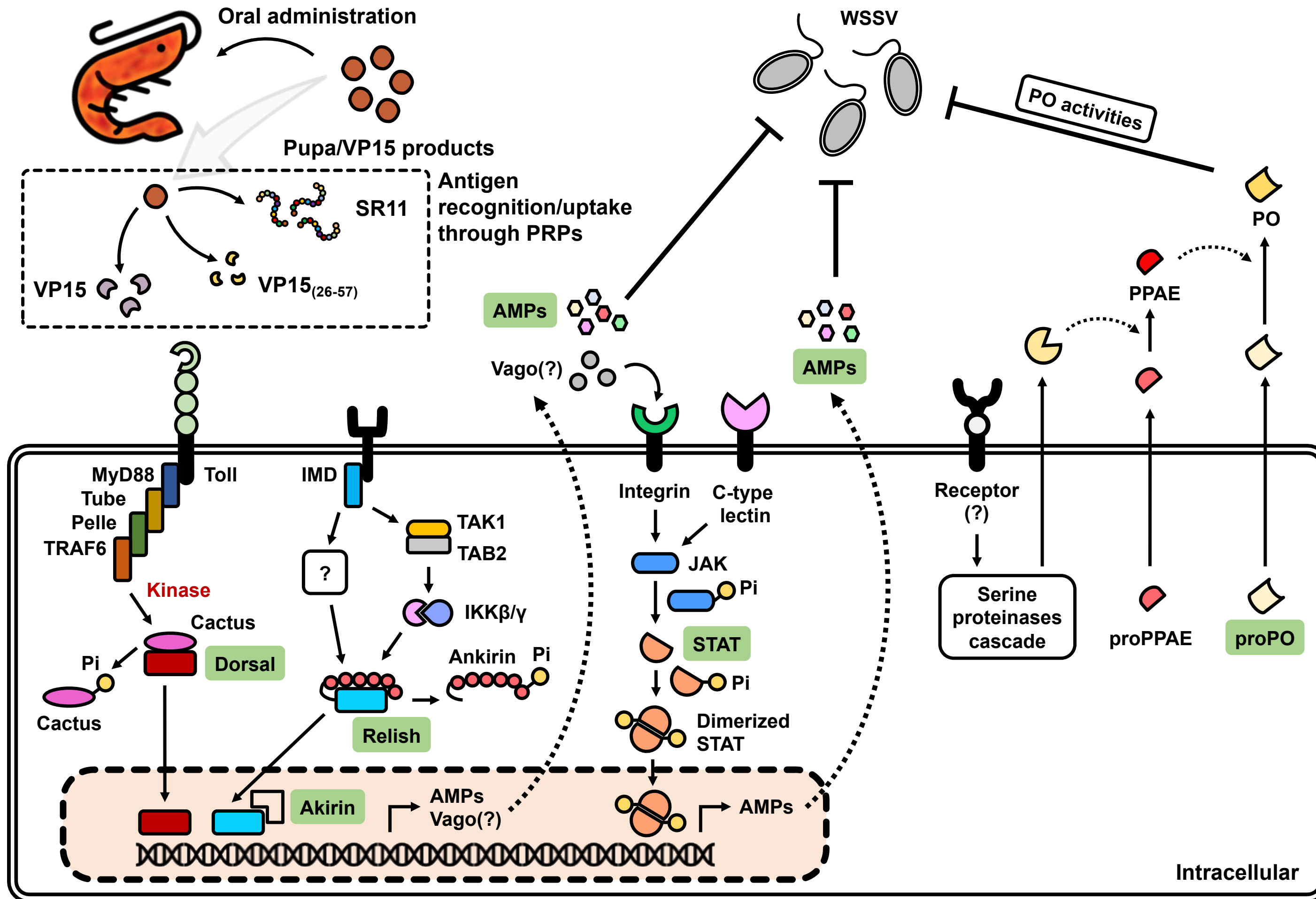

Fig. 8: *Boonyakida* et al.

Supplementary information

Immunostimulation of shrimp through oral administration of silkworm pupae expressing VP15 against WSSV

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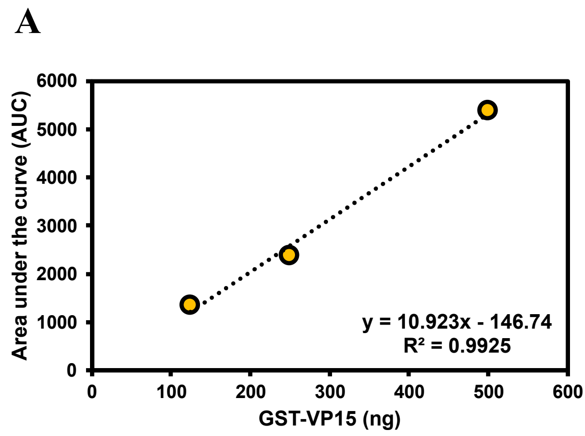

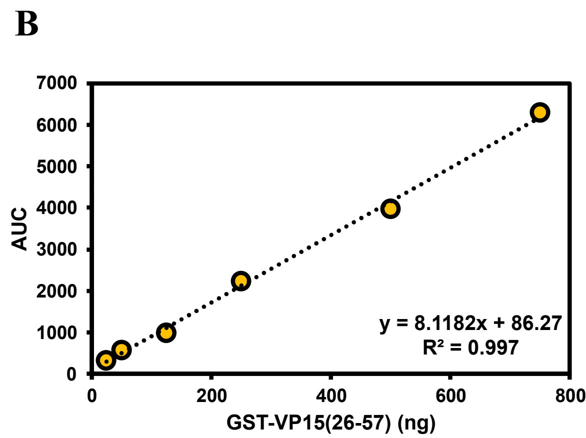

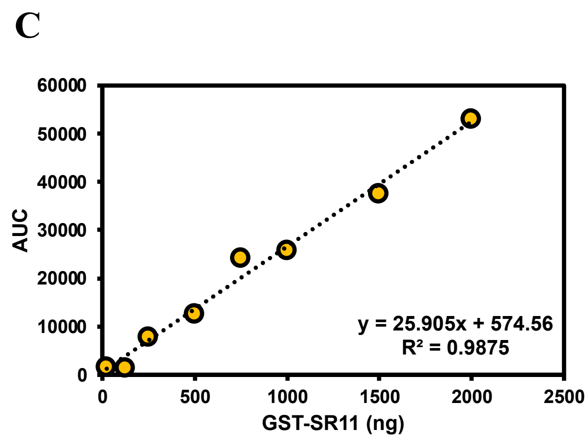

Figure S1. Standard calibration of **(A)** GST-VP15, **(B)** GST-VP15(26-57), and **(C)** GST-SR11 for quantitative western blot analysis of the recombinant protein expressions using silkworm pupae.