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Probiotics expressing double-stranded RNA targeting VP28 efficiently protect shrimps from WSSV infection

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ABSTRACT

Envelope protein VP28 of white spot syndrome virus (WSSV) serves as an excellent target of viral control via RNA interference (RNAi) in shrimps. In this study, double-stranded RNA targeting VP28 (dsVP28) was successfully produced by a hairpin expression vector (pWH1520-VP28) in *Lactobacillus plantarum* and *Lactococcus lactis*. Although leaky expression of xylose-inducible promoter in pWH1520 was detected, it was an application advantage as addition of xylose is not necessary. The probiotics produced dsVP28 at ranges of 0.78–1.82 ng per 10^{12} CFU. Shrimps were submerged in two doses of probiotics (approximately 4.5×10^{8} [1X] CFU/ 2 L seawater or 4.5×10^{10} [100X] CFU/ 2 L seawater) for 5 days prior to oral challenge with WSSV-infected tissues (approximately log copies of 10.2–10.7/tank). Shrimps receiving ds-*L. lactis* showed dose-dependent survival with the lowest mortality rate (33%) and the lowest WSSV copies (2.88 ± 0.18 logs per 100 ng total DNA from shrimp tissue) after receiving 100X ds-*L. lactis*. Both strains of *L. plantarum* could decrease shrimp mortalities and ds-*L. plantarum* could reduce viral loads significantly. To better understand the role of dsVP28 expressing *L. lactis* against WSSV, qRT-PCR assays were performed to assess expression levels of shrimp immune genes. Shrimps receiving 100X of ds-*L. lactis* significantly increased expression of (i) systemic RNA interference defective protein-1 (SID-1) and Dicer-2 (Dcr-2) genes in RNA interference and (ii) peroxinectin (PX) activation, prophenoloxidase-1 (proPO I), and anti-lipopolysaccharide factor-1 (ALF1) innate immune genes. Our overall findings suggest that not only ds-*L. lactis* probiotics can reduce shrimp mortality due to WSSV, the strain can also instigate RNAi and activate shrimp innate immune systems.

1. Introduction

White spot syndrome virus (WSSV) is a causative agent for white spot disease (WSD) that is prevalent in crustaceans, especially shrimps such as *Penaeus monodon*, *P. vannamei*, *Marsupenaeus japonicus*, and *Fenneropenaeus indicus* (Verma et al., 2017). Shrimps infected with WSSV present loss of carapace, dark reddish or pink coloration on a body surface, white spots on the exoskeleton, and eosinophilic inclusion bodies in infected tissues leading to death within 3–10 days (Bir et al., 2017; Verma et al., 2017). Consequently, the annual economic loss due to WSSV infection in global shrimp aquaculture is approximately US$ 1 billion (Stentiford et al., 2012).

To control shrimp viral diseases, RNA interference (RNAi) could be used as a therapeutic tool. Previous studies have demonstrated the use of RNAi approach against yellow head virus (YHV), Laem-Singh virus (LSNV), and WSSV (Attasart et al., 2009; Saksmerprome et al., 2009, 2013; Mejia-Ruiz et al., 2011; Thammasorn et al., 2013, 2017; Escobedo-Bonilla et al., 2015; Sanitt et al., 2016) targeting structural

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and non-structural proteins of shrimp viruses. Typically, the long double-stranded RNAs (dsRNAs) with sequences complement to viral target are introduced and processed through RNAi mechanism in the cell cytoplasm (Krishnan et al., 2009). For WSSV, double-stranded RNA targeting expression of an envelope protein VP28 (dsVP28) is reported as having a potential prophylactic potency in shrimps against WSSV infection (Sarathi et al., 2008; Nilsen et al., 2017). The previous study has demonstrated that introduction of dsVP28 could significantly upregulate systemic RNA interference defective protein-1 (SID-1) which is responsible for systematic uptake of dsRNA into shrimp cells, and Dicer-2 (Dcr-2) and Argonaute-2 (Ago-2) which are involved in RNAi mechanism (Nilsen et al., 2017). Several studies presented protection of shrimps from viral diseases using bacteria as host cells and delivery tools through oral administration. In the 2013, Attasart et al. confirmed that RNAi was induced in shrimps fed with *Escherichia coli* expressing dsRNA targeting shrimp Rab7 and STAT genes. Significant reduction of those target gene transcripts was observed when compared to the group not receiving the recombinant feed. The suppression was found in more than one organ suggesting systemic induction of RNAi via oral delivery of dsRNA (Attasart et al., 2013).

In this study, lactic acid bacteria (LAB) were selected as dsVP28 production and delivery systems in addition to known benefit of LAB stimulation of host immune systems (Chomwong et al., 2018; Li et al., 2018a). LAB are Gram-positive, non-spore forming bacteria that produce lactic acid during fermentation. They have been shown to exhibit probiotic properties including ability to colonize host gut, produce antimicrobial peptide to inhibit pathogenic colonization, enhance digestive enzyme production for feed utilization and promoting growth factors in host, and activate of host immune systems (Zorriehzahra et al., 2016; Hoseinifar et al., 2018). Previous studies reported LAB colonization as part of gut microbiota in *P. vannamei* (Li et al., 2018a; Holt et al., 2020). Common LAB used in aquaculture studies include *Lactobacillus plantarum* (Chiu et al., 2007; Kongnum and Hongpattarakere, 2012; Zheng et al., 2017) and *Lactococcus lactis* (Adel et al., 2017; Won et al., 2020). *Lactobacillus* sp. was reported to protect shrimps against WSSV (Zuo et al., 2019) and *L. plantarum* in YHV (Thammasorn et al., 2017). Therefore, candidate LAB hosts for dsRNA production in this study were *L. plantarum* and *L. lactis*. Our objectives were to develop these probiotics expressing dsVP28, to test their protection efficacy against WSSV infection, and to evaluate shrimp immune gene induction by these probiotics.

2. Materials and methods

2.1. Ethics statement

All applicable institution guideline MUSC-IACUC, protocol no. MUSC63-003-511 for the care and use of animals were followed.

2.2. Bacterial strains and growth condition

Probiotic bacterial strains used in this study included *Lactobacillus plantarum* ATCC 14917 (NSTDA) and lab collection *Lactococcus lactis*. Probiotic bacteria were cultured in MRS medium (Himedia) without shaking at 30 °C for *L. lactis* and at 37 °C for *L. plantarum*. Shuttle vector pWH1520 expressing hpVP28 was constructed in *Escherichia coli* DH5α and the recombinant *E. coli* DH5α was propagated in LB [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] medium at 37 °C under constant shaking at 250 rpm. For selection of pWH1520-hpVP28 plasmid, 100 μg/ml ampicillin (Merck) was used in *E. coli* DH5α, and 30 μg/ml of tetracycline in probiotic bacteria.

2.3. Construction of dsRNA-VP28 hairpin (hpVP28) expression cassette in pWH1520 plasmid (pWH1520-hpVP28)

pWH1520-hpVP28 expression cassette was constructed following the schematic diagram (Fig. S1A) consisting of 433 bp of customized hpVP28 expression cassette contained 182 bp of a forward fragment and inverted fragments from nucleotides 8–189 of *vp28* gene (GenBank no. AY422228.1) according to Thammasorn et al. (2015) linked by a 55 bp loop with overhangs of two restriction sites for *Bam*HI and *Nru*I enzymes. Customized hpVP28 expression cassette located in pUC57 plasmid was digested and cloned into pWH1520 plasmid (MoBiTec GmbH). Plasmids from transformants on LB containing 100 μg/ml ampicillin were extracted with Presto™ mini plasmid kit (Geneaid) and checked by PCR with pWH1520_380 and VP28R1 primers (Table S1). Additional pWH1520-hpVP28 plasmid confirmation by *Pst*I (NEB) digestion was performed.

2.4. Transformation pWH1520-hpVP28 plasmid into probiotic bacteria by electroporation and transformant selection

pWH1520-hpVP28 plasmid was transformed to probiotics *L. plantarum* and *L. lactis*. Competent cell preparation and electroporation of these probiotics were performed according to the method described by the previous study (Thammasorn et al., 2017). Briefly, competent cells were mixed with plasmid in a 0.2 cm Gene Pulser® cuvette (Bio-rad) and electroporated with time constant at 5.0 ms, 10 kV/cm of Gene Pulser XcellTM (Bio-rad). Transformants were selected on MRS containing 30 μg/ml tetracycline. Plasmid was extracted from the transformants using Presto™ mini plasmid kit (Geneaid). Extracted plasmid was detected with PCR using pWH1520_477R and VP28_97R primers (Table S1).

2.5. dsVP28 expression in probiotic bacteria

dsVP28 expression was induced following procedure in the previous study (Thammasorn et al., 2017). Briefly, a single colony of probiotic bacteria containing pWH1520-hpVP28 plasmid was inoculated into 15 ml MRS containing 30 μg/ml tetracycline at 30 °C for *L. lactis* and 37 °C for *L. plantarum* for 48 h and a 1:100 re-inoculation in 15 ml MRS containing antibiotic was incubated overnight. Cells were washed with 15 ml of M-media twice and were diluted (1:3) in M media containing 0.25% xylose for 2.5 h. Probiotics without pWH1520-hpVP28 plasmid were used as negative controls. Additionally, probiotics containing pWH1520-hpVP28 plasmid were investigated for leaky expression in absence of xylose. RNA was extracted with 1 ml TRIzol™ reagent (Invitrogen) followed by cell breaking with 100 μl of 0.1 mm Zirconia glass beads (BioSpec Products, Inc). Supernatant was collected and 0.2X volume of chloroform was added. RNA was precipitated in 1X volume of absolute ethanol and 0.2X volume of NaOAC (pH 5.2). To remove ssRNA and DNA, RNase A and DNase were used. The treated condition consisted of 300 mM NaCl, 1X DNase buffer, 3 units of DNase, 0.07 mg/ml of RNase A, 200 μl of RNA template, and RNase-free water with incubation at 37 °C for 6 h. The amount of dsRNA was measured using NanoDrop™ spectrophotometer (Thermo Scientific) at 260 nm. dsVP28 was initially detected with RT-PCR using VP28F and VP28R1 primers (Table S1). To quantitate dsVP28, 100 ng of extracted dsRNA was used to perform qRT-PCR using VP28F and VP28R1 primers with KAPA SYBR FAST One-Step qRT-PCR Kits (KAPA Biosystems). The data were analyzed with ABI PRISM 7900 software. For generation of standard curve, purified dsVP28 amplicons were used at the range of 10^9–10 copies in parallel qRT-PCR. Quantity of dsVP28 from each probiotic strain was calculated based on the standard curve.

2.6. Efficacy of dsVP28 from probiotic bacteria against WSSV infection in shrimps

Experiments related to shrimps used in this study were approved by MUSC-IACUC, protocol no. MUSC63-003-511. Specific pathogen free (SPF) of white shrimp *P. vannamei* post larvae (0.03 g B.W.) and juvenile (20 g B.W.) were provided by CPF (Thailand) for protection efficiency

assay and for preparation of WSSV inoculum, respectively. The shrimps were cultured in artificial seawater with salinity of 10 parts per thousand (ppt) with aeration and acclimatized for three days before performing experiment. The average water temperature throughout the experiment was 28 ± 0.5 °C. For WSSV inoculum preparation, juvenile shrimps were fed with WSSV-infected tissues at 10% of shrimp body weight. Moribund shrimps were collected and sacrificed for muscle collection. Detection of WSSV in tissues was performed by DNA extraction followed by IQ2000TM kit (Farming IntelliGene Tech. Corp). Infected shrimp muscles were homogenized and quantitated by qPCR for WSSV copies prior kept at −80°C until use. For protection efficiency assay of dsVP28, four strains of probiotics were tested; *L plantarum* without dsVP28 (*L. plantarum*), dsVP28-expressing *L. plantarum* (ds-*L. plantarum*), *L lactis* without dsVP28 (*L. lactis*), and dsVP28-expressing *L. lactis* (ds-*L. lactis*). Two doses of each strain i.e., 1X (4.5×10^8 CFU/ 2 L seawater) and 100X (4.5×10^{10} CFU/ 2 L seawater) based on the previous studies (Thammasorn et al., 2017; Guzmán-Villanueva et al., 2020) were added directly to the seawater. Preparation of dsVP28-expressing probiotics was performed as previously described without xylose.

Post-larvae shrimps were divided into six groups. Each of the four shrimp groups was treated with each of the four probiotic strains with each group containing two doses of probiotics and each dose of probiotics consisting of three replicates (n = 30 shrimps/replicate/2 L seawater). The other two shrimp groups were positive and negative groups, respectively, with three replicates per group (n = 30 shrimps/replicate/2 L seawater). Daily fresh probiotics were provided to shrimps for five days before WSSV infection (Experimental scheme shown in Fig. S2A). Shrimps were fed with shrimp feed two times per day. Water was changed in all groups after five days of probiotic treatments i.e., before WSSV infection. All groups except the negative group were challenged with WSSV by oral feeding with the prepared WSSV-infected tissues at 1 g per each tank (approximately log copies of 10.2–10.7/tank based on qPCR). Water was changed at 100% every two days. Number of survived shrimps were recorded daily until 100% of mortality in the positive group. Results are reported in percentage shrimp cumulative mortality calculated from remaining percentage of cumulative shrimp survival after oral challenge with WSSV-infected tissue. One survived shrimp per replicate was collected at 3-day post infection (3 dpi.) for WSSV detection by qPCR.

2.7. Detection and quantitation of WSSV in shrimps by qPCR

Shrimp DNA was collected and extracted by TF lysis buffer (50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 100 mM EDTA, 2% SDS, 1 μg/ml Proteinase K) followed by phenol-chloroform treatment. DNA was precipitated with cold absolute ethanol at −20 °C overnight until use. For initial detection of WSSV, 100 ng of shrimp DNA was PCR with WSSV447_F and WSSV447_R primers (Table S1). For WSSV quantitation by qPCR, 1X SYBR Green PCR Master Mix (Qiagen), WSSV229_F and WSSV447_R primers, and 100 ng of DNA were prepared and performed on Rotor-Gene® Q PCR machine (Qiagen). The melting curve analysis was performed with Rotor-Gene Q 5plex HRM Platform software. WSSV-specific 448 amplicon at 10^9 to 10 copies was used to perform standard curve.

2.8. Expression analysis of shrimp immune genes for probiotic treatments

White shrimp *P. vannamei* post larvae (average size of 0.03 g) were provided by CPF (Thailand). The shrimps were cultured and acclimated as mentioned earlier. Two probiotic strains consisting of the wild type *L. lactis* and the recombinant ds-*L. lactis* at 100X dose (4.5×10^{10} CFU/ 2 L seawater) were used to investigate expression of selected shrimp RNA interference consisting of genes encoded for systemic RNA interference defective protein-1 (SID-1) and Dicer-2 (Dcr-2) were determined and shrimp innate immune genes, namely genes encoded for peroxinectin (PX), prophenoloxidase-1 (proPO I), and anti-lipopolysaccharide factor-1 (ALF1). A list of qPCR primers is shown in Table S1. Post-larvae shrimps were divided into three groups receiving the wild type 100X *L. lactis*, the recombinant 100X ds-*L. lactis*, or no bacteria (negative control). Each group contained two replicates, n = 30 shrimps/replicate/ 2 L seawater. Shrimps were treated with each probiotic for five days except the negative group and water was not changed during probiotic treatments. After probiotic treatments, water was changed at 100%. Subsequent water changes (100%) were done every two days. At day 6 (or day 1 of post probiotics treatment) and day 8 (or day 3 post probiotics treatment), four shrimps per group were collected (Fig. S2B). Shrimp RNA was extracted from whole body of post-larvae shrimps and 500 ng of total RNA was used to synthesize cDNA by using oligo dT20 primers following SuperScript™ III (Invitrogen) instructions. qPCR was performed using 1X SYBR Green PCR Master Mix (Qiagen), specific shrimp immune gene forward and reverse primers (Table S1), and 2 μl of 1:10 diluted cDNA. qPCR was performed on Rotor-Gene® Q PCR machine (Qiagen) and the melting curve analysis was performed with Rotor-Gene Q 5plex HRM Platform software. For standard curves, qPCR was performed using a range of 10^9 to 10 copies of pGEM-T plasmids containing shrimp immune genes. β-actin RNA was used as a reference gene for normalization. Normalized log copies of each treatment were calculated. Fold changes of shrimp gene expression were quantitated from expression ratios of shrimps treated with probiotic treatments against shrimps without probiotic treatments (negative control).

2.9. Statistical analysis

Student's T-test, Mann-Whitney Test, and one-way ANOVA on SPSS statistics with Duncan post-hoc test were used in data analyses. Statistically significance is reported when p-value ≤ 0.05.

3. Results

3.1. Construction of hpVP28 expression cassette in pWH1520 plasmid (pWH1520-hpVP28) and dsVP28 expression in probiotic bacteria

To construct an hpVP28 expression vector, a previously customized hpVP28 expression cassette on pUC57 plasmid (pUC57-hpVP28) was digested with *Bam*HI and *Nru*I and cloned into pWH1520 vector digested with same enzymes (Fig. S1). pWH1520-hpVP28 plasmid was confirmed by *Pst*I digestion, PCR with chimeric pWH1520_380 and VP28R1 primers, and sequencing. Subsequently, pWH1520-hpVP28 plasmid was transformed into the two probiotics (*L. plantarum* and *L. lactis*).

To measure the level of dsVP28 expression from pWH1520-hpVP28 plasmid in the two probiotics, confirmed recombinants were grown in the presence and the absence of xylose, an inducer for the expression promoter. Total RNA was purified and RT-PCR using VP28F and VP28R1 primers was performed. The results showed that pWH1520-hpVP28 plasmid was able to express dsVP28 in probiotics and leaky expression of a xylose-induced promoter of pWH1520 plasmid was observed (Fig. S3A). Subsequently, amounts of dsVP28 were determined by qRT-PCR. The melting curve analysis revealed peaks at 81.2 °C referring to dsVP28 (Fig. S3B). At 10^{12} CFU (approximately 1 L culture) of each probiotic, expressed dsVP28 amounts were 1.82 ± 0.08 ng from ds-*L. plantarum* and 0.78 ± 0.01 ng from ds-*L. lactis*.

3.2. Evaluation of dsVP28 from probiotic bacteria against WSSV

We evaluated the efficacies of dsRNA produced from the four probiotic strains at 1X and 100X, which were 4.5×10^8 CFU/ 2 L seawater and 4.5×10^{10} CFU/ 2 L seawater, respectively. The test groups received probiotics once daily for five days prior to oral challenge with WSSV-infected shrimp tissues. The positive and negative control groups did not receive probiotics, and only the positive group was fed with WSSV-infected shrimp tissues. At the applied WSSV dose (approximately log copies of 10.2–10.7 per replicate), half of the shrimps in the positive

group died in 2.5 days. Therefore, numbers of survived shrimps were recorded daily until 100% of mortality was observed in the positive group i.e., 5 dpi. The results demonstrated that shrimps treated with *L. lactis* (Fig. 1A) showed dose-dependent manner in protection against WSSV infection. Starting at 3 dpi., significant decrease in mortality was observed with shrimps treated with 100X ds-*L. lactis*. Corresponding to qPCR result, shrimps submerged in 100X ds-*L. lactis* prior to WSSV challenge showed significant decrease in log WSSV copies to 2.88 ± 0.18 (per 100 ng total DNA from shrimp tissue) in comparison to the log WSSV copies of 6.28 ± 0.61 in the positive group and 4.47 ± 0.61 in 100X wild type *L. lactis* group on 3 dpi. corresponding to 1X *L. lactis* treatments at log WSSV copies of 5.44 ± 0.33 (1X *L. lactis*) and 4.95 ± 0.59 (1X ds-*L. lactis*) in Fig. 2A. At the end of the experiment, on 5 dpi., the lowest shrimp cumulative mortality (33%) was observed when treated with 100X ds-*L. lactis*. Submersion with 100X wild type *L. lactis* presented cumulative mortality at 69%. For the lower dose, though not statistically significant from the positive group, 1X ds-*L. lactis* was able to delay the shrimp mortality to the final cumulative mortality of 70%. Submersion with 1X wild type *L. lactis*, however, could not protect the shrimps from WSSV infection showing final cumulative mortality at 93%. In addition, mortality increase rates were also compared over the period of linear increase in death (between 2 dpi. and 4 dpi.). Treatment

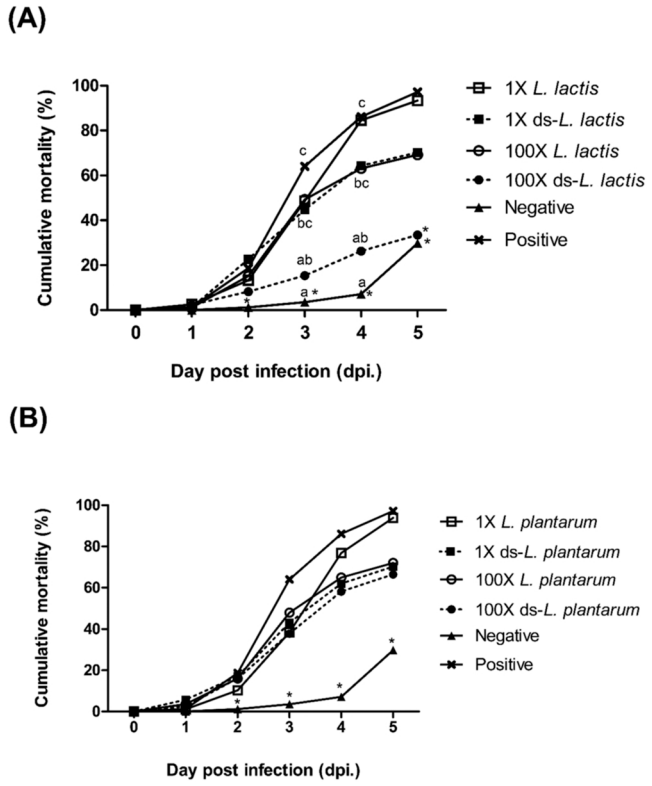


Fig. 1. Efficacy of dsVP28 produced in the recombinant probiotics decreased the shrimp cumulative mortality depending on dose in *L. lactis* treatments and independent on dose in *L. plantarum* treatments after WSSV infection. Average percentages of cumulative mortality in shrimps submerged with 4.5×10^8 [1X] CFU/ 2 L seawater and 4.5×10^{10} [100X] CFU/ 2 L seawater of (A) *L. lactis* treatments: wild type *L. lactis* (*L. lactis*) and dsVP28 expressing *L. lactis* (ds-*L. lactis*), (B) *L. plantarum* treatments: wild type *L. plantarum* (*L. plantarum*) and dsVP28 expressing *L. plantarum* (ds-*L. plantarum*) with n = 30 shrimps per replicate (three replicates/dose/group) while the negative and positive groups without probiotic treatments with n = 30 shrimps per replicate (three replicates/group). Small alphabets (a, b, and c) indicate statistical groups based on ANOVA and Duncan's test at $p < 0.05$ on corresponding day post infection. Asterisks (*) indicate significant differences between the treatment and the positive control on corresponding dpi. at $p < 0.05$ based on independent sample T-test and Mann-Whitney Test.

with 100X ds-*L. lactis* could significantly reduce the mortality increase rate by approximately four folds over the 2-day period in comparison to the positive control group. Our findings suggest that *L. lactis* expressing dsVP28, especially at high dose, could better prolong the shrimp survival.

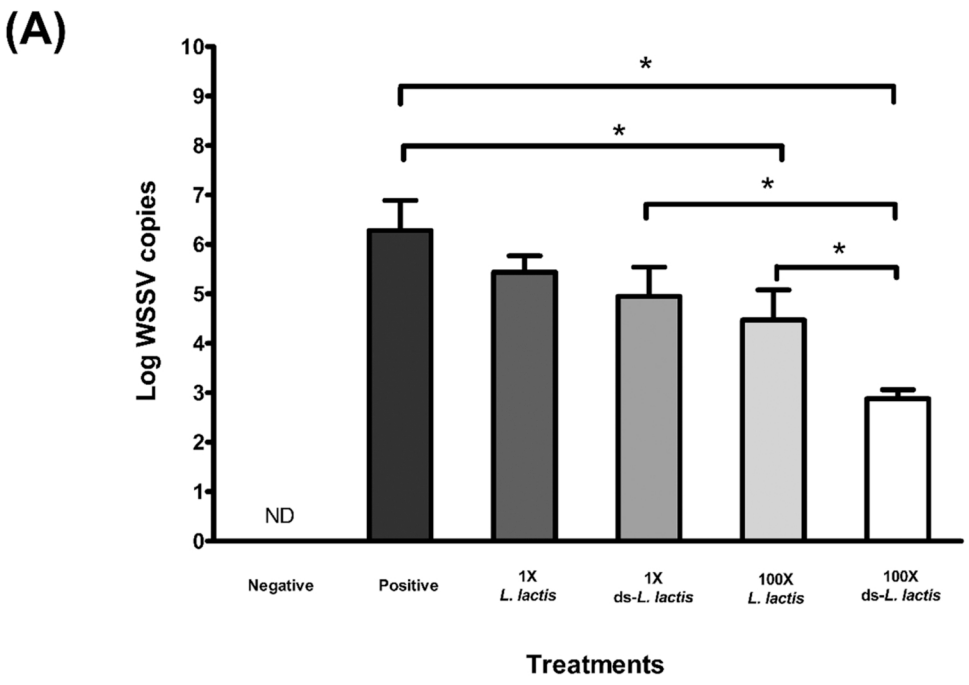
For *L. plantarum* treatments (Fig. 1B), although significant differences were not detected throughout the experiment time course, at 5 dpi., shrimps treated with 100X ds-*L. plantarum* showed 66% of shrimp mortality similar to that of 1X ds-*L. plantarum* (70%) and of 100X wild type *L. plantarum* (72%) when compared to 1X wild type *L. plantarum* (94%). This indicated that, although the effects are not additive, dsVP28 expression in *L. plantarum* or high-dose wild type *L. plantarum* could decrease the shrimp cumulative mortality. Consistently, with assessment of WSSV copies by qPCR from survived shrimps at 3 dpi., the viral copies of shrimps submerged in 1X and 100X ds-*L. plantarum* showed significantly lower log WSSV copies of 3.79 ± 0.09 and 3.33 ± 0.12, respectively, than those submerged with wild type *L. plantarum* (1X and 100X wild type *L. plantarum* at log WSSV copies of 4.13 ± 0.54 and 5.08 ± 0.57, respectively [Fig. 2B]). This finding illustrated that presence of dsVP28 in *L. plantarum*, especially with the high dose, could decrease the viral load in survived shrimps.

3.3. Shrimp immunity activation by probiotics

As 100X ds-*L. lactis* reduced the shrimp cumulative mortality and viral load after viral infection, we investigated the role of dsVP28 produced by probiotic *L. lactis* on shrimp RNA interference and innate immune activation. In this study, we performed qRT-PCR on selected shrimp RNA interference genes i.e., genes encoding in systemic RNA interference defective protein-1 (SID-1) and Dicer-2 (Dcr-2) from shrimps treated with two strains of *L. lactis* at 100X. Log copies of shrimp immune genes normalized to β-actin gene were compared at day 6 and day 8, corresponding to day 1 and day 3 post treatment with probiotics. In other words, day 6 and day 8 represent the immunological states of shrimps during early and late WSSV challenge. Fold changes comparing conditions with probiotics, either the wild 100X *L. lactis* or the recombinant 100X ds-*L. lactis*, over without probiotics (the negative group) were calculated. For both SID-1 and Dcr-2 expression in the wild type 100X *L. lactis*, the levels were the same as in the negative control on both day 6 and day 8. When compared between the recombinant and the wild type, both SID-1 and Dcr-2, on day 6, were expressed at 1.9 folds higher in the recombinant 100X ds-*L. lactis* (Fig. 3). Upregulated Dcr-2 expression on day 6 in shrimps treated with the recombinant 100X ds-*L. lactis* was significantly different from the wild type 100X *L. lactis*. However, both SID-1 and Dcr-2 expression levels were not upregulated in 100X ds-*L. lactis* treatment on day 8. This suggested dsVP28 produced by *L. lactis* could activate shrimp RNAi genes, specifically SID-1 and Dcr-2 during early response. To assess efficacy of dsVP28 on shrimp innate immune gene activation, we also evaluated the levels of selected innate immune genes namely peroxinectin (PX), prophenoloxidase-1 (proPO I), and anti-lipopolysaccharide factor-1 (ALF1) antimicrobial peptide. On day 6, shrimps receiving the recombinant 100X ds-*L. lactis* showed significant upregulations of PX (4.3 folds), proPO I (1.8 folds), and ALF1 (2.6 folds) expression in comparison to the wild type 100X *L. lactis* (Fig. 3). However, on day 8, significant difference in gene expression between wild type 100X *L. lactis* and ds-*L. lactis* was observed only for proPO I at 2.3 folds. Our finding suggested dsVP28 produced by *L. lactis* could activate shrimp innate immune gene during early and later phases of infection.

4. Discussion

Recent studies focused on construction and production of dsVP28 in RNaseIII-deficient *Escherichia coli* and probiotics (Thammasorn et al., 2017; Saelim et al., 2020). In place of coliform host, in the present study, three probiotics namely *Lactococcus lactis* and *Lactobacillus plantarum*



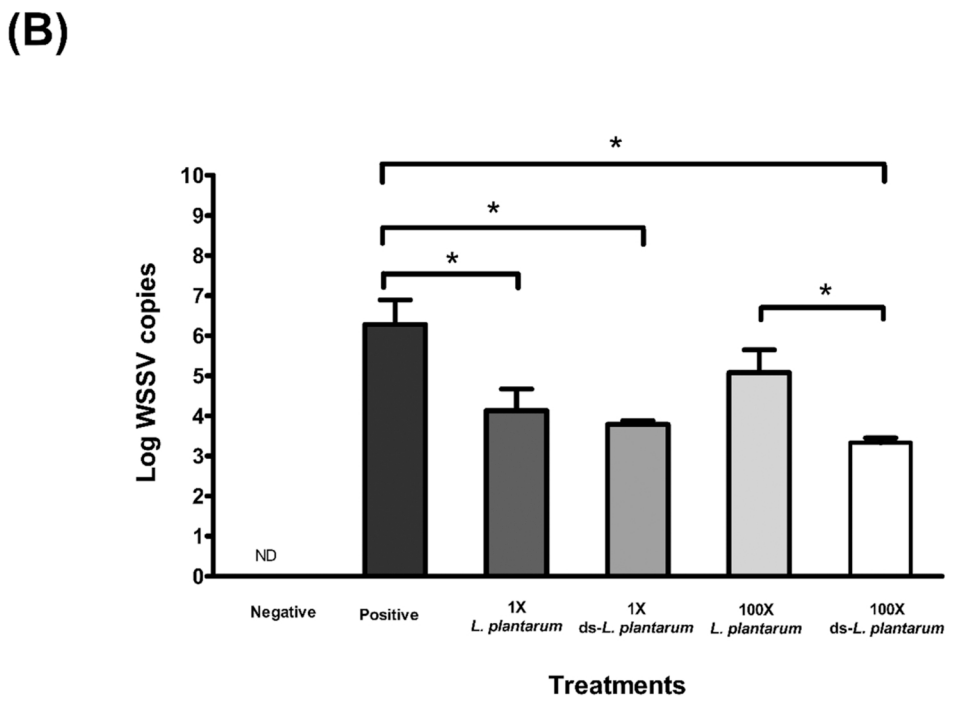


Fig. 2. Log WSSV copies from 100 ng DNA of survived shrimps sampled at 3 dpi. submerged with probiotics at 4.5×10^8 [1X] CFU/ 2 L seawater and 4.5×10^{10} [100X] CFU/ 2 L seawater of (A) *L. lactis* treatments: wild type *L. lactis* (*L. lactis*) and dsVP28 expressing *L. lactis* (ds-*L. lactis*) and (B) *L. plantarum* treatments: wild type *L. plantarum* (*L. plantarum*) and dsVP28 expressing *L. plantarum* (ds-*L. plantarum*). Data are presented as mean ± standard error with n = 3 shrimps per group. Asterisks (*) indicate significant differences ($p < 0.05$) based on ANOVA and Duncan's test. Non-detectable (ND).

were selected and constructed to harbor pWH1520-hpVP28 vector expressing dsVP28 at the range of 0.78–1.82 ng from 10^{12} CFU probiotics without xylose inducers, a comparable yield to the anti-YHV dsRNA produced in *L. plantarum* through pWH1520 expression vector with 0.25% xylose at 2.37 ng per 10^{12} CFU (Thammasorn et al., 2017). Additionally, the current study revealed a leaky expression of xylose-inducible promotor in pWH1520 in probiotics backgrounds. This finding supports the previous report in *L. plantarum* (Heiss et al., 2016) where the basal expression levels of genes under the xylose-inducible promoter derived from *B. megaterium* DSMZ 319 similar to the system of pWH1520 plasmid in absence of xylose were observed. This leaky expression could due to inefficient repressor binding and absence of putative additional sequence encoding repressor proteins (Heiss et al., 2016). Since leaky expression of dsVP28 under xylose-inducible promoter of pWH1520 allowed comparable yield of dsRNA and omitting xylose in probiotics culture preparation could reduce production cost in the long run, the remaining experiments were performed using probiotics grown in the absence of xylose.

To assess the efficacies of probiotics and dsVP28 against WSSV infection, immersion and challenge experiments were performed. Shrimps received daily fresh probiotics at 100X or 1X dose for five days prior to WSSV challenge. On 3 dpi., shrimps receiving 100X ds-*L. lactis* showed a lower shrimp cumulative mortality (15%) compared to 100X-*L. lactis* (49%) and positive control (64%). This finding corresponded to the viral loads in survived shrimps receiving *L. lactis*. Prior exposure to 100X ds-*L. lactis* showed lower WSSV copy numbers than the positive control and the 100X wild type *L. lactis* groups by 3.4 and 1.6 logs, respectively. Congruently, Saelim et al. (2020) also showed that oral

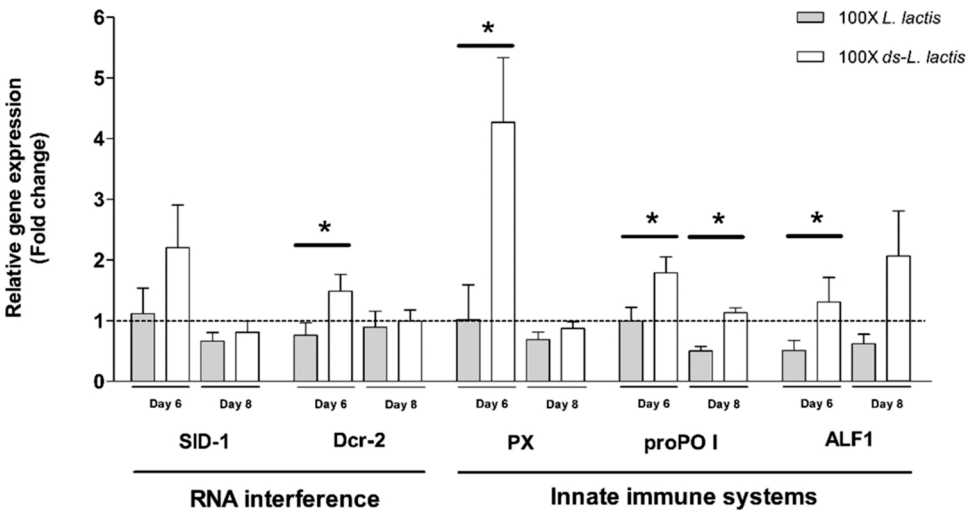


Fig. 3. Probiotic *L. lactis* expressing dsVP28 (ds-probiotic) promotes dual function of shrimp RNA interference and shrimp innate immune activation. Shrimps were treated with each probiotic strain at 4.5×10^{10} [100X] CFU/ 2 L seawater for five days. Mean ± standard error with n = 4 shrimps per group of relative gene expression at day 6 (or one day post-probiotics regimen in treated groups) and day 8 (or three days post-probiotics regimen). Fold difference in expression of shrimp RNAi genes including systemic RNA interference defective protein-1 (SID-1), Dicer-2 (Dcr-2), and Fold difference in expression of shrimp innate immune gene expression consisting of peroxinectin (PX), prophenoloxidase-1 (proPO I), and anti-lipopolysaccharide factor-1 (ALF1). Abbreviations: 100X wild type *L. lactis* (100X *L. lactis*) in gray bars and 100X dsVP28 expressing *L. lactis* (100X ds-*L. lactis*) in white bars. Dotted line at 1 indicates fold change of 1 or no change in gene expression in each probiotic treatment in comparison to without probiotic treatment (negative). Asterisks (*) indicate significant differences ($p \leq 0.05$) based on independent sample T-test and Mann-Whitney Test between the probiotic expressing dsVP28 and the wild type probiotic without dsVP28.

feeding with probiotic *B. subtilis* expressing dsVP28 in *P. vannamei* could lower shrimp cumulative mortality due to WSSV infection i.e., from the positive control level (91%) to 71% on 14 dpi., and, in surviving shrimps, lower expression of VP28 mRNA was detected. As a result, we proposed that, in *L. lactis* background, dsVP28 could decrease the shrimp cumulative mortality through RNAi knock down of viral VP28 leading to incomplete viral assembly and decreased amount of WSSV progeny, respectively. On the other hand, the 100X wild type *L. plantarum* could reduce shrimp cumulative mortality at the same level as the 1X and the 100X recombinant *L. plantarum* i.e., 66–72%. However, 100X recombinant *L. plantarum* could significantly reduce more numbers of viral progenies than the 100X wild type *L. plantarum*, suggesting dsVP28 expressing *L. plantarum* could decrease shrimp mortality and viral load after WSSV infection. Based on our immersion and challenge experiments and quantifications of viral progenies, among the two species, *L. lactis* is a better host for expressing dsVP28 targeting WSSV. The recombinant *L. lactis* is better at reducing the mortality rate at a dose-dependent manner and at reducing the viral propagation.

To further highlight the potential mechanism of shrimp protection against WSSV by the recombinant *L. lactis* at 100X, expression levels of selected genes involving in shrimp RNA interference (RNAi) genes and innate immune system genes were assessed. Shrimps treated with 100X ds-*L. lactis* upregulated early response genes in SID-1 and Dcr-2 on day 6 at range 1.5–2.2 folds over those expressed in the negative group. This corresponds to the previous studies which showed shrimps injected with dsVP28 after 48 h upregulated SID-1, Dcr-2, and Argonaute-2 (Ago2) mRNA expression at approximately range of 3–6-fold changes over shrimps without dsRNA treatment or PBS (Nilsen et al., 2017). Additionally, shrimps fed with *B. subtilis* expressing dsVP28 for 15–30 days showed only a significant upregulation of SID-1 and Ago2 not Dcr-2 approximately range of 1.2–4-fold change over without *B. subtilis* (negative) and shrimps receiving wild type *B. subtilis* (Riet et al., 2021). In this study, we did not observe upregulation of Ago2 expression (data not shown), this might due to our shorter experimental duration (8 days). Nevertheless, our data supported that dsVP28 produced by *L. lactis* that could activate shrimp RNA interference pathway through upregulation of SID-1 and Dcr-2 in early response leading to activate downstream of RNAi pathway.

In addition to RNAi pathway, the effects of dsVP28 produced by *L. lactis* on shrimp innate immune gene expression were also assessed. Specifically, we evaluated prophenoloxidase (proPO)-related genes shown to be activated by probiotics. Generally, pattern recognition receptors of shrimps recognize pathogen-associated molecular patterns such as peptidoglycan of Gram-positive bacteria. This leads to activation of serine proteinase (SP) cascade followed by activation of phenoloxidase (PO) which oxidizes phenols to produce quinones followed by melanin synthesis and pathogen phagocytosis. In addition, expression of peroxinectin (PX), a cell-adhesion protein with a role in pathogen opsonization and phagocytosis (Tassanakajon et al., 2013; Cerenius and Soderhall, 2021) was also quantified. Both PX and proPO I showed significant upregulation in 100X ds-*L. lactis* at 4.3 and 1.8 folds, respectively, over those expressed in the negative group. Chomwong et al. (2018) reported upregulation of proPO I and proPO II mRNA in *P. vannamei* fed with a mix between *L. lactis* and *L. plantarum* for 16 days and Won et al. (2020) demonstrated significant increased expression of SP, PX, and proPO I in shrimps fed with *L. lactis* (10^8 CFU/g feed) for eight weeks. However, our wild type *L. lactis* showed no upregulation of genes encoding PX and proPO I. Again, this might due to our shorter experimental duration (8 days). Nevertheless, our data showed that, in our setting, *L. lactis* expressing dsVP28 could activate PX and proPO I faster than the wild type 100X *L. lactis* i.e., at day 6 after treatment.

Lastly, expression of an immune gene encoding anti-lipopolysaccharide factor 1 (ALF1) known to be activated through Toll-signaling by Toll4 and immune deficiency pathways (Li et al., 2018b, 2019a; Tassanakajon et al., 2018) was evaluated. Li et al. (2018b) showed that ALF1 interacts with structural viral proteins such as VP16, VP19, VP26, VP28, and WSSV-189 and interferes with viral infection. It is possible that dsVP28-producing *L. lactis* could inhibit viral production by activation of ALF1. Li et al. (2019b) also proposed a cross-link of RNAi pathway to activate interferon-regulatory factor (IRF) and Vago cascade that, in turn, is able to activate the JAK-STAT pathway to upregulate antiviral genes such as ALF encoding genes. Upregulation of ALF1 in shrimps receiving 100X ds-*L. lactis* was found on day 6 while downregulation of ALF1 was observed in shrimps receiving the wild type 100X *L. lactis*, suggesting dsVP28 produced by *L. lactis* could activate the mechanism for ALF1 production. We hypothesize that the presence of dsVP28 could activate Toll-signaling and IRF-Vago cascade signaling leading to a high ALF1 upregulation in the recombinant 100X ds-*L. lactis*.

5. Conclusion

The main purpose of this study was to develop ability of probiotics to inhibit WSSV-infected shrimps. In this study, probiotic *L. lactis* and *L. plantarum* expressing dsRNA targeting VP28 enveloped of WSSV (dsVP28) through pWH1520 expression vector reduced the shrimp cumulative mortality and viral progeny in WSSV-infected shrimps in comparison to shrimps receiving the wild type. Dose dependency was evident especially in 100X (or 4.5×10^{10} CFU/ 2 L seawater) ds-*L. lactis* treatment. However, both strains of *L. plantarum* showed no effects on shrimp mortality but the recombinant ds-*L. plantarum* reduced viral progeny after viral infection. To clarify the role of dsVP28 expressing *L. lactis* at 100X to inhibit WSSV infection in comparison to the wild type *L. lactis* at same dose, we evaluated activation of gene involving in shrimp RNA interference and shrimp innate immune systems. Shrimps treated with the recombinant dsVP28 expressing *L. lactis* induced shrimp RNA interference through upregulation of gene encoding systemic RNA interference defective protein-1 and Dicer-2. Additionally, shrimp innate immune genes encoding peroxinectin, prophenoloxidase-1, and anti-lipopolysaccharide factor-1 were upregulated in shrimps receiving dsVP28 produced by *L. lactis*, the levels significantly higher than those of the wild type *L. lactis.* Overall, we proposed dual function of *L. lactis* expressing dsVP28 with combination of RNA interference and shrimp innate immune activation to decrease shrimp mortality and viral dissemination from WSSV infection. Further assessment of this recombinant bacterium in dsRNA delivery and in shrimp immune activation will be performed to ensure safety and efficacy against WSSC infection in shrimp aquaculture.

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CRediT authorship contribution statement

Kanokwan Dekham: Methodology, Data analysis, Writing. **Sarocha Jitrakorn:** Methodology. **Patai Charoonnart:** Methodology. **Duangnate Isarangkul:** Providing bacterial strains, Methodology. **Vanvimon Saksmerprome:** Design, Writing, Editing. **Soraya Chaturongakul:** Design, Writing, Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

All data generated or analyzed during this study are included in this published article (and its Supplementary Information file).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2022.101067.

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