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Ingestion of food pellets containing *Escherichia coli* overexpressing the heat-shock protein DnaK protects *Penaeus vannamei* (Boone) against *Vibrio harveyi* (Baumann) infection

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Abstract

Feeding aquatic animals with bacterial encapsulated heat-shock proteins (Hsps) is potentially a new method to combat vibriosis, an important disease affecting aquatic animals used in aquaculture. Food pellets comprised of shrimp and containing *Escherichia coli* overexpressing either DnaK-DnaJ-GrpE, the prokaryotic equivalents of Hsp70-Hsp40-Hsp20, or only DnaK were fed to juveniles of the white leg shrimp *Penaeus vannamei*, and protection against pathogenic *Vibrio harveyi* was determined. Maintaining pellets at different temperatures for varying lengths of time reduced the number of live adhering *E. coli*, as did contact with sea water, demonstrating that storage and immersion adversely affected bacterial survival and attachment to pellets. Feeding *P. vannamei* with *E. coli* did not compromise their survival, indicating that the bacteria were not pathogenic to shrimp. Feeding *P. vannamei* with pellets containing bacteria overproducing DnaK (approximately 60 cells g^{-1} pellets) boosted *P. vannamei* survival twofold against *V. harveyi*, suggesting that DnaK plays a role in *Vibrio* tolerance. Pellets containing DnaK were effective in providing protection to *P. vannamei* for up to 2 weeks before loss of viability and that DnaK encapsulated by these bacteria enhanced shrimp resistance against *Vibrio* infection.

Keywords: DnaK, *heat-shock proteins*, Hsp70, *P. vannamei*, shrimp, *Vibrio*.

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Introduction

Heat-shock proteins (Hsps), commonly referred to as stress proteins or molecular chaperones, occur constitutively in all living cells. Major Hsp families include Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps (sHsps), with monomers of the latter having molecular masses less than 40 kDa. Some Hsps are highly conserved with amino acid sequence similarities reaching 60–70%, as is true for members of the Hsp70 family. As with other Hsps, induction of Hsp70 occurs upon exposure to stressors which denature proteins, and intracellular Hsps may increase 2–3 times in amount during temperature stress, hypoxia, desiccation and infection (Sung *et al.* 2011). Hsp70 functions as a molecular chaperone,

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preventing cell proteins from denaturation and aggregation by assisting in the folding, repair and localization of proteins during stress (Hartl & Hayer-Hartl 2009; Roberts *et al.* 2010; Sung & MacRae 2011). Hsp70 also plays a significant role in immunity. In mammals, for example, Hsp70 elicits cytokine production and delivers maturation signals and peptides to antigen-presenting cells through receptor-mediated interactions, and it influences the production of cell surface peptides that are presented to the immune system (Sung *et al.* 2011).

The relationship between Hsps and immunity in aquatic organisms is important to aquaculture, and there is evidence that Hsp70/DnaK plays a role in the protection of fish, shrimp and molluscs against disease (Basu *et al.* 2002; Roberts *et al.* 2010; Sung & MacRae 2011; Sung *et al.* 2011). Methods using Hsps to enhance the protection of aquatic organisms against pathogens include accumulation of endogenous Hsp70 upon heat shock, administration of stimulants which boost the synthesis of Hsp70, and provision of exogenous Hsps (Sung *et al.* 2007, 2009a,b; Roberts *et al.* 2010; Ryckaert *et al.* 2010; Baruah *et al.* 2011; Loc *et al.* 2013; Hu *et al.* 2014). Injection represents an effective method to deliver Hsps to organisms, and an intracoelomal injection with 1 mg GroEL and DnaK reduces the mortality of platyfish by 20–25% during *Yersinia ruckeri* infection (Ryckaert *et al.* 2010). Injection of white leg shrimp *Penaeus vannamei* (Boone) with full-length DnaK upregulates mRNA for transglutaminase-1 and prophenoloxidase-2 (proPO-2), proteins that are crucial components of the shrimp innate immune system, but the effect on bacterial tolerance was not measured in this study (Hu *et al.* 2014).

Immersion of gnotobiotic *Artemia franciscana* (Kellogg) in sea water containing the Gram-negative bacterium *Escherichia coli* (Migula) overproducing DnaK yielded a twofold survival upon challenge with *Vibrio campbellii* (Baumann), a common bacterial pathogen of brine shrimp (Sung *et al.* 2009a). Protection may be due to the activation of prophenoloxidase (ProPO), a major defence mechanism of crustaceans responsible for melanization of pathogens and damaged tissue (Baruah *et al.* 2011). Immersion of *P. vannamei* in sea water containing *E. coli* overproducing DnaK enhanced tolerance against bacteria *Vibrio harveyi* (Baumann), with primary evidence indicating that protection is associated with increased expression of crustin, an antimicrobial peptide (Sung 2014).

To summarize, feeding *P. vannamei* with pellets containing bacteria overexpressing DnaK increased survival twofold against *V. harveyi*, indicating that DnaK has a role in the protection of shrimp against disease. Pellets retained their protective effect for 2 weeks, with decreasing effectiveness perhaps due to immune fatigue. This work evaluated a food-based method for delivery of Hsp70 to *P. vannamei* and indicated that Hsps may be used to enhance shrimp resistance against *Vibrio* infection, thus potentially serving as an alternative to antibiotic use in aquaculture.

Materials and methods

Maintenance of experimental animals

Juveniles of *P. vannamei*, measuring approximately 5 cm in length, were a generous gift from Agrobest (M) Sdn. Bhd., Pekan, Pahang, and they were acclimatized in the hatchery of Universiti Malaysia Terengganu for 1 week prior to use in experiments. Two individuals were maintained per L of sea water at 28 °C ± 1 °C, pH 7.8–8.0 and salinity of 32‰ in 500-L fibreglass tanks. Animals were fed twice daily to satiation with high-protein shrimp pellets.

Induction of recombinant Hsp synthesis

Escherichia coli strains P3 and YS2, respectively capable of overproducing DnaK-DnaJ-GrpE and DnaK (Table 1), were grown as described (Sung *et al.* 2009b). Induction of Hsps in *E. coli* P3 and YS2 was performed by adding 0.5 mg mL^{-1} L-arabinose for 1 and 4 h, respectively. *E. coli* were harvested by centrifugation at 2200 ***g*** for 15 min, and the pellets were washed once with autoclaved sea water and suspended in 30 mL of sterile H_2O. Culture densities were determined spectrophotometrically at 550 nm, and the bacterial numbers calculated according to the McFarland standard (bioMerieux, Marcy L'Etoile, France) where an optical density of 1.0 corresponds to 1.2×10^9 cells mL^{-1} (Sung *et al.* 2009a). Hsps were detected after SDS–polyacrylamide gel electrophoresis and immunoblotting with the induction of Hsps in strains P3 and YS2 consistent with previous results (Sung *et al.*, 2009a).

Table 1 Transformed bacterial strains and Hsps induced by arabinose

Bacterial strain	Plasmid	Induction	Hsps encoded by plasmids
P3	pKJE7	L-arabinose 0.5 mg mL^{-1} for 1 h	DnaK-DnaJ-grpE
YS2	pDnaK	L-arabinose 0.5 mg mL^{-1} for 4 h	DnaK

Vibrio harveyi, previously isolated from diseased shrimps (Loc *et al.* 2013), was grown at 28 °C on Marine Agar 2216 (Difco Laboratories). Colonies were transferred individually to Marine Broth 2216 (Difco Laboratories) and grown to stationary phase by incubation overnight with constant shaking at 28 °C. Bacteria were harvested by centrifugation at 1800 ***g*** for 10 min at 28 °C, the supernatants were removed, and pellets were suspended in filtered autoclaved sea water. Cell number was determined as just described.

Preparation of food pellets containing *E. coli* strains P3 and YS2

Twenty grams of food pellets with approximately 30% protein (Gold Coins Holding, Malaysia) was manually sprayed with 20 mL of bacterial suspensions containing 1×10^7 *E. coli*, and steps were taken to ensure pellets received equal amounts of bacteria (Sarathi *et al.* 2008; Ning *et al.* 2011; Valdez *et al.* 2014) yielding pellets containing *E. coli* overexpressing DnaK which were termed P3+ and YS2+, and those not overproducing DnaK, termed P3- and YS2-. Pellets were air-dried for 30 min at room temperature (RT) prior to use in experiments. Pellets without bacteria served as control.

Quantitating *E. coli* on pellets upon storage and immersion in sea water

The number of *E. coli* on pellets upon storage for 72 h at RT, 4 °C and −80 °C was determined. One gram of pellets was homogenized in 1 mL of sterile water, and the suspension was plated on Luria–Bertani (LB) agar with 20 mg L^{-1} chloramphenicol for *E. coli* P3 and 50 mg L^{-1} ampicillin for *E. coli* YS2 (Sung *et al.* 2009a). Plates were incubated at 37 °C, and colonies were counted after 24 h of incubation. Each treatment was performed in triplicate, and the experiment was performed twice.

To determine the number of bacteria on pellets as immersion time increased, 1 g of pellets containing *E. coli* P3 and YS2 was separately placed in sterile 1.5-mL centrifuge tubes containing 1 mL sea water. Pellets were collected, and the number of bacteria on the pellets was determined at 2-h intervals for 8 h following the methods just described. At corresponding times, the sea water in which the pellets were immersed (1 mL) was plated to determine bacterial number. Each treatment was performed in triplicate with experiments performed twice.

Feeding *P. vannamei* with pellets

Twenty *P. vannamei* juveniles were placed in 50-L fibreglass tanks and fed to satiation twice daily with pellets containing *E. coli* strains P3 and YS2, either induced or non-induced. Survival was determined weekly for 4 weeks by counting actively swimming animals. Shrimp fed with pellets without *E. coli* served as control. Survival was calculated as $N_t \times 100/N_o$, where N_t and N_o are final and initial numbers of shrimp, respectively (Loc *et al.* 2013). Each treatment was performed in triplicate, and the experiment was performed twice.

Challenge of *P. vannamei* with vibrios

Twenty *P. vannamei* juveniles fed twice daily with pellets containing *E. coli* either overproducing or not overproducing DnaK were challenged weekly for 4 weeks with 100 μL of 1.0×10^7 *V. harveyi* per mL via intramuscular injection (Loc *et al.* 2013). Survival was determined 24 h after injection as just described. Moribund shrimp were counted as dead. Shrimp injected with saline were used to show that the injection was not lethal to the shrimp. Treatments were performed in triplicate, and the experiment was performed twice.

Data analysis

Values of larval survival were arcsine to satisfy normality and homoscedasticity requirements whenever necessary. Significant differences in terms of survival were investigated by performing one-way ANOVA, followed by Tukey's test at a significance level of 0.05. All analysis was performed using statistical analysis software SPSS® version 20.0 for Windows®.

Results

Prolonged storage reduced the number of live *E. coli* on pellets

One gram of homogenized pellets in 1 mL sea water contained approximately 60 *E. coli* per mL immediately after coating, and it decreased to 30–40 *E. coli* per mL upon 12-h storage at either RT, 4 or −80 °C and then decreased again by 24 h. After 48 h, there were <5 *E. coli* per mL (Fig. 1). Thus, pellets were used in experiments 30 min after coating.

Prolonged immersion in sea water reduced the number of live *E. coli* on pellets

Upon immersion for 2 h in sea water, the number of *E. coli* on 1 g homogenized pellets in 1 mL sea water decreased from 60 to 20 cells mL^{-1} and then to approximately 5 cells mL^{-1} at 4 h (Fig. 2a). *E. coli* were not detected in the sea water immediately upon immersion, but approximately 40 *E. coli* per mL were found after 2-h incubation, suggesting that bacteria leached from pellets but were not dead (Fig. 2b).

Ingestion of pellets containing *E. coli* did not affect the survival of *P. vannamei*

Approximately 95% of *P. vannamei* survived ingestion of pellets coated with *E. coli* strains P3 and YS2, indicating that these *E. coli* strains were not pathogenic to shrimp (Fig. 3a, b).

Ingestion of pellets containing *E. coli* overproducing DnaK protected *P. vannamei* against *V. harveyi*

Survival was approximately 50% when *P. vannamei* were fed with pellets containing *E. coli* not overproducing DnaK and then exposed for 24 h to *V. harveyi* challenge. Feeding *P. vannamei* with pellets coated with *E. coli* overproducing DnaK boosted survival to 70–90% in the first week, and 85% in the second week, but protection was not apparent on the third and fourth weeks (Fig. 4a, b). Mortality was not observed in the saline-injected shrimp (data not shown).

Discussion

Heat-shock proteins such as DnaK are potent immunomodulators (Sung *et al.* 2009a), a characteristic reflecting their potential as therapeutic agents and leading to their application in combating infection in aquaculture. Feeding with *E. coli* overproducing different prokaryotic Hsps enhances the resistance of gnotobiotic *Artemia* to *V. campbellii*. Specifically, the immunoprobing of Western blots demonstrated that the enhanced resistance to *V. campbellii* correlates with DnaK

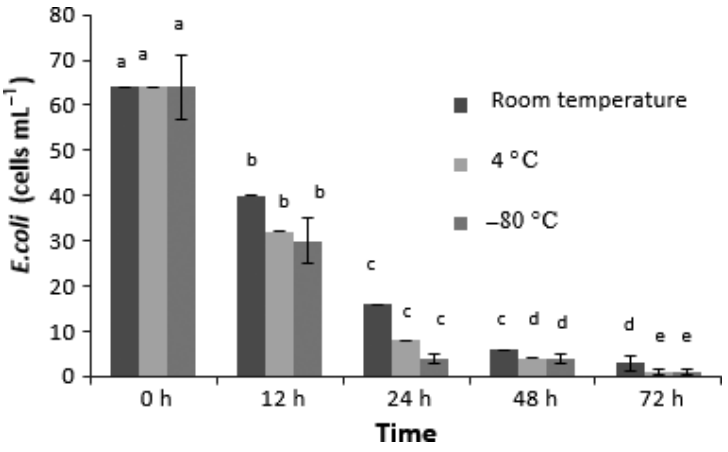

Figure 1 *Escherichia coli* P3 and YS2 decreased upon storage of pellets. The number of *E. coli* on pellets upon storage for 72 h at RT, 4 °C and −80 °C was determined. One gram of pellets was homogenized in 1 mL of sterile water, and the suspension was plated on Luria–Bertani (LB) agar with either 20 mg L^{-1} chloramphenicol for *E. coli* P3 and 50 mg L^{-1} ampicillin for *E. coli* YS2. Plates were incubated at 37 °C for 24 h and colonies were then counted. Pellets without bacteria coating served as controls. Data presented are mean ± SD. Bars showing the same superscript letter for each treatment are not significantly different ($P > 0.05$). Each treatment was performed in triplicate, and the experiment was repeated once.

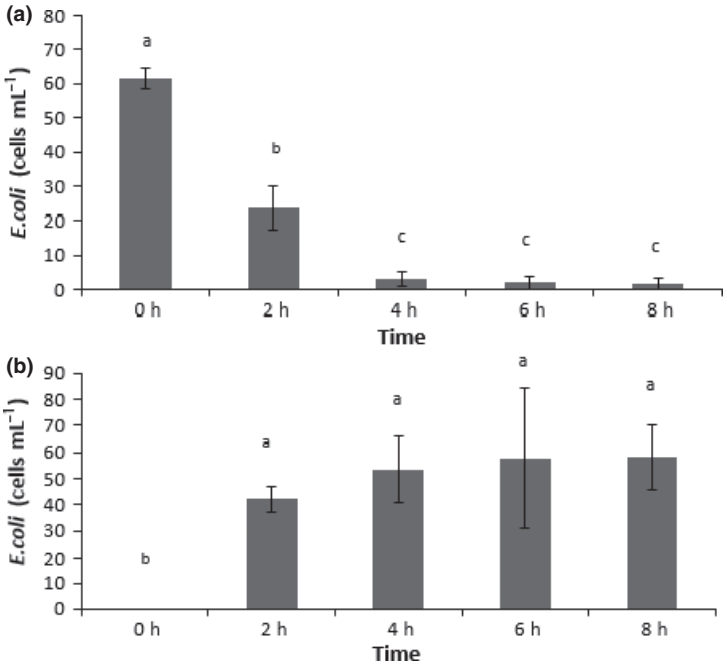

Figure 2 Immersion in seawater-leached *E. coli* strains P3 and YS2 from pellets. One gram of pellets containing *E. coli* P3 and YS2 was separately immersed in 1 mL sea water. (a) Pellets were collected, and the number of bacteria on the pellets was determined at 2-h interval for 8 h by plating on LB agar with either 20 mg L^{-1} chloramphenicol for *E. coli* P3 or 50 mg L^{-1} ampicillin for *E. coli* YS2. (b) At corresponding times, the sea water in which the pellets were immersed (1 mL) was plated to determine bacterial number. Plates were incubated at 37 °C for 24 h, and colonies were counted. Data presented are mean ± SD. Bars showing the same superscript letter for each treatment are not significantly different ($P > 0.05$). Each treatment was performed in triplicate, and the experiment was repeated once.

production in *E. coli* (Sung *et al.* 2011). Protection may be linked to the C-terminal fragment of DnaK, a conserved peptide-binding domain of the *Artemia* Hsp70, which activates the prophenoloxidase (proPO) system (Baruah *et al.* 2013).

In this study, pellets containing *E. coli* overproducing DnaK were fed to *P. vannamei* juveniles to test whether delivery of molecular chaperones by this method protects against bacterial infection in shrimp. Prior to the feeding experiments, the number of viable bacteria on pellets after spraying and upon either storage at different temperature or immersion in sea water was determined, demonstrating that prolonged exposures to these conditions significantly reduced the number of live *E. coli* on pellets. Reductions in the number of *E. coli* on pellets and their increase in sea water used for immersion were probably due to leaching whereby bacteria slowly detach from pellets (Pusparaj, Ramesh & Ambika 2012). Leaching can be overcome using binders or coating agents such as sodium alginate, but most binders alter bacteria content (Zhou *et al.* 2007). To prevent the possibility of changes in DnaK accumulation, binders were not used as coating agent in this study. Rather, pellets were sprayed with 1×10^7 *E. coli* per mL and allowed to dry for 30 min at RT to facilitate bacterial attachment. This coating method has been used to attach probiotic bacteria to pellets to examine their efficacy in boosting the tolerance of aquatic organisms to disease and infection (Fu *et al.* 2011).

Feeding *P. vannamei* with pellets containing *E. coli* with or without induced DnaK for 4 weeks did not cause shrimp mortality, indicating that these *E. coli* were not pathogenic to *P. vannamei*, observations similar to those made for other shrimp species (Sung *et al.* 2009a,b; Sung 2014). The bacterial strains used herein were constructed previously with *E. coli* K-12 strain CAG 626, and they are described in the NIH Recombinant DNA Advisory Committee (RAC) guidelines (Federal Register 1986) as non-pathogenic.

While *P. vannamei* fed with the non-coated control pellets experienced 50% mortalities in median lethal *V. harveyi* challenge assays (LD50), the survival of shrimp fed with pellets containing *E. coli* P3+ for one and 2 weeks was boosted to 70–80% upon *V. harveyi* challenge. Similar trends occurred with pellets containing *E. coli* YS2+, the latter overexpressing only DnaK, where survival was enhanced from 50% to 70–90%, respectively, upon 1 and 2 weeks of feeding followed by (LD50) exposure to *V. harveyi*. The effectiveness of *E. coli* P3 and YS2 was enhanced upon Hsp induction which increased the amount of DnaK in the bacteria, suggesting that DnaK plays a functional role in protection, an observation

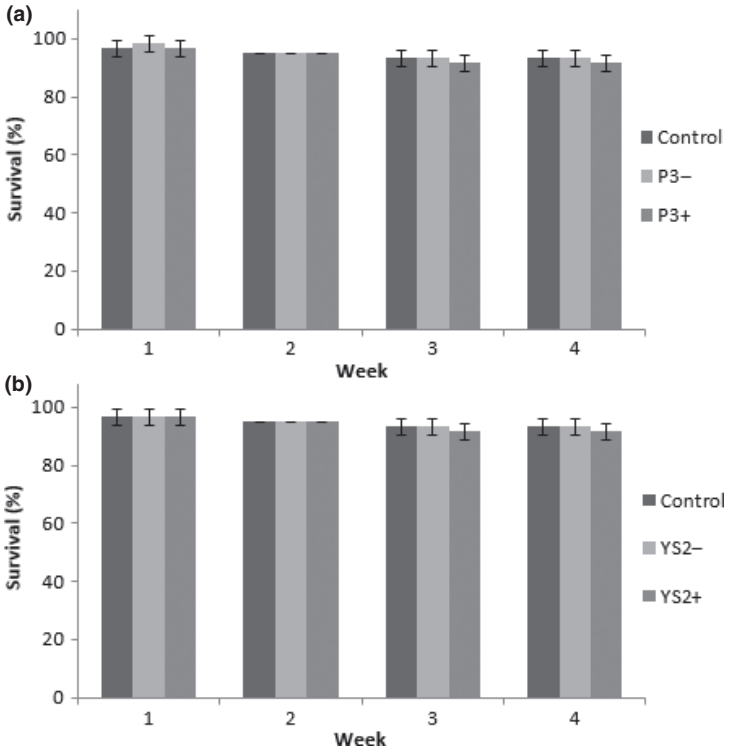

Figure 3 Pellets containing *E. coli* P3 and YS2 were not toxic to *P. vannamei* juveniles. Twenty *P. vannamei* juveniles were fed to satiation twice daily with pellets containing either induced or non-induced *E. coli* strains (a) P3 and (b) YS2. Survival was determined weekly for 4 weeks by counting actively swimming animals. Shrimp fed with pellets without *E. coli* served as control. P3+, *E. coli* P3 overproducing DnaK-DnaJ-GrpE; P3-, *E. coli* P3 not overproducing DnaK-DnaJ-GrpE; YS2+, *E. coli* YS2 overproducing DnaK; YS2-, *E. coli* YS2 not overproducing DnaK; Control, shrimp fed pellets without *E. coli*. Each treatment was performed in triplicate, and the experiment was repeated once.

similar to that made for other shrimp species. Supplying gnotobiotic larvae of *Artemia* with *E. coli* overproducing DnaK enhanced survival approximately two to threefold upon challenge with *V. campbellii* (Sung *et al.* 2009a). Similar results were obtained when larvae were fed with heat-shocked bacterial strains producing increased amounts of DnaK when compared to non-heated controls. Immunoprobing of Western blots and quantification by ELISA demonstrated that improvement in larval resistance to *V. campbellii* infection correlated with increasing amounts of DnaK, suggesting a protective role for this protein, either via chaperoning or immune enhancement (Sung *et al.* 2009b). In another example, feeding *P. vannamei* larvae with induced *E. coli* P3 + increased the immune-related protein crustin mRNA approximately sevenfold and significantly increased the protection of animals against *V. harveyi*, suggesting immune activation (Sung 2014). Support for an immunoregulatory role of DnaK in *P. vannamei* is strengthened by the observation that injection with a full-length recombinant DnaK enhances expression of prophenoloxidase and transglutaminase mRNA, potent immune-related genes of the shrimp (Hu *et al.* 2014). Taken together, these studies indicate that the resistance of shrimp against infection is enhanced by DnaK.

Similar to other shrimp species, *P. vannamei* depends on its innate immune system to protect against disease. Hsps stimulate the innate immune system (Pockley 2003) serving as ligands for Toll-like receptors (TLR) by activating TLR to transfer inflammatory signals to the immune cells upon binding to the TLR in order to respond to disease and infection (Vabulas *et al.* 2002; Gobert *et al.* 2003; Kulkarni, Behboudi & Sharif 2011; Sung & MacRae 2011). Pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) are two examples of receptors for Hsps (Vazquez *et al.* 2009). Hsp70/DnaK binds with PAMPs such as endotoxin lipopolysaccharide (LPS), potent antimicrobial substances which suppress pathogen proliferation (Hu *et al.* 2014). Methods of pathogen attenuation include melanization, haemolymph coagulation, phagocytosis and encapsulation of foreign particles (Soderhall & Cerenius 1998; Breloer *et al.* 2001).

That protection occurred only in the first and second week of feeding with DnaK may be due to 'immune fatigue', referring to the exhaustion of

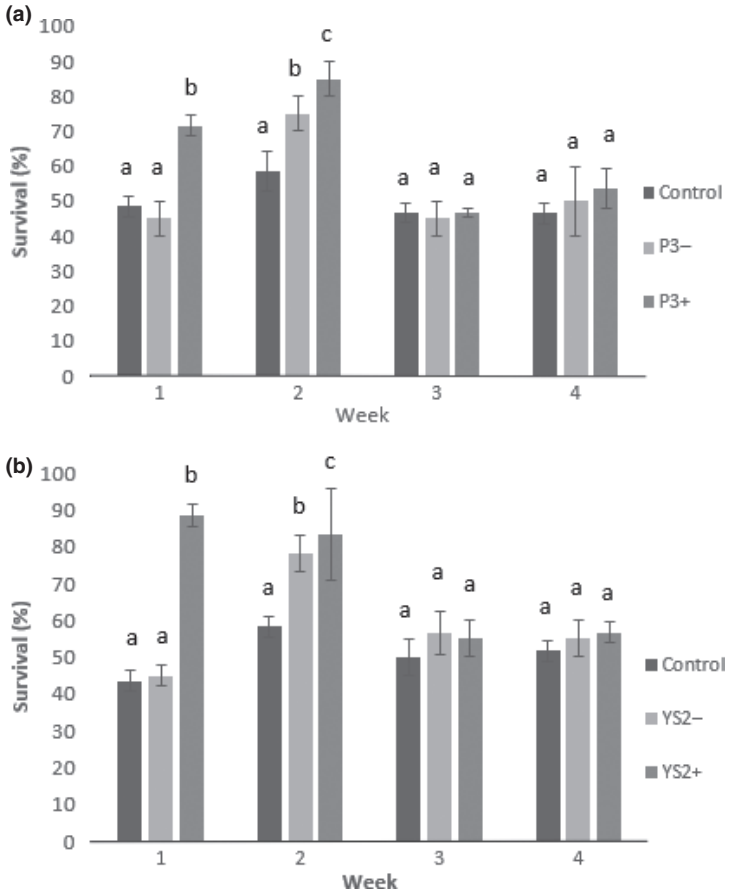

Figure 4 Feeding pellets coated with *E. coli* P3 and YS2 enhanced the protection of *P. vannamei* against *V. harveyi*. Twenty *P. vannamei* juveniles fed twice daily with pellets containing *E. coli* (a) P3 and (b) YS2 either overproducing or not overproducing DnaK were challenged weekly for 4 weeks with 1.0×10^7 *V. harveyi* per mL via intramuscular injection in the caudal region. Survival was determined 24 h after injection. P3+, *E. coli* P3 overproducing DnaK-DnaJ-GrpE; P3-, *E. coli* P3 not overproducing DnaK-DnaJ-GrpE; YS2+, *E. coli* YS2 overproducing DnaK; YS2-, *E. coli* YS2 not overproducing DnaK; Control, shrimp fed with pellets without added *E. coli*. Bars showing the same superscript letter for each treatment are not significantly different ($P > 0.05$). Each treatment was performed in triplicate, and the experiment was repeated once.

the shrimp immune system upon prolonged ingestion of an immunostimulant (Chang *et al.* 2000). In this study, shrimp were fed twice daily with pellets that were freshly coated with bacteria. In post-larvae of the Indian white shrimp *Fenneropenaeus indicus* (H. Milne-Edwards), continuous intake of a diet containing glucan for 40 days significantly reduced survival and protection against white spot syndrome virus (Sajeevan, Philip & Singh 2009). It is therefore suggested that DnaK, as with other immunostimulants, provides short-term protection to shrimp larvae which can be extended upon optimization of feeding regimes. Whatever the outcome might be, this study demonstrated that feeding of pellets containing DnaK encased in *E. coli* promoted the tolerance of *P. vannamei* against *V. harveyi*. This method represents a potential biocontrol strategy to counter disease in aquatic organisms, thereby serving as a promising alternative to the use of antibiotics during aquaculture.

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