



Melanin-containing feedstuffs protect *Litopenaeus vannamei* from white spot syndrome virus

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Abstract Viral diseases are a serious issue for the shrimp aquaculture industry. White spot syndrome virus (WSSV) has been considered one of the most dangerous pathogens infecting cultured shrimp, causing a mortality rate as high as 100% within 7–10 days of viral infection. So far, several protocols have been applied to protect shrimp against virus attacks, but their protection efficiency is very limited. In this study, for the first time, three melanin-containing feedstuffs (F1, F2, and F3) were formulated and fed to cultured shrimp to investigate the ability of melanin to protect shrimp from WSSV. The obtained results showed that F2 had a protection rate of 64% at day 7 and 62% at day 10 after virus challenge. The protection ability of the feedstuff depended on the amount of melanin consumed by shrimp. Moreover, our results also demonstrated that the transcription level of the *VP28* gene, which codes for the VP28 protein, a representative for the presence of WSSV, was significantly decreased in shrimp fed F2. Taken together, our study suggests that melanin-containing diets may be applied in aquaculture to protect shrimp against WSSV infection; further, combined protocols with the simultaneous use of melanin-containing diets and other protectants should be studied and applied to increase the protection efficiency.

Keywords *Litopenaeus vannamei* · White spot syndrome virus (WSSV) · VP28 · Protection rate · Melanin-containing feed stuff

Introduction

Litopenaeus vannamei is mainly cultured in South African and Asian countries, especially Brazil, Ecuador, Mexico, China, Thailand, Indonesia, and Vietnam (Weidner and Rosenberry 1992; Jiravanichpaisal et al. 2001; Guzman et al. 2009; Gunalan et al. 2014). Approximately 3.7 million tons of *Litopenaeus vannamei* were produced in 2014. Shrimp export is considered a major sector providing considerable economic benefits to these countries. However, shrimp aquaculture continues to face the serious problem of infectious diseases caused by fungi, bacteria, and especially viruses. So far, more than 20 types of viruses have been reported as shrimp pathogens. Among these, white spot syndrome virus (WSSV) has been considered one of the worst

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pathogens because of its strong dissemination and infectious abilities. More seriously, WSSV can lead to a 100% mortality rate among cultured shrimp within 3–10 days after infection (Jiravanichpaisal et al. 2001; Jiravanichpaisal et al. 2006a, b; Guzman et al. 2009).

VP28 (28 kDa) is an important structural protein of WSSV that can be used as a marker to determine the presence of WSSV in water or/and shrimp samples (Hoffmann et al. 1999; Lin et al. 2002; Tang et al. 2007; Nguyen et al. 2014). Shrimp immunity heavily relies on the innate immune system rather than an adaptive immune system (Hoffmann et al. 1999; Nguyen et al. 2014). The innate immunity of shrimp includes cellular barriers and humoral barriers. These barriers play critical roles to protect shrimp from attacks of pathogens via mediating phagocytosis and initiating the apoptosis pathway (Van de Braak et al. 2002; Jiravanichpaisal et al. 2006a, b; Balasubramanian et al. 2008; Liu et al. 2009; Tassanakajon et al. 2010). In addition, melanization is also an important immune mechanism in shrimp (Jiravanichpaisal et al. 2001).

Many studies have been conducted to find solutions that protect shrimp from viral attacks; however, no real effective protocol has been reported. So far, several products including plant extracts and protein-/DNA-based vaccines have been developed, but these protectants have many limitations such as low protection efficiencies (Balasubramanian et al. 2006; Pholdaeng and Pongsamart 2010; Sudheer et al. 2011; Vaseeharan et al. 2006) and problems related to genetically modified organisms and genetically modified feeds (GMO/GMF) (Witteveldt et al. 2004; Rout et al. 2007; Sutthangkul et al. 2017).

Melanin is a natural polymer with very low toxicity (Garcia Borron et al. 2014). Melanin has many important roles in the human body including UV-radiation absorbent (Brenner and Hearing 2008), antioxidant (Vate and Benjakul 2013), and heavy-metal absorbent properties (Cuong et al. 2018). Melanization and melanin secretion help shrimp protect themselves by encapsulating pathogens and excreting them into the environment (Charoensapsri et al. 2014; Sutthangkul et al. 2015; Harikrishnan et al. 2011). However, no studies have examined the role of melanin as a supplement in protecting shrimp from pathogen attacks. Therefore, in this study, for the first time, we created melanin-containing feedstuffs and applied them to cultured *Litopenaeus vannamei* to investigate their abilities to protect shrimp against WSSV.

Materials and methods

Laboratory-scale shrimp culture

An experimental model for shrimp culture was set up based on a previous report (Nguyen et al. 2014). The designed culturing pool had a size of 45 × 25 × 30 cm with air-supplier and water filtration–circulation pump systems. Each pool was filled with 15 L of artificial seawater adjusted to 16‰ salinity, and seeded with 25 shrimp. The filter was washed every day and the cultured water was replaced with 70% freshwater every 3 days. The culturing temperature was maintained at 24–28 °C. Feedstuffs were supplied four times per day, at 10 g each feeding.

Determination of dose of WSSV for 75% shrimp death (LD₇₅)

WSSV was isolated from WSSV-infected shrimp in a previous study (Nguyen et al. 2014). After replacing with freshwater, WSSV solutions with viral doses of 1×10^4 , 5×10^4 , 1×10^5 , 5×10^5 , and 1×10^6 copies/mL were added to culturing pools, and shrimp were challenged with WSSV for 3 days. During virus challenge, water filtration–circulation pumps were stopped to avoid virus loss. After challenge, culturing systems were activated as usual and cultured for next 7 days. Every day, dead shrimp were collected and counted for mortality rate calculation to determine the LD₇₅ value.

Preparation of melanin-containing feedstuffs

Melanin was extracted from Splendid Squid (*Loligo formosana*) as reported in our previous study (Cuong et al. 2018). Commercial feed in the bead form with a diameter of 2 mm (containing 45–50% starch) was used as the core feed to produce melanin-containing feedstuffs with different formulas. Formula 1 (F1): melanin was coated on the surface of the commercial beads using cod oil as a binding agent. At first, melanin powder



(< 150 μm in size) was distributed into cod oil and evenly coated on the commercial feed beads at different ratios (w/w) of 1/500, 1/200, and 1/50. Formula 2 (F2): melanin powder was well mixed with commercial powder, and starch solution (3%) was used as cohesive agent. First, commercial feed beads were ground into a powder and sieved through a 150- μm pore. Then commercial feed powder and melanin powder were well mixed at different ratios of 1/500, 1/200, and 1/50 (w/w) and evenly distributed in 3% starch solution. Next, the mixtures were pushed through a tube to form beads with a diameter of 2 mm. Formula 3 (F3): commercial feed beads were directly dipped into squid ink-sac liquid at a 1/1 (w/v) ratio. All feedstuffs were dried at room temperature (25 °C) and kept in a 4 °C incubator before use. Commercial feedstuff (NURTRA-BEST, No. 880) for shrimp was purchased from Tongwei Vietnam Ltd. The ingredients of the feedstuff were recorded as follows: humid max. of 11.0%, total protein min. of 40.0%, digestible protein min. of 37%, energy (Kcal/kg) of 3200, total lipid min. of 5.0%, total fiber max. of 3.0%, calcium content max. of 2.3%, total phosphor content of 1.25–2.5%, lysine content of 1.8%, and methionine content of 0.6%.

Experimental design to investigate the ability of melanin-containing feedstuffs to protect shrimp against WSSV attack

Shrimp were divided into five groups: Group 1 (negative control group) shrimp were supplied with commercial feed without virus challenge; Group 2 (positive control group) shrimp were supplied with commercial feedstuff and challenged with virus (1×10^6 copies/mL); Groups 3, 4, and 5 (experimental groups) shrimps were supplied with feedstuff formula 1, 2, or 3, respectively, and challenged with virus (1×10^6 copies/mL). All the experiments were repeated three times. After challenge, shrimp were continuously cultured for 7 days and dead shrimp were collected and counted for mortality rate calculation to determine the protection rates (PRs) of the various feedstuff formulations. The PR was calculated based on the following formula:

$$\text{PR (\%)} = 1 - \frac{\text{mortality rate of experimental group}}{\text{mortality rate of positive group}} \times 100.$$

Real-time-PCR analysis

Total RNA was isolated from mouse tissue using the GeneJET RNA Purification (Thermo Scientific, Singapore), according to the manufacturer's instructions. Then cDNA was synthesized from total RNA by reverse transcriptase reaction, according to the protocol supplied with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Singapore). Real-time quantitative RT-PCR was performed in a LightCycler[®] 96 Instrument (Roche Diagnostics GmbH, Mannheim, Germany). Expression levels of VP28 were measured by quantitative RT-PCR, and adjusted through the transcript expression level of β -actin (an internal control). The primer sequences of VP28 and β -actin were presented as below:

VP28-Forward: 5'-CCCAAGCTTGATCTTTCTTTCACTCTTTCGG-3'; and VP28-Reverse: 5'-AAAGAA TTCTTACTCGGTCTCAGTGCCAGAG-3', (615 bp) (Nguyen et al. 2014).

β -Actin-Forward: 5'-TGTGTGACGACGAAGTAGCC-3'; and β -actin-Reverse: 5'-TGGTCGTGAAGG TGTAACCA-3' (605 bp) (Wang et al. 2007).

PCR products were used for electrophoresis running on agarose gel.

Results

High WSSV dose results in the death of 75% of shrimp (LD₇₅)

The obtained results showed that shrimp began to die on day 3 after challenging with virus at concentrations of 5×10^5 and 1×10^6 copies/mL, and the mortality increased rapidly over the next 5 days. Particularly, at day 7, the cumulative number of dead shrimp in the pools challenged with 5×10^5 and 10^6 copies/mL virus were 30% and 80%, respectively; but, no shrimp had died by day 7 in the pools challenged with low viral concentrations (1×10^4 , 5×10^4 , and 1×10^5 copies/mL). Based on this result, the LD₇₅ of WSSV for shrimp was determined to be 10^6 copies/mL for a 7-day challenge (Fig. 1a). The results also indicated that



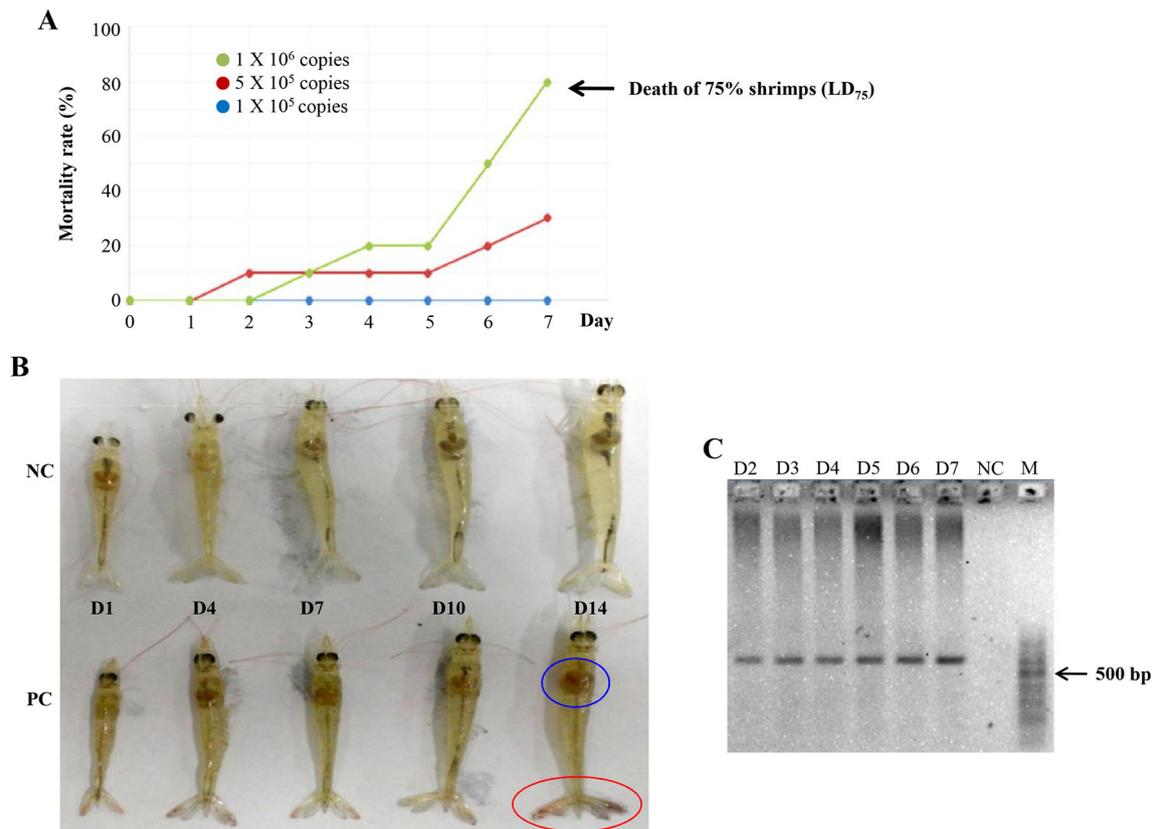


Fig. 1 Lethal dose of WSSV for 70% (75%) shrimp death. **a** Mortality rates of shrimps challenged with various WSSV concentrations; **b** morphologies of negative control (NC) shrimps and WSSV-infected shrimps (positive control—PC) at day 1 (D1), day 4 (D4), day 7 (D7), day 10 (D10), and day 14 (D14) post-viral infection; **c** transcript expressions of VP28 gene in PC and NC shrimps at day 2 (D2), day 3 (D3), day 4 (D4), day 5 (D5), day 6 (D6) and day 7 (D7) post-WSSV infection. The blue and the red circle signals indicated the necrotic head and red tail occurred on the WSSV-infected shrimps, respectively

shrimp in the WSSV-challenged pool had typical signs of viral infection such as red tails and necrotic heads, while shrimp in the control pool did not have these signs (Fig. 1b). Further, the PCR results confirmed the presence of virus in the WSSV-infected shrimp via *VP28* expression (Fig. 1c).

Melanin-containing feedstuffs protect shrimp from WSSV infection

Three melanin-containing feedstuffs named F1, F2, and F3 were produced and are presented in Fig. 2a. Although F1, F2, and F3 feedstuffs were initially made with different melanin/feed ratios, including 1/500, 1/200, and 1/50, our preliminary results indicated that the 1/200 ratio was the most suitable one (data not shown) and thus we used the feedstuff with this ratio for further experiments. Shrimp were cultured and fed with F1, F2, or F3 feedstuffs, then challenged with WSSV for 3 days. The dead shrimp were collected and counted. The results showed that while the mortality rate of shrimp in the negative control pool was always stable at a low level (around 2–4%), that of the positive control pool was very high: generally, in the positive pool, shrimp started to die at day 3, the death rate quickly increased over the next 5 days, and the mortality rate reached about 70–80% at day 7 and 96–100% at day 10. The protection rates of melanin-containing feedstuffs varied widely depending on the type of feedstuff. In general, while the F3 feedstuff was not able to protect shrimp from WSSV attack, F1 and F2 had protection rates of 57% and 64% at day 7 and 42% and 62% at day 10, respectively (Fig. 2b).



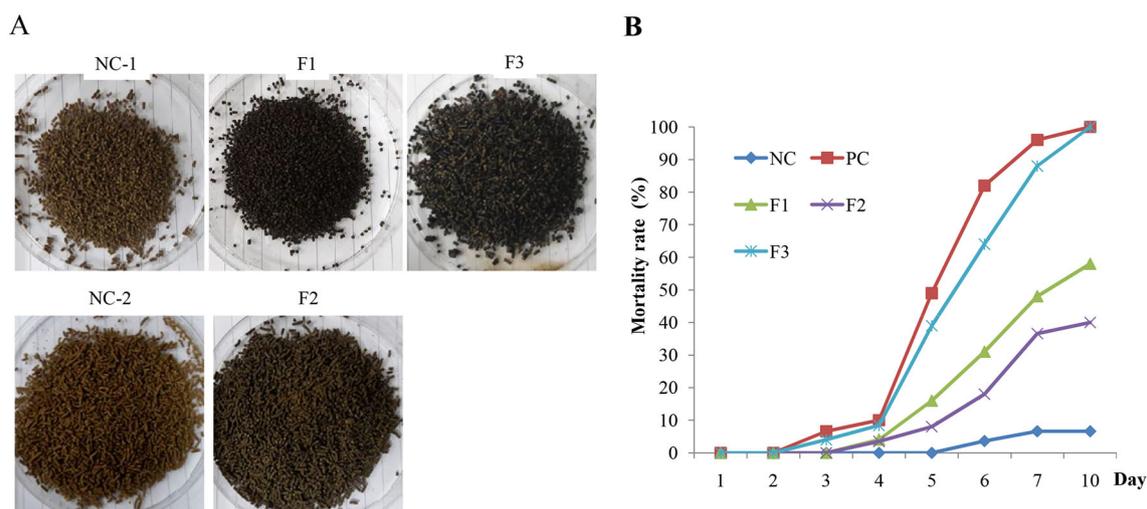


Fig. 2 Protection rates of different feed stuffs on WSSV-infected shrimps. **a** Appearances of commercial feed (NC1); commercial feed coated with melanin at ratio of 1/200 (w/w) and with the presence of cod oil as binding agent (F1), modified commercial feed (mixed with 3% starch solution) (NC2), commercial feed well mixed with melanin at ratio of 1/200 (w/w) and with the presence of starch 3% as cohesive agent (F2), and commercial feed directly coated with squid ink liquid at ratio 1/1 (w/v); **b** the accumulative mortality rates of shrimps fed with various feed stuffs (NC1, NC2, F1, F2, F3) at different days post-WSSV infection

Melanin-containing feedstuffs reduced the transcription level of VP28

Transcription levels of VP28 in shrimp were analyzed at day 7 and day 10 after virus challenge. The results showed that at day 7, the transcription level of VP28 in shrimp of the positive control (PC) pool and F1 pool were 4.6- and 5.0-fold higher than that in shrimp of the F2 pool, respectively (Fig. 3a). More impressively, at day 10, VP28 levels in shrimp of PC and F1 pools were almost twice those at day 7, while levels in shrimp of the F2 pool at day 10 were only 1.4-fold higher than those at day 7 (Fig. 3b).

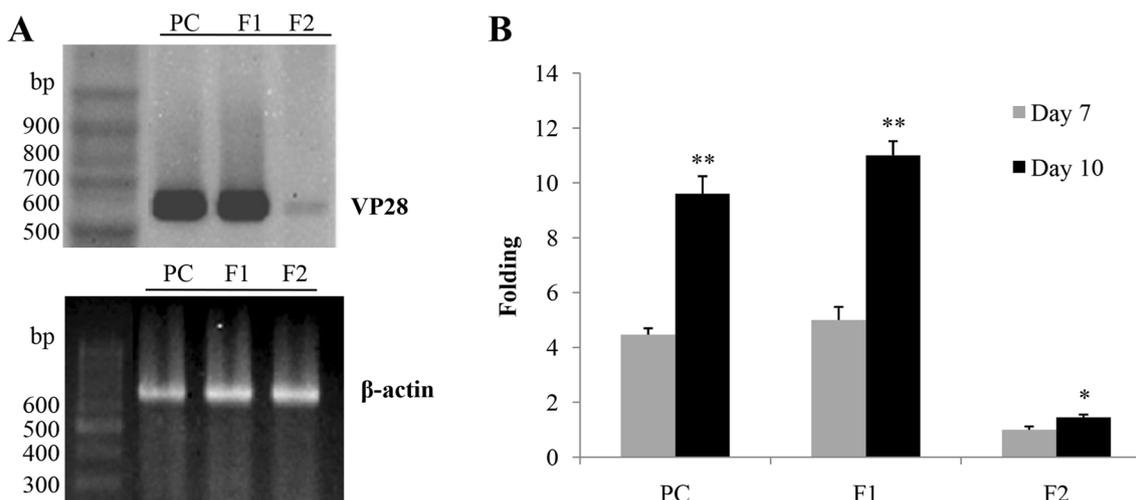


Fig. 3 Transcription levels of VP28 on WSSV-infected shrimps. **a** Transcription levels of VP28 on positive control (PC), F1-fed (F1) and F2-fed (F2) shrimps at day 7 post-WSSV infection. Beta-actin was used as internal control; **b** transcription levels of VP28 were quantitatively measured and presented in a graph. *,**Significant differences ($p < 0.05$, and 0.01 , respectively) by the Student’s t test

Protection rates of melanin-containing feedstuffs depended on amount of melanin consumed by shrimp

The same amount of melanin was supplemented in F1, F2, and F3 feedstuffs. During the experimental period, shrimp in all tanks were fed the same amount of feedstuffs and challenged with WSSV at day 3; representative images of the shrimp are shown in Fig. 4. The morphological differences among these three groups of shrimp were easily observed even with the naked eye (Fig. 4a–c). Consumed melanin was present in the shrimp intestines. Although the same amount of melanin was supplemented in F1 and F2 feedstuffs and the same amount of feedstuffs were fed to shrimp, the melanin consumed melanin by the shrimp of the F2 pool was much higher than that by the shrimp of the F1 pool. To explain this, the amount of melanin that released from the melanin-containing feedstuffs (F1 and F2) into water was examined using a spectrophotometer at a wavelength of 415 nm. The results demonstrated that while the melanin in the F2 feedstuff was strongly bound inside the feed beads, melanin in the F1 feedstuff was easily released into water. Particularly, at 1, 2, 3 and 4 h after watering, the melanin amounts of F2 released in water were only 1/3.6, 1/2.5, 1/2.1, and 1/2.1 of those of F1, respectively (Fig. 4d).

Discussion

In general, shrimp mainly rely on innate immunity for protection against pathogen attacks (Witteveldt et al. 2004; Rout et al. 2007; Sutthangkul et al. 2017). However, melanin biosynthesis and release are also primary mechanisms that shrimp use to encapsulate pathogens (Tassanakajon et al. 2013; Nguyen et al. 2014). Melanin is a product created from the reaction of the shrimp immune system to envelop and excrete the pathogen into the environment (Tassanakajon et al. 2013; Nguyen et al. 2014). So far, there is no information about the quantity of melanin that shrimp produce in normal conditions and in virus-infected conditions. Therefore, it is unknown whether shrimp can produce enough melanin to sufficiently eliminate pathogens. More importantly, although the effects of endogenous melanin in shrimp have been identified, those of supplemented melanin (in the diet, for example) have not been investigated. Therefore, we presumed that supplying extrinsic melanin by oral administration may help shrimp capture and remove WSSV, which enters the intestinal tract of the shrimp.

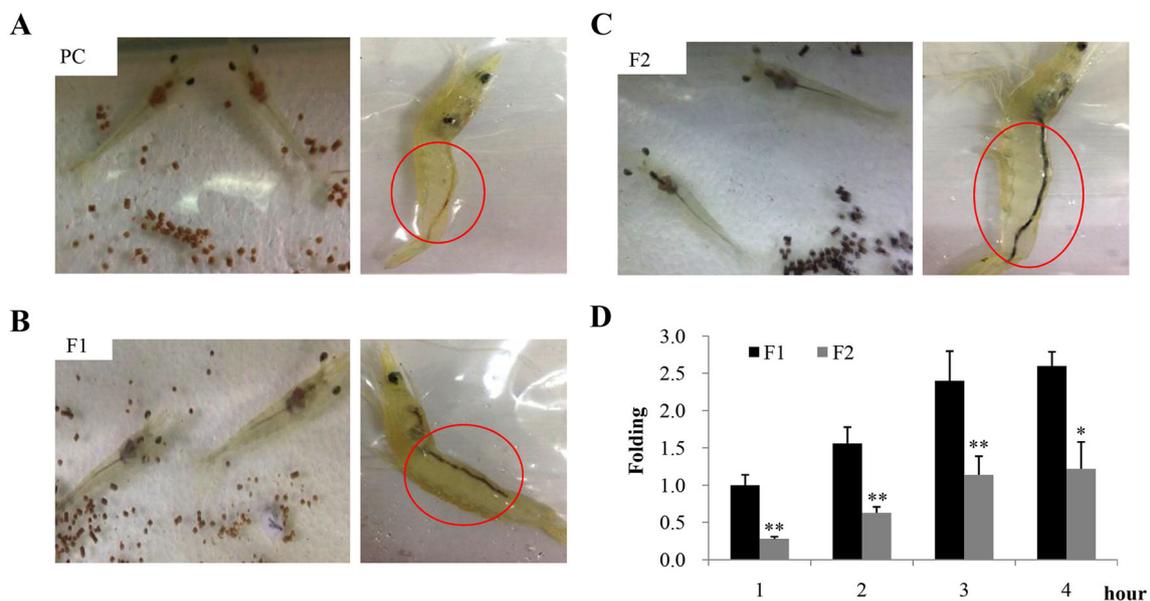


Fig. 4 Amount of melanin consumed by shrimps depended on the type of feed stuff. **a–c** The WSSV-infected shrimps fed with NC1, F1 and F2 feed stuffs, respectively. The circle reds indicated the different amount of melanin in intestines of shrimps. **d** Melanin released from feed stuffs into environment. *,**Significant differences ($p < 0.05$, and 0.01 , respectively) by the Student's t test

In this study, natural melanin extracted from squid ink-sac liquid, which is considered waste by seafood-processing companies, was supplemented as a feedstuff ingredient and fed to shrimp to investigate its ability to protect shrimp against WSSV attacks. Our results demonstrate that the formula used for preparing melanin-containing feedstuffs affects the protection capabilities of melanin supplementation in cultured shrimp. The melanin well-mixed feedstuff (F2) had a significantly higher protection rate compared with those of melanin-coated feedstuffs (F1 and F3). The well-mixed form of F2 was able to capture melanin inside feed beads, whereas the coated formulas, F1 and F3, were not good at retaining melanin on their surfaces when dipped into water; thus, the amount of melanin consumed by shrimp fed F2 was much higher than that by shrimp fed F1 or F3. This result might consequently lead to differences in their protection abilities. Results of this study showed that the protection rate depended on the amount of melanin that shrimp had consumed; therefore, supplying a mixture of F2 and F3 feedstuffs to shrimp is unlikely to have a synergistic effect on the protection rate.

The results of this study indicate that the protection rate for shrimp fed the F2 feedstuff was around 60–65%. This result was underwhelming, yet reasonable; melanin supplementation could only partially help shrimp capture and encapsulate viruses that entered via the digestive system, but it could not help eliminate viruses that infected shrimp by other modes of entry. Thus, the simultaneous use of other substances that can stimulate the activity of the immune system might be necessary. So far, many agents have been developed to protect shrimp against virus attacks, such as plant extracts and protein/DNA vaccines. However, while protection efficiencies of plant extracts are only around 40–50% (Balasubramanian et al. 2006; Pholdaeng and Pongsamart 2010; Sudheer et al. 2011; Vaseeharan et al. 2006), recombinant protein/DNA-based vaccines, which might have protection rates as high as 70–75%, are always treated with hesitancy associated with GMOs/GMFs (Witteveldt et al. 2004; Rout et al. 2007; Sutthangkul et al. 2017; Nguyen et al. 2014). Moreover, this study used natural melanin extracted from squid ink sacs, which is considered waste from seafood-processing factories. Therefore, utilizing this waste source to produce melanin would offer great benefits because of the low cost.

Based on the results of this study, we suggest that a protocol that simultaneously uses both melanin (as a supplement in feedstuffs) and plant extracts or vaccines be developed to create a synergistic effect on protecting shrimp against viral attacks. However, in farms, shrimp are always threatened with viral and bacterial infections. In the case of WSSV infection, it is very difficult to protect the shrimp pool when several individuals are infected. Therefore, farmers should periodically feed the shrimp melanin-containing feedstuff at appropriate intervals (depending on practical conditions) during the culturing period. Melanin-containing feedstuff is not expensive; thus, this protocol is likely to be cost-effective to farmers.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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