Aquaculture 430 (2014) 120–127

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

A plant-based heat shock protein inducing compound modulates host–pathogen interactions between *Artemia franciscana* and *Vibrio campbellii*

Yufeng Niu [a,b,1], Parisa Norouzitallab [a,1], Kartik Baruah [a], Shuanglin Dong [b], Peter Bossier [a,*]

[a] Laboratory of Aquaculture & Artemia Reference Center, Faculty of Bioscience Engineering, Ghent University, Rozier 44, Gent 9000, Belgium
[b] The Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, China

ARTICLE INFO

Article history:
Received 27 March 2014
Received in revised form 31 March 2014
Accepted 1 April 2014
Available online 12 April 2014

Keywords:
Artemia franciscana
Heat shock protein (Hsp) 70
Vibrio
Virulence factor
Tex-OE®
Heat shock protein inducing compound (Hpsi)

ABSTRACT

Induction of heat shock proteins (e.g. Hsp70) has been considered as a potential disease control and health management strategies in aquaculture. Recently, the compound Tex-OE®, a patented extract from the skin of the prickly pear fruit, *Opuntia ficus indica*, was reported to enhance the production of Hsp70 in fish and shrimp tissues without any adverse effect, protecting the animals against various abiotic stressors. Most of the studies on this compound primarily focused on the positive effects at the level of the host while the effects on the microorganisms in the culture environment were often overlooked. In our study, we aimed at evaluating the effect of this compound on the interaction of the host brine shrimp *Artemia franciscana* with pathogenic *Vibrio*. Pretreatment of axenically-grown *Artemia* with Tex-OE® conferred significant protection against *V. campbellii* in a conventional rearing system. However, continuous exposure of axennicly-grown *Artemia* to Tex-OE® in an open system negatively affected their survival. *In vitro* experiments provided unequivocal evidence that continuous exposure of Tex-OE® modulated the growth and virulence (such as hemolytic and caseinase activity) of *V. campbellii*. Pretreatment of *Vibrio* with the compound also increased their virulence in an *Artemia* challenge assay. Based on the overall results, it can be suggested that the compound Tex-OE® has the potential to be used as a disease control tool in (shrimp) aquaculture systems. However, the possibility that such kind of compounds also modulate the microbial activity cannot be ignored making the outcome dependent on the composition of the standing microbial community.

1. Introduction

According to reports by the United Nations' Food and Agriculture Organization (FAO), vibriosis – bacterial disease caused by vibrios – has been one of the most constraining factors for sustainable (shrimp) aquaculture, causing economic losses amounting to billions of dollars (Defoirdt et al., 2007; FAO, 2012). In intensive aquaculture systems, fishes and shrimps especially at their larval stages, are often exposed to a multitude of stressful conditions, which eventually make them more susceptible to microbial infections (Smith et al., 2003). The traditional application of antibiotics initially brought great help in combating vibriosis in aquaculture animals. However, the emergence of antibiotic-resistant bacteria due to indiscriminate use of antibiotics has made this traditional approach ineffective and unsustainable due to their potential risk to the surrounding environment as well as to the human health (Hoa et al., 2011). As an alternative to antibiotics, various anti-infective strategies, such as probiotics and immunostimulants, have been suggested and many are in the process of development. Experimental studies (including farm trials) have shown that they are effective in protecting aquaculture animals against bacterial infections (Merrifield et al., 2010; Roberts et al., 2010).

The molecular chaperone heat shock protein 70 (Hsp70) has been shown to control bacterial diseases, including vibriosis, in experimental aquaculture animals (Baruah et al., 2010, 2011; Sung et al., 2013). Hsp70 is a highly conserved protein which is expressed constitutively under normal physiological conditions, however, their expression is up-regulated by various stressors, such as cold, heat, UV radiation, toxins, pathogens, nutritional deficiency, protein degradation, hypoxia, acidosis, microbial damage or indeed any cellular stress (Roberts et al., 2010). It performs essential biological functions under normal and stressful physiological conditions and these functions include the folding of nascent polypeptides, assembly/disassembly of multi-subunit oligomers, translocation of proteins across intracellular membranes, regulation of apoptosis and cytoskeletal organization, repair of partially denatured proteins, degradation of irreversibly denatured proteins and inhibit protein aggregation, and other processes that enhance the survival of normal and diseased cells (Parsell and Lindquist, 1993). Previous studies in a wide variety of experimental animal models have

* Corresponding author. Tel.: +32 926 43754; fax: +32 926 44193.
E-mail address: Peter.Bossier@Ugent.be (P. Bossier).
[1] The first and second authors contributed equally to this work.

http://dx.doi.org/10.1016/j.aquaculture.2014.04.001
0044-8486/

reported that Hsp70 elicits protective immune responses against many (bacterial) diseases (Joly et al., 2010; Tsan and Gao, 2009). For instance, feeding of recombinant Hsp70 to the brine shrimp *A. franciscana* larvae (Baruah et al., 2011, 2013) or *in vivo* induction of Hsp70 within *Artemia* by pretreating the shrimp with the Hsp-inducing compound Tex-OE®, extracted from the skin of the prickly pear fruit, *Opuntia ficus indica* (Baruah et al., unpublished data), was shown to increase the prophenoloxidase innate immune response in the larvae and provide sequential protection against pathogenic vibrios (Baruah et al., 2011). Besides vibrios, this Hsp70-inducing compound was also shown to protect fish and shrimp against various abiotic stressors including lethal temperature, hypersalinity, ammonia toxicity and transportation stress (Baruah et al., 2012; Roberts et al., 2010; Sung et al., 2012). These findings suggest that Hsp70 or the compounds inducing Hsp70, pending thorough verification, could be used as a disease control agent in aquaculture. It is to be noted that most of the earlier studies on the Hsp70-inducing compound focused primarily on the positive effects at the level of the host. Only on rare occasions, the effects of these compounds on the microbial communities in the culture environment were described. On occasion, its effect on host-associated vibrios has been investigated. The impact of the Hsp70-inducing compound on the phenotypic characteristics (such as virulence factor production) of the pathogen and on microbial–host interaction is often overlooked. In this study, we aimed to evaluate (i) whether the Hsp70-inducing compound Tex-OE® affects the *Artemia* host in inducing resistance to *V. campbellii* in a conventional rearing system and (ii) the impact of this compound on the pathogen response, focusing mainly on their growth, and production of Hsp70 (DnaK) and virulence factors.

2. Materials and methods

2.1. Bacteria strains and culture conditions

Two bacterial strains were used in this study. The pathogenic strain *V. campbellii* LMG21363 (Defoirdt et al., 2006; Gomez-Gil et al., 2004; Soto-Rodriguez et al., 2003) was used for *Artemia* challenge assays and for determining the effect of Hsp70-inducing compound on the *Vibrio* response. *Aeromonas* sp. strain LVS3 (Verschuere et al., 1999; Marques et al., 2005), after autoclaving, was used as feed for *Artemia*. All strains were preserved at −80 °C in Marine Broth 2216 (Difco Laboratories, Detroit, MI, USA) with 20% sterile glycerol. The bacterial strains, LMG 21363 and LVS3 were initially grown at 28 °C for 24 h on Marine Agar (Difco Laboratories, Detroit, MI. USA) and then to log phase in Marine Broth by incubation at 28 °C with continuous shaking prior to use. Bacterial cell numbers were determined spectrophotometrically at 550 nm according to the McFarland standard (BioMerieux, Marcy L'Etoile, France), assuming that an OD of 1.000 corresponding to 1.2×10^9 cells ml^{-1}.

2.2. Artemia rearing water

The rearing water for *Artemia* used in this experiment was obtained from a whiteleg shrimp *Penaeus vannamei* recirculating culture system. The water quality parameters were analyzed using standard protocols and were found within the optimum range for *P. vannamei* rearing.

2.3. Axenic Artemia as model organism

Axenic *Artemia* were obtained following decapsulation and hatching (Baruah et al., 2010). Briefly, 2.5 g of *Artemia* cysts originating from the Great Salt Lake, Utah, USA (EG Type, batch 21452, INVE Aquaculture, Dendermonde, Belgium) were hydrated in 89 ml of distilled water for 1 h. Sterile cysts and nauplii were obtained via decapsulation using 3.3 ml NaOH (32%) and 50 ml NaOCl (50%). During the reaction, 0.22 μm filtered aeration was provided. All manipulations were carried out under a laminar flow hood and all tools were autoclaved at 121 °C for 20 min. The decapsulation was stopped after about 2 min by adding 50 ml $Na_2S_2O_3$ at 10 g l^{-1}. The aeration was then terminated and the decapsulated cysts were washed with filtered (0.2 μm) and autoclaved artificial seawater (FASW) containing 35 g 1 l^{-1} of instant ocean synthetic sea salt (Aquarium Systems, Sarrebourg, France). The cysts were suspended in 1–l glass bottles containing FASW and incubated for 28 h on a rotor at 4 rpm at 28 °C with constant illumination of approximately 27 μE m^{-2} sec^{-1}. The emerged nauplii reaching stage II, at which time they start ingesting bacteria, were collected.

2.4. In vivo treatment of Artemia with Hsp-inducing compound

The compound Tex-OE® (hereinafter referred to as 'T-Hspi' for Tex-OE®-based Hsp-inducing compound), supported in food grade ethanol, was kindly provided by Bradan Ltd. Campbeltown. It was stored at room temperature until use.

Two species of the brine shrimp larvae, *Artemia franciscana* and *Artemia* Megalon Embolon, were used as testing organisms. In total, three separate studies were performed. In the first study, the protective effect of T-Hspi on *Artemia* against *V. campbellii* in the conventional rearing system was evaluated. Two different strategies for administration of T-Hspi were followed: pretreatment and continuous exposure. In the pretreatment strategy, the *Artemia* were initially treated with T-Hspi (20, 40, 80, 160 μl l^{-1}) or with absolute ethanol (160 μl l^{-1}) alone as negative control in sterile sea water for 1 h. Then the pretreated *Artemia* were rinsed repeatedly with sterile sea water, allowed to recover in sterile sea water for 1 h, transferred to shrimp rearing water and then challenged with *V. campbellii* at 10^7 cells ml^{-1} for 36 h. While in the continuous exposure strategy, the *Artemia* were treated continuously (meaning that the T-Hspi compound was not washed away) with the same concentration of T-Hspi or absolute ethanol as in the pretreatment strategy in the shrimp rearing water. In addition, the *Artemia* were challenged with *V. campbellii* at 10^7 cells ml^{-1}. For both the administration strategies, controls were maintained without the addition of T-Hspi or ethanol. Dead LVS3 at 10^7 cells ml^{-1} were fed to the *Artemia* of all groups. The survival of the *Artemia* was scored 36 h after challenge.

The second study aimed to determine the toxicity of T-Hspi. To this end, *Artemia* were distributed in the shrimp rearing water and then T-Hspi was added at increasing concentrations (20, 40, 80 and 160 μl l^{-1}). *Artemia* that received absolute ethanol (160 μl l^{-1}) alone served as (negative) control. No *Vibrio* challenge was performed. The survival of the *Artemia* was scored 36 h after addition of the compound.

In the third experiment, the impact of T-Hspi on the phenotypic characteristics of *V. campbellii* was determined by carrying out *in vivo* and *in vitro* (see sections below) studies. In the in *vivo* study, the *Artemia* hatched under gnotobiotic conditions were challenged with *V. campbellii* (10^7 cells ml^{-1}) that were previously treated with different concentrations of T-Hspi (20, 40, 80 and 160 μl l^{-1}) or with absolute ethanol (160 μl l^{-1}) alone for 6 h. The *Vibrio* that received no T-Hspi or ethanol was kept as control. Dead LVS3 at 10^7 cells ml^{-1} were fed to the *Artemia* of all groups. The survival of the *Artemia* was scored 36 h after challenge.

In all the above three studies, the final ethanol concentration (160 μl l^{-1}) in the control and T-Hspi-treated groups corresponded to the amount added in the treatment with highest Hspi concentration.

2.5. Effect of T-Hspi on the growth and bioluminescence of V. campbellii in Marine Broth medium

Aliquots of the overnight grown *V. campbellii* were transferred to three separate flasks containing 5 ml of fresh Marine Broth to obtain an optical density at 550 nm (OD_{550}) of approximately 0.1. In one flask containing the *Vibrio* culture, an optimized dose of T-Hspi (160 μl l^{-1}) was added. In the other two flasks, which were used as control groups, *Vibrio* were grown in Marine Broth added with absolute

ethanol (160 μl l^{-1}) or not. All the cultures were incubated at 28 °C on an orbital shaker at 20 rev min^{-1}. *Vibrio* samples were taken from each flask, each in triplicates, at every 2 h interval for determination of growth, by measuring OD_{550} with a Tecan Infinite 200 micro plate reader (Tecan, Belgium). From the same sample, *V. campbellii* luminescence was simultaneously measured as described previously (Defoirdt and Sorgeloos, 2012).

2.6. Effect of T-Hspi on the growth of total heterotrophic bacteria in the shrimp rearing water and V. campbellii in sterile sea water

Shrimp rearing water was inoculated with 160 μl l^{-1} of T-Hspi or absolute ethanol. Total bacterial growth was checked at 0, 4, 8, 12 and 16 h after T-Hspi or ethanol addition by plating the water samples on Marine Agar. The shrimp water was diluted with a 10-fold dilution series and 2 dilution series were used for plating with 3 replicates for each dilution.

Overnight grown *V. campbellii* was diluted to an OD_{550} of approximately 0.1 with Marine Broth. The bacteria were added to 30 ml of FASW at 10^7 cells ml^{-1} concentration. Simultaneously, T-Hspi or absolute ethanol at 160 μl l^{-1} was added and the culture was incubated at 28 °C. The growth of *V. campbellii* was checked at regular intervals in a similar fashion as described above for total heterotrophic bacteria.

2.7. Virulence factor assay

2.7.1. Lipase and phospholipase assays

The lipase and phospholipase activities were measured by a modification of the method described by Liuxy et al. (1996) and Austin et al. (2005), respectively. Agar plates for lipase and phospholipase assays were prepared by supplementing Marine Agar with 1% Tween 80 (Sigma-Aldrich, Belgium) and 1% egg yolk emulsion (Sigma-Aldrich, Belgium), each sterilized separately at 121 °C for 5 min prior to mixing. The development of opalescent zones around the colonies was observed and the diameter of the zones was measured after 2–4 days of incubation at 28 °C.

2.7.2. Caseinase, gelatinase and hemolysin assays

The caseinase, gelatinase and hemolysin assays were performed as described previously (Austin et al., 2005; Liuxy et al., 1996; Zhang and Austin, 2000) with some modifications. The caseinase assay plates were prepared by mixing double strength Marine Agar with a 4% skim milk powder suspension (Oxoid, Hampshire, UK), each sterilized separately at 121 °C for 5 min. Clearing zones surrounding the bacterial colonies were measured after 2 days of incubation. Gelatinase assay plates were prepared by mixing 0.5% gelatin (Sigma-Aldrich, Belgium) with Marine Agar. After incubation for 7 days, saturated ammonium sulfate (80%) was poured over the plates and after 2 min, the diameters of the clearing zones around the colonies were measured. Hemolytic assay plates were prepared by supplementing Marine Agar with 5% defibrinated sheep blood (Oxoid, Hampshire, UK) and clearing zones were measured after 2 days of incubation.

2.8. Analysis of DnaK production

V. campbellii was initially grown at 28 °C for 24 h on Marine Agar and then to log phase in Marine Broth. The culture medium was supplemented with an increasing concentration of T-Hspi (20, 40, 80 or 160 μl l^{-1}) or with 160 μl l^{-1} of absolute ethanol, followed by incubation at 28 °C for 6 h. The culture medium without supplement was used as a control. *V. campbellii* culture (1 ml) from each treatment was collected and centrifuged at 4000 ×*g* for 15 min. The pellets obtained were rinsed once with FASW and then homogenized by rapid agitation with 0.1 mm diameter glass beads in cold buffer K (150 mM sorbitol, 70 mM potassium gluconate, 5 mM $MgCl_2$, 5 mM NaH_2PO_4, 40 mM HEPES, pH 7.4) containing protease inhibitor cocktail (Sigma-Aldrich, Inc. USA). Subsequent to centrifugation at 2200 ×*g* for 1 min at 4 °C, supernatant protein concentrations were determined by the Bradford method (Bradford, 1976). Supernatants were then combined with loading buffer, vortexed, heated at 95 °C for 5 min, centrifuged at 4000 ×*g* for 1 min and then electrophoresed in 10% SDS-PAGE gels, with each lane receiving equivalent amounts of protein. Gels were transferred to polyvinylidene fluoride membrane (BioRad Immun-BlotTM PVDF) for antibody probing. Membrane was incubated with blocking buffer [50 ml of 1× phosphate buffered saline containing 0.2% (v/v) Tween-20 and 5% (w/v) bovine serum albumin] for 1 h at room temperature and then with rabbit polyclonal antibody raised against the ATPase domain of *E. coli* DnaK (a generous gift from Dr. Bernd Bukau, ZMBH) at the recommended dilution of 1:2500. Horseradish peroxidase conjugated goat anti-rabbit IgG was used as the secondary antibody at the recommended dilution of 1:5000. Detection was done with 0.7 mM diaminobenzidine tetrahydrochloride dihydrate (DAB) in association with 0.01% (v/v) H_2O_2 in 0.1 m Tris–HCl (pH 7.6).

2.9. Statistical analysis

Survival data were arcsin transformed to satisfy normality and homocedasticity requirements as necessary. Data on survival, virulence factor and bacterial growth were then subjected to one-way analysis of variances followed by Duncan's multiple range tests using the statistical software Statistical Package for the Social Sciences version 14.0 to determine significant differences among treatments. Significance level was set at $p < 0.05$.

3. Result

3.1. Test 1: Effect of T-Hspi pretreatment on the survival of Artemia reared in a conventional system and challenged with V. campbellii

To determine the pretreatment effect of T-Hspi on the protection of *Vibrio*-challenged *Artemia* in a conventional (shrimp rearing water) system, the *Artemia* were pretreated with different doses of T-Hspi and then challenged with *V. campbellii.* As shown in Fig. 1, *A. franciscana* larvae pretreated with T-Hspi at concentrations ranging between 20 and 80 μl l^{-1} did not exhibit significant ($p > 0.05$) improvement in the survival compared with the ethanol control. However, at a dose of 160 μl l^{-1}, a significant increase in the survival of the *Artemia* compared with the controls was observed. In case of the *Artemia* strain Megalon Embolon, a significant improvement in the survival of the *Vibrio*-challenged *Artemia* compared with the (ethanol) control was noted already at the low dose of T-Hspi (20 μl l^{-1}). Further increase in the T-Hspi dose (>20 μl l^{-1}) did not further increase the larval survival compared with the group pretreated with 20 μl l^{-1} T-Hspi, however, the survival was significantly higher than the controls.

3.2. Test 2: effect of continuous exposure of T-Hspi on the survival of Artemia reared in a conventional system and challenged with V. campbellii

Next, the possibility that continuous exposure of T-Hspi protects the *Artemia* against *V. campbellii* was verified using the same doses of T-Hspi as in the test described above. Continuous exposure of the *Artemia* to T-Hspi at concentration between 20 and 160 μl l^{-1} did not confer protection to the *Vibrio*-challenged *Artemia* (Fig. 2). In fact, at doses between 20 and 80 μl l^{-1}, T-Hspi had a negative effect on the survival of the *Artemia*.

3.3. Test 3: toxicity of T-Hspi on the Artemia

To determine if continuous exposure to T-Hspi has a toxic effect on *Artemia*, the larvae were incubated continuously with an increasing concentration of T-Hspi in the shrimp rearing water as described in test 1. No additional *Vibrio* challenge was performed. T-Hspi appeared to have no adverse impact on the survival of the *Artemia*, indicating

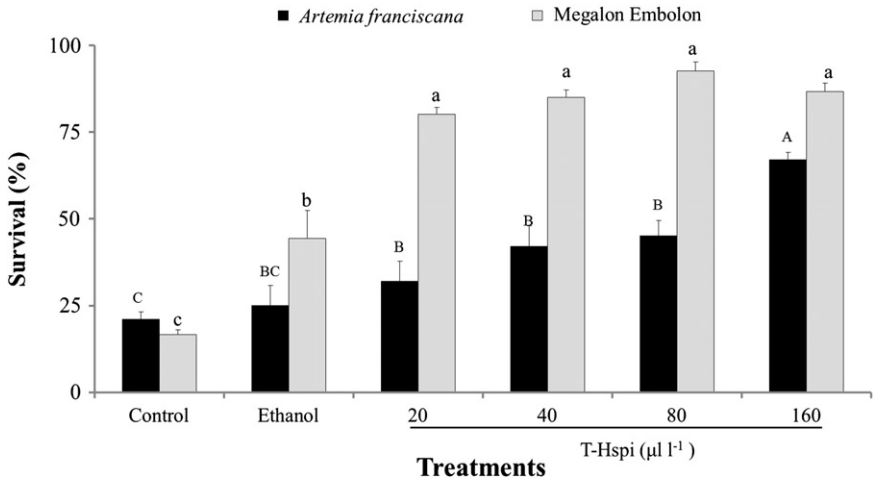

Fig. 1. Survival (%) of *A. franciscana and Artemia* Megalon Embolon 36 h after challenge with *V. campbellii*. Axenic *Artemia* were pretreated with different doses of T-Hspi (20, 40, 80 and 160 µl l^{-1}) or with 160 µl l^{-1} absolute ethanol (as one of the controls) for 1 h. They are then rinsed repeatedly with sterile seawater and allowed to recover for 1 h. *Artemia* not pretreated with T-Hspi and/or ethanol served as another control. The *Artemia* were then challenged with *V. campbellii* at 10^7 cells ml^{-1} in shrimp rearing water. Dead LVS3 (10^7 cells ml^{-1}) were provided as feed for the *Artemia*. Data in each survival test represent the mean ± standard error of five replicates. Error bars with different alphabet letters (capital and small letters for *A. franciscana* and Megalon Embolon, respectively) indicate significant difference ($p < 0.05$).

that it is non-toxic to the *Artemia* among the doses tested (data not shown).

3.4. Microbial responses to T-Hspi treatment

3.4.1. Effect of T-Hspi on the growth and bioluminescence of V. campbellii in Marine Broth medium

Next, we investigated the direct impact of T-Hspi incubation on the growth and bioluminescence of *V. campbellii* in the Marine Broth medium. To this end, *V. campbellii* culture was incubated with the optimal dose of T-Hspi (160 µl l^{-1}) for a period of 16 h. T-Hspi exposure had no significant impact on the growth and bioluminescence of *V. campbellii* (data not shown).

3.4.2. Effect of T-Hspi on the growth of V. campbellii in sterile sea water and of heterotrophic bacteria in shrimp rearing water

In the following experiment, *V. campbellii* was incubated with T-Hspi or absolute ethanol at a concentration of 160 µl l^{-1} in sterile sea water. In addition to this, shrimp rearing water was inoculated with T-Hspi or absolute ethanol at 160 µl l^{-1} and then the growth of *V. campbellii* and total heterotrophic bacteria was measured at specific time interval by plating the culture water on Marine Agar. As shown in the figures, incubation of the bacteria with T-Hspi significantly stimulated the growth of *V. campbellii* in sterile sea water (Fig. 3) and of heterotrophic bacteria in shrimp rearing water (Fig. 4).

3.4.3. Impact of T-Hspi on in vitro production of virulence factors in V. campbellii

To determine the effect of T-Hspi on the production of virulence factors in *V. campbellii*, an *in vitro* study was carried out by incubating *V. campbellii* with different doses of T-Hspi for a period of 6 h in Marine Broth medium, followed by plating the culture on specific agar. To continuously incubate the *V. campbellii* with T-Hspi, the same concentrations of T-Hspi were also added into the agar. As shown in Fig. 5, the caseinase and hemolytic activities of *V. campbellii* incubated with T-Hspi at concentrations ranging between 20 and 80 µl l^{-1} were not significantly different from that of the (ethanol) control. However, a significant increase in the activity of caseinase (Fig. 5A) and hemolysin (Fig. 5C) was recorded due to exposure of *V. campbellii* to a higher dose of T-Hspi (160 µl l^{-1}) compared to the control and ethanol control groups. Conversely, the lipase activity was significantly decreased at the highest dose (160 µl l^{-1}) of T-Hspi treatment (Fig. 5B). No significant

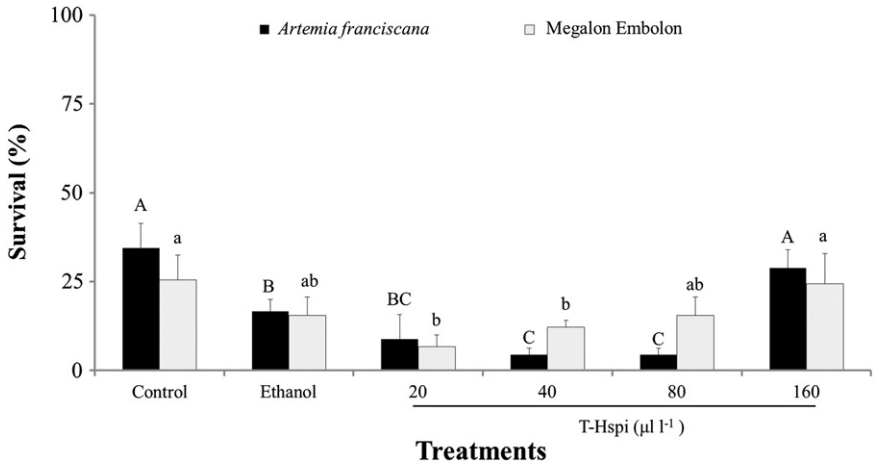

Fig. 2. Survival (%) of *A. franciscana and Artemia* Megalon Embolon 36 h after challenge with *V. campbellii*. Axenic *Artemia* were incubated with either T-Hspi (20, 40, 80 and 160 µl l^{-1}) or with 160 µl l^{-1} of absolute ethanol (as one of the controls) for 1 h. *Artemia* not incubated with T-Hspi and/or ethanol served as another control. The *Artemia* were then challenged with *V. campbellii* at 10^7 cells ml^{-1} in shrimp rearing water. Dead LVS3 (10^7 cells ml^{-1}) were provided as feed for the *Artemia*. Data in each survival test represent the mean ± standard error of five replicates. Error bars with different alphabet letters (capital and small letters for *A. franciscana* and Megalon Embolon, respectively) indicate significant difference ($p < 0.05$).

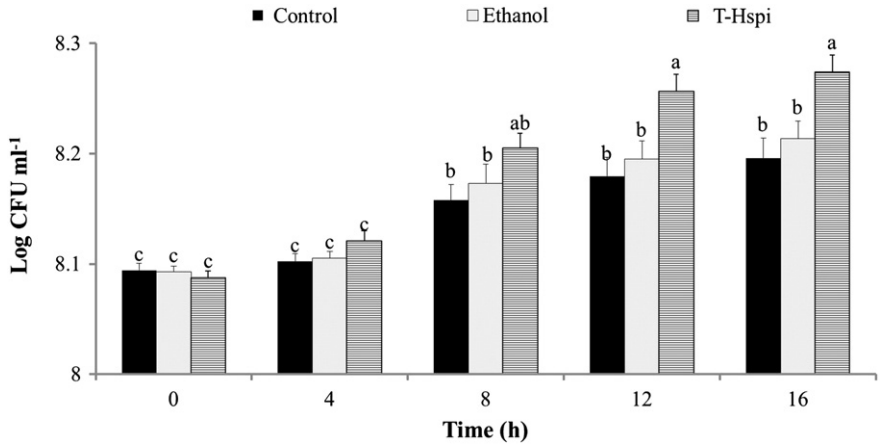

Fig. 3. Growth of *V. campbellii* in sterile sea water with or without T-Hspi or absolute ethanol, added at a concentration of 160 μl l^{-1}. Results are expressed as mean ± standard error of three replicates. Error bars with different alphabet letters indicate significant difference ($p < 0.05$).

differences in the activity of gelatinase and phospholipase of *V. campbellii* due to T-Hspi exposure were observed (Fig. 5 D and E).

3.4.4. Induction of DnaK by T-Hspi in V. campbellii

To further study the response of *V. campbellii* to T-Hspi, we investigated the induction of DnaK in *V. campbellii in vitro* by carrying out Western Blot analysis on the protein extracts from the *V. campbellii* cultured for 6 h in the presence of different doses of T-Hspi. As shown in Fig. 6, there was a constitutive production of DnaK in the control and the production was almost the same to that in the ethanol-treated control group. However, T-Hspi treatment at a concentration as low as 20 μl l^{-1} markedly increased DnaK production compared with the controls. The maximal effect was observed at a dose of 160 μl l^{-1}.

3.5. Virulence of T-Hspi-pretreated V. campbellii towards Artemia

To further verify the effect of T-Hspi on the virulence of *V. campbellii*, we next determined the *in vivo* effect of T-Hspi-pretreated *V. campbellii* on the survival of *Artemia*. As illustrated in Fig. 7, pretreatment of *V. campbellii* with T-Hspi at a dose ranging from 20 to 80 μl l^{-1} did not significantly affect the survival of the *Artemia*. However, at the dose of 160 μl l^{-1}, T-Hspi-pretreated *V. campbellii* significantly reduced the survival of both *Artemia franciscana* and Megalon embolon compared to the controls.

4. Discussion

Artificial manipulation of Hsp70 levels within an organism has been demonstrated to be a promising approach for combating a variety of abiotic and pathogenic biotic stressors in cultured fishes and shrimps (Baruah et al., 2011; Roberts et al., 2010; Sung et al., 2007, 2012). Despite the success of Hsp70 in the treatment of diseases in experimental aquaculture animals to date, the development of a non-invasive approach to induce Hsps within the host or to deliver Hsps into the animals (in farm levels) remains an impediment. In our previous study, we tested a (patented) plant-based compound Tex-OE® (referred to as T-Hspi) for its Hsp70-inducing property and protective effects against abiotic stressors using the model organism *A. franciscana* in a germ-free system. We showed explicitly that T-Hspi pretreatment induced endogenous Hsp70 within axenically-reared *Artemia* and markedly protected the shrimp against abiotic stressor — lethal heat shock or salinity stress (Baruah et al., 2012). Further study carried out to validate our above findings exhibited that T-Hspi conferred similar Hsp70-mediated protection to common carp *Cyprinus carpio* against acute ammonia toxicity in a conventional rearing system (Sung et al., 2012). In our present study, we extended the work of our previous study (Baruah et al., 2012) with the aim of investigating the effect of T-Hspi on the interaction of host–bacteria in a conventional rearing system. Here, by using two strains of *Artemia* (larvae) as model organisms, we clearly demonstrated that T-Hspi pretreatment (of axenic *Artemia*) significantly protected the *Artemia* against subsequent challenge by pathogenic

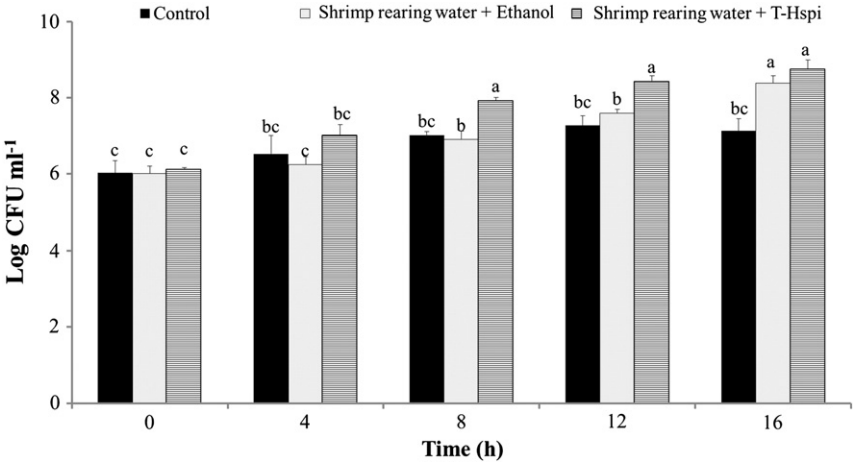

Fig. 4. Growth of total heterotrophic bacteria in shrimp rearing water with or without T-Hspi or absolute ethanol, added at a concentration of 160 μl l^{-1}. Results are expressed as mean ± standard error of three replicates. Error bars with different alphabet letters indicate significant difference ($p < 0.05$).

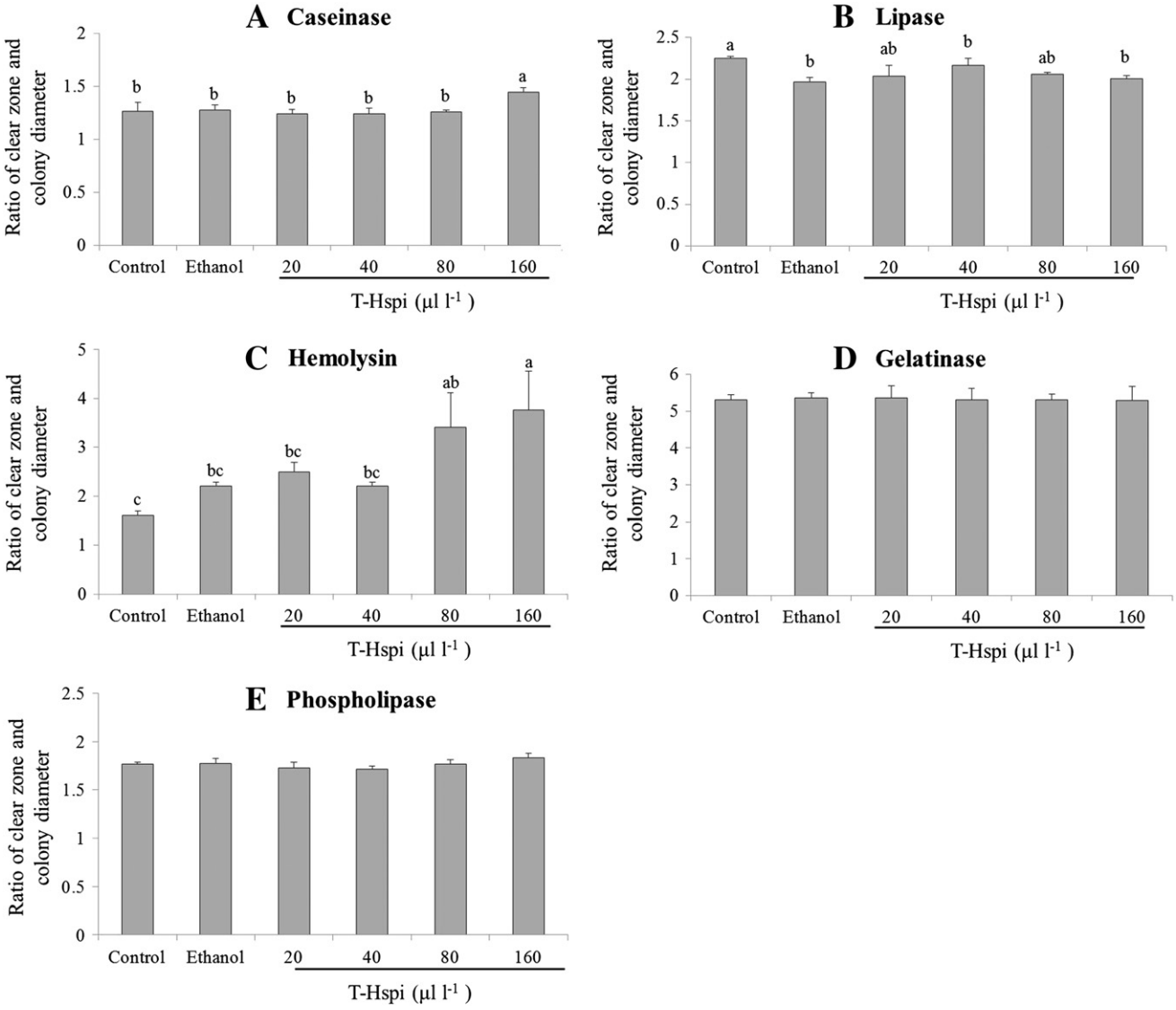

Fig. 5. Activities of (A) caseinase, (B) lipase, (C) hemolysin, (D) gelatinase, and (E) phospholipase of *V. campbellii* incubated with T-Hspi for 48 h. The activities of the virulent determinants were expressed as the ratio of clear zone (mm) and colony diameter (mm). Results are expressed as mean ± standard error of three replicates. Error bars with different alphabet letters indicate significant difference ($p < 0.05$).

V. campbellii in a conventional (i.e., shrimp rearing water) system. This finding is in agreement with previous studies in which pre-stimulation of fishes, salmon and gilthead sea bream, with T-Hspi were reported to provide protection against *V. anguillarum* infection to both fishes (El Fituri, 2009; Roberts et al., 2010). However, when the delivery strategy of T-Hspi was switched from pretreatment exposure to continuous exposure, no protective effect of T-Hspi against *V. campbellii* was observed. On the contrary, an adverse effect of T-Hspi was observed. These observations could be explained by the fact that T-Hspi at the tested doses might have an (direct) effect on the standing microbial communities, including *V. campbellii,* in the rearing water. For instance, it might have increased the growth of bacteria and/or stimulated virulence factors production, which in turn might have negatively interfered with the performance of *Artemia*.

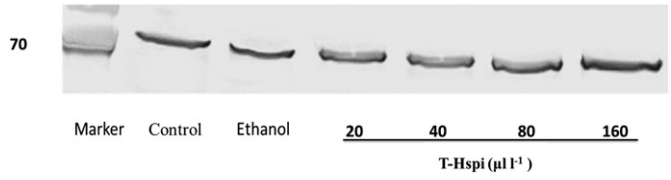

Fig. 6. Induction of DnaK in *V. campbellii* by T-Hspi. *V. campbellii* were incubated with different doses of T-Hspi (20, 40, 80 or 160 μl l^{-1}) or with absolute ethanol alone (160 μl l^{-1}) as ethanol control (ethanol) for 6 h. *V. campbellii* not pretreated with T-Hspi and/or ethanol served as control. Thirty microgram of bacterial protein was loaded in each lane. Molecular mass standards (M) in kilodaltons on the left.

In a further series of experiments, we investigated the impact of T-Hspi on the pathogen responses in terms of growth and virulence factors production. T-Hspi appeared to have (to a limited extent) a growth stimulating effect on *V. campbellii* in sterile sea water and also on the heterotrophic bacteria in the shrimp rearing water, suggesting that the T-Hspi compound might have been utilized by the microbes to proliferate. One may also argue that the ethanol content and not the T-Hspi compound in the plant extract might have contributed to the growth of bacteria in the culture water. In our study, the growth of heterotrophic bacteria appeared to be higher in the T-Hspi-treated group than that in the ethanol-treated group at most of the time points tested. These results indicated that the higher and faster bacterial growth was mainly associated with the active T-Hspi compound in the plant-based extract. However, as observed in this study, no adverse effect was observed on the non-challenged *Artemia* continuously exposed to T-

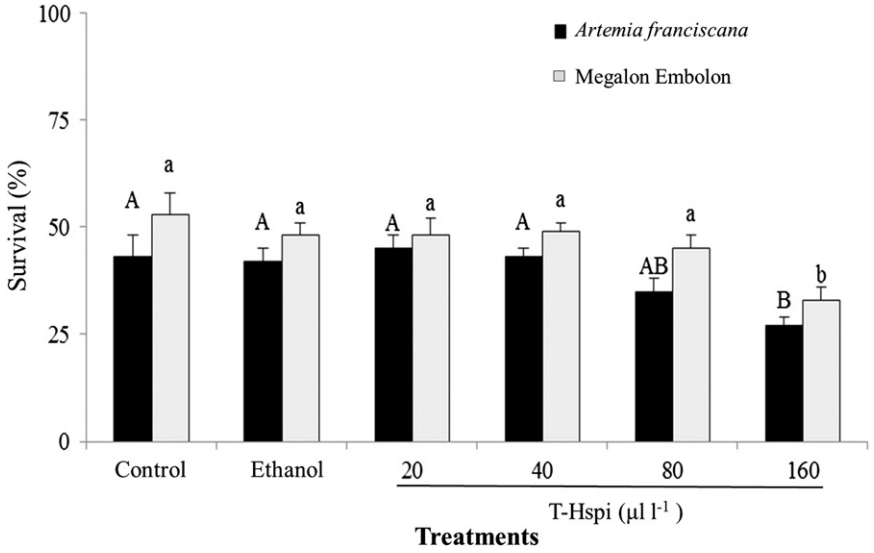

Fig. 7. Survival (%) of *A. franciscana* and Megalon Embolon 36 h after challenge with T-Hspi-pretreated *V. campbellii*. *Artemia* were challenged with *V. campbellii* (10^7 cells ml^{-1}) that were pretreated with different concentrations of T-Hspi (20, 40, 80 or 160 μl l^{-1}). The larvae challenged with untreated *V. campbellii* or those challenged with ethanol-treated *V. campbellii* served as controls. The nauplii were fed with autoclaved LVS3 at 10^7 cells ml^{-1}. Results are expressed as the mean of five replicates ± standard errors. Error bars with different alphabet letters indicate significant difference ($p < 0.05$).

Hpi in the shrimp rearing water (Test 3, data not shown). Thus, the higher bacterial load in the rearing water due to T-Hspi may not be the primary cause for the ineffectiveness of T-Hspi treatment. Alternatively, T-Hspi might affect V*ibrio* activity, negatively affecting *Artemia*.

To substantiate these assumptions, we investigated the effect of T-Hspi on the production of virulence factors (such as caseinase, lipase, hemolysin, gelatinase and phospholipase) in *V. campbellii*. Caseinase and gelatinase are a group of proteases that are able to degrade casein and gelatin, respectively, and the production of these proteases facilitates the infection of pathogens by damaging the host tissue (Ruwandeepika et al., 2012). Previous studies have shown that the production of proteases by vibrios was linked to pathogenesis towards fish and shrimp (Kahla-Nakbi et al., 2009; Rui et al., 2009). Consistent with this, we observed a significant increase in the activity of caseinase (while the activity of gelatinase was not affected) of *V. campbellii* due to continuous exposure to T-Hspi. Besides having an effect on caseinase activity, T-Hspi also markedly enhanced the production of hemolysin in *V. campbellii*. Hemolysin is arguably the most important and widely distributed virulence factor among pathogenic vibrios (Wong et al., 2012). There is ample evidence that hemolysin is toxic to erythrocytes and other cell types as well (Rowe and Welch, 1994) and that this toxin infects the host cells either by forming pores or by breaking down cell membranes (Sun et al., 2007). It is also reported to be involved in pathogenesis of aquatic animals due to the anemic response that has been observed in infected fish (Clauss et al., 2008; Zhang et al., 2001). In addition to the impact on the virulence factors mentioned above, there was also a decrease in the activity of lipase, another putative virulence factor, in *V. campbellii* due to continuous exposure to T-Hspi. However, the role of lipases in the pathogenesis of vibrios towards aquatic animals is unclear yet (Ruwandeepika et al., 2012). Based on the observations made in this study, it can be suggested that the decrease in the survival of *Vibrio*-challenged *Artemia* continuously exposed to T-Hspi is associated with the interference of T-Hspi with production of the virulence determinants in *V. campbellii*, particularly, enhancing the activity of caseinase and hemolysin. The increment of these lytic enzymes may contribute to inflicting host tissues damages, allowing the pathogen to obtain nutrients and to spread through the tissues. In our study, only few virulence factors, such as lytic enzymes, of *V. campbellii* were investigated. It is possible that together with caseinase and hemolysin, production of other virulence factors due to continuous T-Hspi exposure might contribute to the pathogenesis of *V. campbellii* and subsequently lower the survival of *Vibrio*-challenged *Artemia*.

A previous study reported that T-Hspi induces Hsp70 production in *Artemia* (Baruah et al., 2012). In our study, we demonstrated that T-Hspi induces Hsp70 in *V. campbellii* (commonly known as DnaK) as well. There are evidences showing that when induced by environmental stressors, Hsp70 are involved in the homeostasis and biogenesis of protein, thereby protecting cells from harmful environmental stressors (Guisbert and Morimoto, 2013; Parsell and Lindquist, 1993). Accordingly, the induced DnaK, as observed in our study, might have contributed to the robustness of *V. campbellii*, resulting in stronger tolerance of the pathogen towards the arsenals of the host defense system. Several lines of studies have reported that Hsps also play an important role in regulating the virulence of pathogens (Chakrabarti et al., 1999; Gophna and Ron, 2003). For instance, Hoffman and Garduno (1999) reported that surface associated Hsp60 and Hsp70 of an ulcer-causing bacterium, *Helicobacter pylori*, mediate attachment to gastric epithelial cells. The increased expression of these Hsps following acid shock correlates with both increased bacterial adhesion and inflammation of the gastric mucosa. Yamaguchi et al. (1997) also showed that Hsp60 mediates the adhesion of *H. pylori* to human gastric epithelial cells. In addition, Chakrabarti et al. (1999) also suggested that DnaK production may interfere with virulence factor production of *V. cholera*. Based on these compendiums of evidences, it can be suggested that bacterial Hsp70 (DnaK) induced by continuous exposure of *V. campbellii* to T-Hspi may lead to a higher stress tolerance and virulence of *V. campbellii* rendering the *Artemia* more susceptible to infection.

Having seen that T-Hspi induced the expression of virulent determinants in *V. campbellii in vitro*, we next carried out an *in vivo* survival assay to confirm the impact of T-Hspi on the virulence of *V. campbellii*. Interestingly, our results showed that *V. campbellii* pretreated with T-Hspi (160 μl l^{-1}) led to a significantly higher mortality of *Artemia* compared to *Artemia* challenged with non-treated *V. campbellii*. One may argue that the higher dose of T-Hspi may lead to faster *V. campbellii* growth and result in higher mortality. However, it should be noted that all the *Artemia* were challenged with the same dose of *V. campbellii*, thus, the higher mortality is more likely to be associated with an enhanced virulence and robustness of *V. campbellii*.

In conclusion, the overall results provide strong evidence that administration of the T-Hspi compound by pretreatment of (axenic)

Artemia confers significant protection against *V. campbellii* in a subsequent conventional rearing system. In contrast, continuous and simultaneous exposure to this compound appeared to have a detrimental effect on *Artemia*, probably due to the enhanced virulence of certain pathogens or opportunists in the rearing system. Thus, when applying this compound for disease control or for stress mitigation purpose, its interaction with the microbiota in the fish/shrimp culture system needs to be considered. To lower the risk of stimulating pathogenic bacteria in aquaculture system or their activity, the rearing water is recommended to be disinfected prior to the addition of such compound. To our knowledge, this is the first experimental evidence indicating that bio-treatments, such as the application of compound inducing Hsps interacts with the microbes in the rearing system and negatively affects the welfare of the animal. Further research should focus on investigating how such type of compounds, affect microbiota in the rearing environment or those associated with the host. It can be stated that an Hspi compound may affect the host but also the standing associated microbial community, modulating the effectiveness of such treatment.

Acknowledgments

The authors acknowledge financial support from Ghent University, Belgium (PhD-BOF scholarship to Niu Yufeng, BOF12/DC1/013; 01DI1312 and Parisa Norouzitallab, BOF10/DOS/031; 01W03110), GOA project entitled 'Host microbial interaction in aquatic production' (project number: 01G02212) and the Belgian Science Policy Office (Belspo) project entitled 'AquaStress', project number: IUAPVII/64/ Sorgeloos. Dr. Kartik Baruah is a postdoctoral fellow of Research Foundation Flanders (FWO), Belgium.

References

Austin, B., Austin, D., Sutherland, R., Thompson, F., Swings, J., 2005. Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia* nauplii. Environ. Microbiol. 7, 1488–1495.

Baruah, K., Ranjan, J., Sorgeloos, P., Bossier, P., 2010. Efficacy of heterologous and homologous heat shock protein 70s as protective agents to *Artemia franciscana* challenged with *Vibrio campbellii*. Fish Shellfish Immunol. 29, 733–739.

Baruah, K., Ranjan, J., Sorgeloos, P., Macrae, T.H., Bossier, P., 2011. Priming the prophenoloxidase system of *Artemia franciscana* by heat shock proteins protects against *Vibrio campbellii* challenge. Fish Shellfish Immunol. 31, 134–141.

Baruah, K., Norouzitallab, P., Roberts, R.J., Sorgeloos, P., Bossier, P., 2012. A novel heat-shock protein inducer triggers heat shock protein 70 production and protects *Artemia franciscana* nauplii against abiotic stressors. Aquaculture 334–337, 152–158.

Baruah, K., Norouzitallab, P., Shihao, L., Sorgeloos, P., Bossier, P., 2013. Feeding truncated heat shock protein 70s protect *Artemia franciscana* against virulent *Vibrio campbellii* challenge. Fish Shellfish Immunol. 34, 83–191.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Chakrabarti, S., Sengupta, N., Chowdhury, R., 1999. Role of DnaK in in vitro and in vivo expression of virulence factors of *Vibrio cholerae*. Infect. Immun. 67, 1025–1033.

Clauss, T.M., Dove, A.D., Arnold, J.E., 2008. Hematologic disorders of fish. Vet. Clin. N. Am. Exotic Anim. Pract. 11, 445–462.

Defoirdt, T., Sorgeloos, P., 2012. Monitoring of *Vibrio harveyi* quorum sensing activity in real time during infection of brine shrimp larvae. ISME J. 6, 2314–2319.

Defoirdt, T., Halet, D., Sorgeloos, P., Bossier, P., Verstraete, W., 2006. Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. Aquaculture 261, 804–808.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2007. Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends Biotechnol. 25, 472–479.

El Fituri, A.A., 2009. The Possible Role of TEX-OE in the Pathogenesis of Vibriosis. (MSc thesis) University of Malta p. 97.

FAO, 2012. State of World Fisheries and Aquaculture. FAO Fisheries Department, Food and Agriculture Organisation of the United Nations, Rome, Italy.

Gomez-Gil, B., Soto-Rodriguez, S., García-Gasca, A., Roque, A., Vazquez-Juarez, R., Thompson, F.L., Swings, J., 2004. Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. Microbiology 150, 1769–1777.

Gophna, U., Ron, E.Z., 2003. Virulence and the heat shock response. Int. J. Med. Microbiol. 292, 453–461.

Guisbert, E., Morimoto, R.I., 2013. The Regulation and Function of the Heat Shock Response. Springer, pp. 1–18.

Hoa, P.T.P., Managaki, S., Nakada, N., Takada, H., Shimizu, A., Anh, D.H., Viet, P.H., Suzuki, S., 2011. Antibiotic contamination and occurrence of antibiotic-resistant bacteria in aquatic environments of northern Vietnam. Sci. Total Environ. 409, 2894–2901.

Hoffman, P., Garduno, R., 1999. Surface-associated heat shock proteins of *Legionella pneumophila* and *Helicobacter pylori*: roles in pathogenesis and immunity. Infect. Dis. Obstet. Gynecol. 7, 58–63.

Joly, A.-L., Wettstein, G., Mignot, G., Ghiringhelli, F., Garrido, C., 2010. Dual role of heat shock proteins as regulators of apoptosis and innate immunity. J. Innate Immun. 2, 238–247.

Kahla-Nakbi, A.B., Chaieb, K., Bakhrouf, A., 2009. Investigation of several virulence properties among *Vibrio alginolyticus* strains isolated from diseased cultured fish in Tunisia. Dis. Aquat. Org. 86, 21–28.

Liuxy, P.C., Lee, K.K., Chen, S.N., 1996. Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon*. Lett. Appl. Microbiol. 22, 413–416.

Marques, A., Dinh, T., Ioakeimidis, C., Huys, G., Swings, J., Verstraete, W., Dhont, J., Sorgeloos, P., Bossier, P., 2005. Effects of bacteria on *Artemia franciscana* cultured in different gnotobiotic environments. Appl. Environ. Microbiol. 71, 4307–4317.

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R., Bøgwald, J., Castex, M., Ringø, E., 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture 302, 1–18.

Parsell, D., Lindquist, S., 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu. Rev. Genet. 27, 437–496.

Roberts, R., Agius, C., Saliba, C., Bossier, P., Sung, Y., 2010. Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a review. J. Fish Dis. 33, 789–801.

Rowe, G.E., Welch, R.A., 1994. Assays of hemolytic toxins. Methods Enzymol. 235, 657–667.

Rui, H., Liu, Q., Wang, Q., Ma, Y., Liu, H., Shi, C., Zhang, Y., 2009. Role of alkaline serine protease asp in *Vibrio alginolyticus* virulence and regulation of its expression by luxO-luxR regulatory system. J. Microbiol. Biotechnol. 19, 431.

Ruwandeepika, H.A., Sanjeewa Prasad Jayaweera, T., Paban Bhowmick, P., Karunasagar, I., Bossier, P., Defoirdt, T., 2012. Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the Harveyi clade. Rev. Aquac. 4, 59–74.

Smith, V.J., Brown, J.H., Hauton, C., 2003. Immunostimulation in crustaceans: does it really protect against infection? Fish Shellfish Immunol. 15, 71–90.

Soto-Rodriguez, S., Roque, A., Lizarraga-Partida, M., Guerra-Flores, A., Gomez-Gil, B., 2003. Virulence of luminous vibrios to *Artemia franciscana* nauplii. Dis. Aquat. Org. 53, 231–240.

Sun, B., Zhang, X.-H., Tang, X., Wang, S., Zhong, Y., Chen, J., Austin, B., 2007. A single residue change in *Vibrio harveyi* hemolysin results in the loss of phospholipase and hemolytic activities and pathogenicity for turbot (*Scophthalmus maximus*). J. Bacteriol. 189, 2575–2579.

Sung, Y., Van Damme, E.J.M., Sorgeloos, P., Bossier, P., 2007. Non-lethal heat shock protects gnotobiotic *Artemia franciscana* larvae against virulent Vibrios. Fish Shellfish Immunol. 22, 318–326.

Sung, Y., Roberts, R., Bossier, P., 2012. Enhancement of Hsp70 synthesis protects common carp, *Cyprinus carpio* L., against lethal ammonia toxicity. J. Fish Dis. 35, 563–568.

Sung, Y.Y., Liew, H.J., Bolong, A.M.A., Wahid, E.A., MacRae, T.H., 2013. The induction of Hsp70 synthesis by non-lethal heat shock confers thermotolerance and resistance to lethal ammonia stress in the common carp, *Cyprinus carpio* (Linn). Aquac. Res. 1–7.

Tsan, M.-F., Gao, B., 2009. Heat shock proteins and immune system. J. Leukoc. Biol. 85, 905–910.

Verschuere, L., Rombaut, G., Huys, G., Dhont, J., Sorgeloos, P., Verstraete, W., 1999. Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. Appl. Environ. Microbiol. 65, 2527–2533.

Wong, S.K., Zhang, X.-H., Woo, N., 2012. *Vibrio alginolyticus* thermolabile hemolysin (TLH) induces apoptosis, membrane vesiculation and necrosis in sea bream erythrocytes. Aquaculture 330, 29–36.

Yamaguchi, H., Osaki, T., Kurihara, N., Taguchi, H., Hanawa, T., Yamamoto, T., Kamiya, S., 1997. Heat-shock protein 60 homologue of *Helicobacter pylori* is associated with adhesion of *H. pylori* to human gastric epithelial cells. J. Med. Microbiol. 46, 825–831.

Zhang, X.H., Austin, B., 2000. Pathogenicity of *Vibrio harveyi* to salmonids. J. Fish Dis. 23, 93–102.

Zhang, X.-H., Meaden, P., Austin, B., 2001. Duplication of hemolysin genes in a virulent isolate of *Vibrio harveyi*. Appl. Environ. Microbiol. 67, 3161–3167.