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White faeces syndrome caused by *Vibrio alginolyticus* and *Vibrio fluvialis* in shrimp, *Penaeus monodon* (Fabricius 1798) - multimodal strategy to control the syndrome in Sri Lankan grow-out ponds

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Abstract

Additional mortality of 20–30 % and total rejection of harvest have been recorded for shrimp *Penaeus monodon* (Fabricius 1798) in Sri Lanka due to white faeces syndrome (WFS). Occurrence of WFS in relation to *Vibrio* count in culture water, isolation, identification and confirmation of the causative *Vibrio*, antagonism assay of a locally isolated strain of *Bacillus subtilis* (Ehrenberg 1835) against the pathogens and the possibility of controlling WFS by a multimodal strategy were carried out. White faeces syndrome was first observed at the 7th week of post stocking when mean total *Vibrio* count (TVC) in culture water was $3.1\pm0.17 \times 10^3$ CFU mL^{-1}; by the 12th week mean percentage occurrence of WFS was 45.0±1.07 %. The major causative pathogen is a strain of *V. alginolyticus* (Miyamoto et al. 1961) while a strain of *Vibrio fluvialis* (Lee et al. 1981) also contributes (accession numbers KU 891054 and KX 361118). *Bacillus subtilis* showed profound inhibitory activity against both *Vibrio* species. The tested multimodal strategy (proper disinfection of culture water, zero water exchange and the use of a locally isolated strain of *B. subtilis* as a bioremediator and a probiotic) could successfully control WFS in grow-out ponds of *P. monodon* in Sri Lanka.

Keywords: white faeces syndrome, *Vibrio alginolyticus*, *Vibrio fluvialis*, *Penaeus monodon*

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Introduction

Although the white spot disease caused by white spot virus (WSV) has been identified as the major disease in cultured shrimp *Penaeus monodon* (Fabricius 1798) there are other diseases that contribute to low survival and growth retardation reducing the crop value (Lavilla-Pitogo et al. 1998). Since 2010, shrimp processing plants in Sri Lanka have been recognising a loose shell condition in cultured shrimp and a significant number of shrimp grow-out ponds have been harvested for the local market at an average body weight of 10 g due to white faeces syndrome (WFS) every year.

Some Sri Lankan farmers have recorded 20–30 % additional mortality due to WFS and total rejection of harvested shrimp has been reported by the processing plants due to the loose shell condition produced by WFS. Strings of white faecal matter floating on the water surface of grow-out ponds with suddenly reduced feed consumption are early signs of the syndrome followed by mortality (Jayasree et al. 2006). Somboon et al. (2012) reported that *Vibrio* sp. were the major bacterial species responsible for WFS in cultured *Penaeus vannamei* (Boone 1931) in Thailand. According to these authors the effect of the syndrome was prominent after the second month of culture at 10–12 g body weight of shrimp and the severity increased when temperature of the culture water was high and decreased with the commencement of the rainy period during which the water temperature decreases in the tropical climate.

The objectives of the present study were to monitor the occurrence of the syndrome in randomly selected shrimp ponds located in the North Western Province of Si Lanka in relation to *Vibrio* count in culture water and to isolate and identify the *Vibrio* species that cause WFS in cultured shrimp *P. monodon.* The antagonism of the locally isolated strain of *Bacillus subtilis* against the *Vibrio* species that causes WFS in cultured *P. monodon* would be investigated to see whether the bacterium could be used as a bioremediator and a probiotic. A multimodal strategy, viz. combined effect of proper disinfection of culture water, zero water exchange and the use of the locally isolated strain of *B. subtilis* as a bioremediator and a probiotic would be tested to determine if it is able to control the syndrome so that farmers could apply the same strategy to protect their cultured shrimp stocks.

Materials and Methods

Monitoring occurrence of WFS in relation to total Vibrio count in culture water

Sixty randomly selected shrimp grow-out ponds (4000–5000 m^2) located in the North Western Province of Sri Lanka were monitored over the first 12 weeks of one production cycle (from August 2013 to November 2013). Random samples of shrimp were collected weekly from each pond and the number of affected individuals (shrimp with white mid gut) was recorded to obtain the mean percentage occurrence of WFS for each affected pond.

Weekly water samples were also randomly collected from each pond and mean total *Vibrio* count (MTVC) was enumerated using the spread plate technique on thiosulphate citrate bile salts sucrose (TCBS) agar while wet mounts of gut of juvenile shrimp in random samples were observed under the light microscope (x10 and x40) for parasites, occlusion bodies of monodon baculo type virus (MBV) and/or any other abnormality.

Quantification of Vibrio in shrimp tissues

Samples of juvenile shrimp were collected randomly from 20 WFS affected ponds and 20 unaffected ponds in April 2014. A subsample of ten shrimp was randomly taken from each grow-out pond and was used for quantification of *Vibrio* in shrimp tissues, viz. haemolymph, hepatopancreas and gut. TCBS agar plates were inoculated separately with 0.1mL of haemolymph taken from each shrimp under aseptic conditions. Hepatopancreas and gut of each shrimp were then dissected out aseptically, weighed and homogenised separately in sterilised saline (10 mL of 1.5 % NaCl); 0.1mL samples of inocula taken after 10-fold dilution of the homogenised hepatopancreas and gut were inoculated on TCBS agar separately. After incubation at 28 ^{o}C for 24 hours, mean total *Vibrio* count (TVC) was estimated for each shrimp tissue.

Isolation and identification of Vibrio causing WFS

The predominant morphologically different two types of colonies that grew on TCBS agar plates that were inoculated aseptically with 0.1 mL haemolymph from WFS affected shrimp were used for the identification of pathogenic *Vibrio* that contribute to the occurrence of WFS. The isolated two types of colonies were re-cultured on nutrient agar supplemented with 1.5 % NaCl to obtain pure cultures. The isolated pure colonies were identified using standard microbiological and biochemical tests (API-20E; Biomerieux, France); stock cultures of both species were prepared and stored at 4 ^{o}C for further studies.

Identification of strains of isolated Vibrio species using 16s rRNA partial sequence analysis

The two types of isolated predominant colonies were identified by microbiological and biochemical tests as *Vibrio alginolyticus* and *Vibrio fluvialis* Table 2); from their stock cultures fresh colonies were obtained separately on nutrient agar supplemented with 1.5 % NaCl. DNA was extracted from a single colony of each isolate of *Vibri*o and 5µl of extracted DNA was subjected to polymerase chain reaction (PCR) using 27F / 800R and 518F/1492R forward and reverse primers according to the instructions of the manufacturer. Amplified DNA was subjected to DNA sequencing using 518 F and 800 R primers (nucleotide sequencing was done by the commercial service of Macrogen Inc. Co. Ltd., Seoul, Korea). The 16s rRNA sequence was then compared with already existing DNA sequences in the GenBank database to confirm the identification of species and to obtain an accession number for each strain.

Experimental infection with Vibrio species isolated from haemolymph of WFS affected shrimp

The two *Vibrio* species (*V. alginolyticus* and *V. fluvialis*) that were isolated from haemolymph of affected shrimp with WFS were pre-cultured in Luria Broth (LB) and incubated at 28 ^{o}C under constant shaking for 24 hours to be used for the experimental infection.

In a preliminary experiment, pelleted shrimp feed incorporated with each species of *Vibrio* separately at concentration of 8.2 x 10^3 to 8.2 x 10^{10}CFU.g^{-1} were fed to separate groups of healthy juvenile shrimp to determine the suitable concentration for experimental infection; the lowest concentration that produced gross clinical signs within 3 days was chosen for experimental infection. Apparently healthy juvenile shrimp (12.57±0.87 g body weight) were disinfected with formalin (100 mgL^{-1} for 30 seconds), randomly distributed into twelve aquaria with UV treated brackish water (200 L) to have 20 juveniles in each aquarium and were acclimatised for 5 days (salinity 15 g.L^{-1}, pH 8.0 and fed with a pelleted shrimp feed at a rate of 3.5 % of body weight per day). Pure cultures of *V. alginolyticus* and *V. fluvialis* were then mixed separately with pelleted shrimp feed to have the chosen concentration, 8.2 x 10^9 CFU.g^{-1}and were fed to healthy, acclimatised *P. monodon* juveniles separately only for two meals of a single day (after that normal feed was provided) while the control group was not exposed to any of the *Vibrio* species; four replicates were arranged for each experimental group and for the control group. The gross clinical signs of WFS that were developed in shrimp, *Vibrio* count in haemolymph, hepatopancreas and gut of infected individuals and daily mortality in each aquarium were recorded over 2 weeks after challenge; Koch's postulate test was performed with haemolymph of experimentally infected, moribund shrimp.

Antagonism assay of Bacillus subtilis on V. alginolyticus and V. fluvialis

Antagonism assay of a strain of *B. subtilis* that was locally isolated (from guts of healthy, wild *P. monodon* captured from a few estuaries located in the North Western Province, Sri Lanka) was performed on the two species of *Vibrio* isolated from haemolymph of WFS affected shrimp. Isolated *V. alginolyticus* and *V. fluvialis* were pre-cultured in LB broth, incubated at 28 ^{o}C for 2 days and 50 µL of each culture was spread separately over nutrient agar (supplemented with 1.5 % NaCl) plates. *Bacillus subtilis* was grown at 28 ^{o}C in LB broth and a 3 days old culture was centrifuged at 9600 g for 15 minutes at 28 ^{o}C to take the supernatant fluid which was filtered through a 0.45 µm membrane filter to obtain cell-free extract; 100 µL of cell-free extract was then introduced into the wells of the agar medium on which the *Vibrio* was spread-plated while using sterilised culture medium of *B. subtilis* as the control. After incubating the agar plates with *Vibrio* and cell-free extract of *B. subtilis*, for a period of 24–48 hours at 28 ^{o}C, the antagonism assay was performed following Baucer et al. (1966). The mean diameter (mm) of clear inhibitory zones formed around the wells due to antibacterial activity of *B. subtilis* on the two species of *Vibrio* was taken as antagonism of the former bacterium on *V. alginolyticus* and *V. fluvialis*.

Multimodal strategy to control WFS

The combined effect of proper disinfection of water (with chlorine at 30 $mg.L^{-1}$), zero water exchange and the use of *B. subtilis* (which is antagonistic to the causative *Vibrio* of WFS) as a bioremediator and a probiotic was studied as a multimodal strategy to control WFS. Healthy post larvae of *P. monodon* (free of WSV, MBV and pathogenic *Vibrio* species) were stocked in experimental, positive control and negative control ponds with 16 replicate ponds for each group. The size of each pond was 4,500 m^2 and each was stocked with 12 days old post larvae at a stocking density of 20 $PL.m^{-2}$.

A multimodal strategy was employed for experimental ponds, viz. culture water to be filled into the ponds was first disinfected with chlorine (30 $mg.L^{-1}$), managed with zero water exchange and regular application of *B. subtilis* (10^4 $CFU.mL^{-1}$ per week as a bioremediator) while feed offered to shrimp was incorporated with the same bacterium (10^6 $CFU.g^{-1}$ as a probiotic). Positive control ponds were managed by simulating the usual practice of Sri Lankan grow-out farmers, viz. the culture water to be filled into the grow-out ponds was first disinfected with chlorine (30 $mg.L^{-1}$), managed with the application of hydrated lime or agricultural lime and an antibiotic in recommended dosage (by the National Aquaculture Development Authority in Sri Lanka) as required with limited water exchange.

Water in negative control ponds was not disinfected before filling and did not receive hydrated lime, agricultural lime or any antibiotic over the production cycle but water was exchanged frequently. Shrimp in all three groups of ponds (experimental, positive control and negative control) were fed with a commercially available pelleted shrimp feed containing 36–38 % crude protein at the feeding rate of 6.5–2.5 % of body weight from the first month of stocking to the month of harvesting (the same feed was incorporated with *B. subtilis* before offering to the shrimp in experimental ponds over the production cycle).

Random samples of culture water were used to enumerate mean total bacterial count (on nutrient agar) and mean total *Vibrio* count (on TCBS agar) in culture water of each grow-out pond. Similarly, random samples of shrimp were obtained from each pond and mean total bacterial count and mean total *Vibrio* counts were recorded for haemolymph, hepatopancreas and gut of shrimp on nutrient agar and TCBS agar by the spread plate technique. Presumptively identified predominant *Vibrio* colonies from haemolymph of WFS affected shrimp (from positive and negative control ponds) were re-cultured on nutrient agar (supplemented with 1.5 % NaCl), identified using standard microbiological and biochemical tests (by API-20E; Biomerieux, France) and were confirmed by the analysis of 16s rRNA partial sequence. In addition, physiochemical parameters of culture water in each grow-out pond (water pH, unionised ammonia, alkalinity, salinity, transparency and dissolved oxygen) were recorded weekly while random samples of shrimp were used to observe their status of health and to estimate mean weekly growth rate of shrimp for each grow-out pond. Mean body weight of shrimp at harvest was recorded for the three groups of ponds by taking random samples.

Results

Monitoring the occurrence of WFS

Mean percentage occurrence of WFS by the 12th week of the production cycle was 45±1.07 % (Fig. 1). The occurrence of WFS, the white gut that could be easily observed through the cuticle (Fig. 2A), was first recorded by the 7th week of post-stocking the grow-out ponds with post larvae of shrimp; those ponds had significantly ($p < 0.05$) higher mean total *Vibrio* count in culture water ($3.1 \pm 0.17 \times 10^3$ CFU.mL^{-1}) than that of the culture water of ponds that were unaffected by WFS (Fig. 1). Figure 2B shows the strings of white faeces floating on the water surface when juvenile shrimp were 60–70 days old in grow-out ponds. Affected shrimp lost their appetite, had loose shell, discoloured hepatopancreas and white colour gut compared to hepatopancreas and gut of normal shrimp (Fig. 3A & B). Severely affected individuals died while others exhibited growth retardation. Gut contents of affected, moribund, juvenile shrimp had numerous lipid globules (Fig. 4A & B); parasites such as gregarines were not observed in the gut, abnormalities were not seen, MBV occlusion bodies or fungal infections were not present but enormous numbers of bacterial cells were observed in the gut under the light microscope (x40).

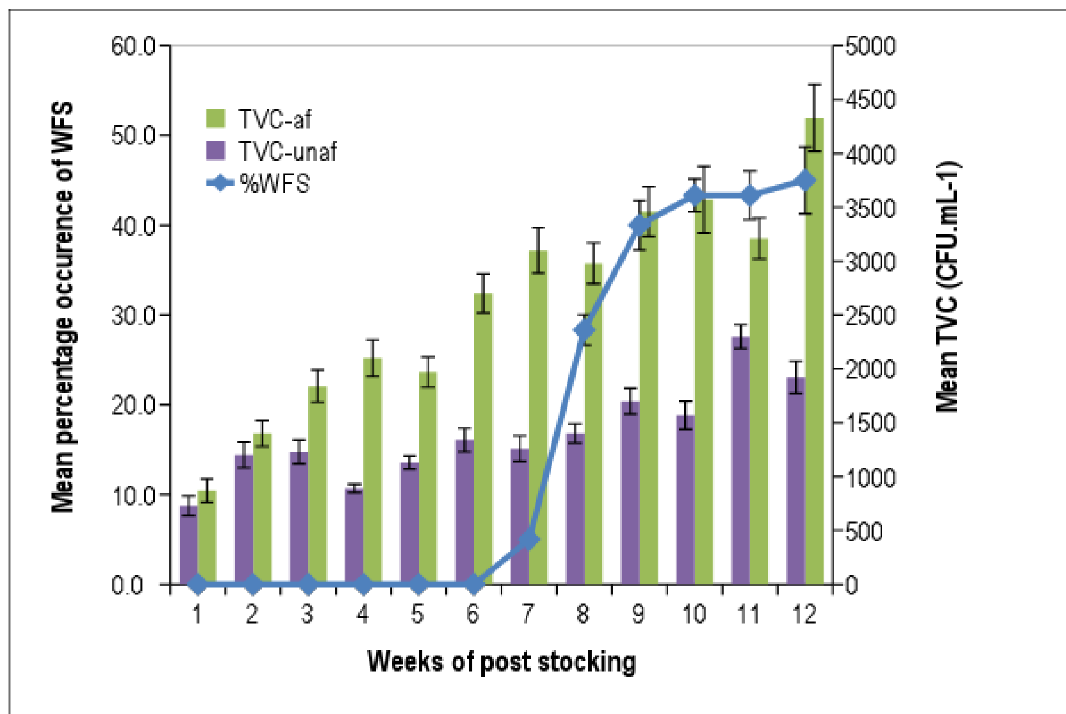

Fig. 1. Mean percentage occurrence of WFS in shrimp over the first twelve weeks of post stocking with the mean total *Vibrio* count (TVC) in culture water of grow-out ponds (n = 60; data are provided as mean ± standard error).
TVC-af: Mean total *Vibrio* count in WFS-affected ponds; TVC-unaf: Mean total *Vibrio* count in WFS-unaffected ponds; % WFS: Mean percentage occurrence of WFS

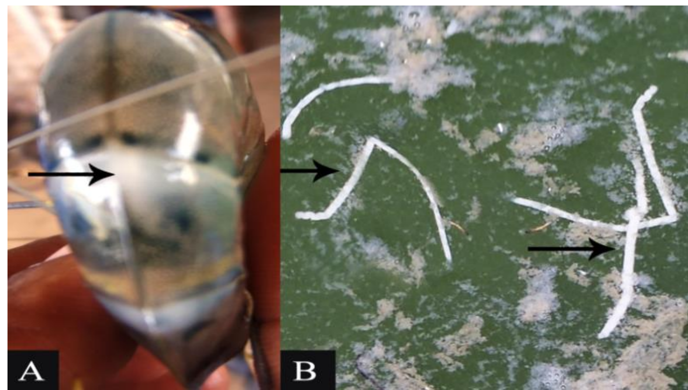

Fig. 2. White gut that could be observed through the cuticle by the 7th week of post stocking (A) and white faecal matter floating on the water surface of grow-out ponds when juvenile shrimp were 60-70 days old (B).

Fig. 3. White coloured gut with discoloured hepatopancreas of WFS-affected shrimp (A) and normal appearance of gut & hepatopancreas of a healthy shrimp (B).

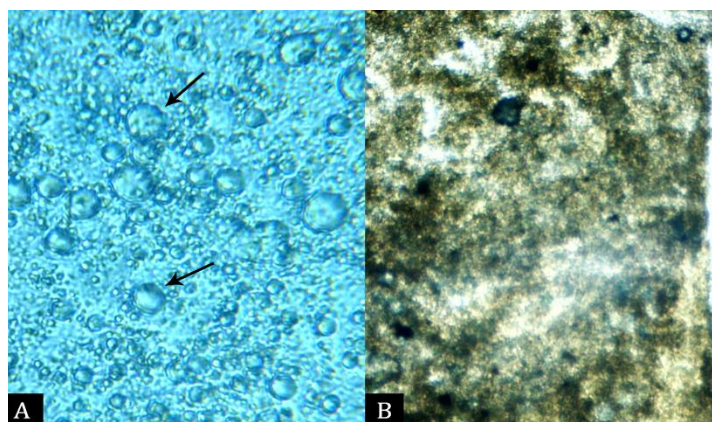

Fig. 4. Accumulation of lipid globules in the gut contents of WFS-affected shrimp (A; ×40) and appearance of gut contents of a healthy shrimp (B; ×40)

Quantification of Vibrio in shrimp tissues

Significantly higher log mean total *Vibrio* count was recorded in haemolymph, hepatopancreas and gut of shrimp that were randomly collected from WFS-affected grow-out ponds compared to respective values recorded for the tissues of shrimp obtained from WFS-unaffected ponds ($p < 0.05$; Fig. 5). Shrimp collected from ponds affected with WFS had a very high mean TVC in haemolymph, hepatopancreas and gut ($3.2\pm0.53\times10^5$ CFU.mL^{-1}, $9.32\pm0.41\times10^6$ CFU.g^{-1} and $7.54\pm0.17\times10^7$ CFU.g^{-1} respectively).

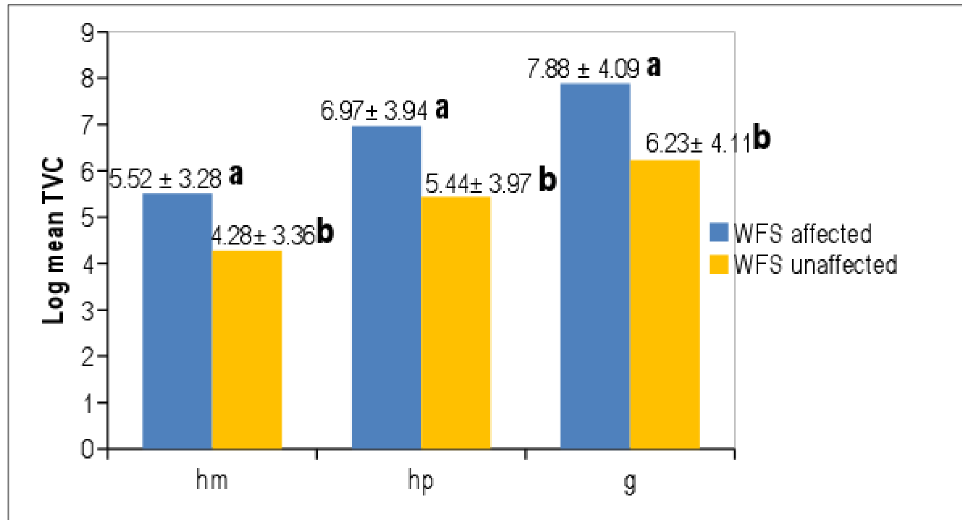

Fig. 5. The log value of mean total *Vibrio* count (TVC) in haemolymph (hm), hepatopancreas (hp) and gut (g) of shrimp collected from WFS-affected ponds (n=20) compared to respective tissues of shrimp collected from WFS-unaffected ponds (n=20; different superscripts on adjacent bars indicate significant difference, $p< 0.05$; data are provided as mean ± standard error)

Isolation and identification of Vibrio that cause WFS

Four types of *Vibrio* colonies were identified growing on TCBS agar based on colony morphology, viz. yellow large, yellow small, green large and green small. However, the number of yellow colonies that grew on TCBS agar from inocula of all three types of tissues (haemolymph, hepatopancreas and gut) obtained from WFS-affected shrimp was significantly higher ($p < 0.05$) than the number of green colonies recorded for respective tissues. The mean *Vibrio* count of yellow large colonies from haemolymph, hepatopancreas and gut of WFS-affected shrimp was significantly higher ($p < 0.05$; $1.97\pm0.78 \times 10^5$ CFU.mL^{-1}, $6.69\pm0.87 \times 10^6$ CFU.g^{-1}and $5.11\pm0.38 \times 10^7$ CFU.g^{-1} respectively) than the values recorded for respective tissues of WFS-unaffected shrimp ($7.3\pm0.54 \times 10$ CFU.mL^{-1}, $7.89\pm0.17 \times 10^3$ CFU.g^{-1} and $7.30\pm0.42 \times 10^5$ CFU.g^{-1} respectively; Table 1). A similar observation was made with the mean *Vibrio* count of yellow small colonies in tissues of WFS affected and unaffected shrimp (Table 1). The yellow large colonies were identified as *V. alginolyticus* and yellow small colonies were identified as *V. fluvialis* using microbiological and biochemical characteristics (Table 2).

Table 1. Mean count of *Vibrio* with different colony morphology from different tissues of WFS affected shrimp (20 ponds) and WFS unaffected shrimp (20 ponds) collected from grow-out ponds located in the North Western Province, Sri Lanka

	Mean count of *Vibrio* with different colony morphology from different shrimp tissues					
	Haemolymph (CFU.mL^{-1})		Hepatopancreas (CFU.g^{-1})		Gut (CFU.g^{-1})	
CM	WFS-af	WFS-un	WFS-af	WFS-un	WFS-af	WFS-un
YL	$1.97 \pm 0.78 \times 10^{5a}$	$7.3 \pm 0.54 \times 10^{b}$	$6.69 \pm 0.87 \times 10^{6a}$	$7.89 \pm 0.17 \times 10^{3b}$	$5.11 \pm 0.38 \times 10^{7a}$	$7.30 \pm 0.42 \times 10^{5b}$
YS	$1.22 \pm 0.91 \times 10^{5a}$	$4.1 \pm 0.32 \times 10^{b}$	$2.62 \pm 0.74 \times 10^{6a}$	$1.41 \pm 0.91 \times 10^{3b}$	$2.45 \pm 0.52 \times 10^{7a}$	$9.09 \pm 0.71 \times 10^{5b}$
GL	$1.12 \pm 0.23 \times 10^{b}$	$5.7 \pm 0.35 \times 10^{a}$	$2.7 \pm 0.32 \times 10^{3b}$	$5.37 \pm 0.62 \times 10^{3a}$	$9.1 \pm 0.45 \times 10^{3b}$	$2.97 \pm 0.18 \times 10^{4a}$
GS	$1.21 \pm 0.62 \times 10^{b}$	$2.1 \pm 0.20 \times 10^{a}$	$1.5 \pm 0.80 \times 10^{3a}$	$1.40 \pm 0.08 \times 10^{3a}$	$7.47 \pm 0.37 \times 10^{3b}$	$2.9 \pm 0.52 \times 10^{4a}$

WFS-af: WFS affected shrimp, WFS-un: WFS unaffected shrimp, CM: colony morphology, YL: yellow large colonies, YS: yellow small colonies, GL: green large colonies, GS: green small colonies. Mean *Vibrio* count with same colony morphology recorded for a particular shrimp tissue given in a row with different superscripts are significantly different from each other; $p < 0.05$, Student T-test

Table 2. Microbiological and biochemical characteristics of *Vibrio* species isolated from haemolymph of juvenile *Penaeus monodon* with symptoms of white faeces syndrome.

Tests	*Vibrio* sp	*Vibrio* sp
Colony morphology on TCBS agar	Yellow large colonies (2-2.5 ± 0.06 mm)	Yellow small colonies (0.5 ± 0.02 mm)
Motility	+	+
ONPG	-	+
ADH	-	+
LDC	+	+
ODC	+	+
CIT	-	-
H_2S	-	-
URE	-	-
TDA	-	+
IND	+	+
VP	+	-
GEL	+	+
GLU	+	+
MAN	+	+
INO	-	-
SOR	-	-
RHA	-	-
SAC	+	+
MEL	-	-
AMY	-	+
ARA	-	-
OX	+	+
Identified as	***Vibrio alginolyticus***	***Vibrio fluvialis***

Identification of strains of isolated V. alginolyticus and V. fluvialis by 16s rRNA partial sequence analysis

Blast analysis of the partial 16s ribosomal RNA gene (16s rRNA) indicated the identification of the isolates as strains of *V. alginolyticus* and *V. fluvialis* (showing 99–100 % similarity to other relevant strains of the two species of *Vibrio*). Isolated sequences for 16s rRNA genes were deposited into the NCBI database and accession numbers KU891054 and KX 361118 were obtained from Genbank for the strains of *V. alginolyticus* and *V. fluvialis* respectively.

Experimental infection with Vibrio species isolated from haemolymph

All juvenile shrimp (100 %) that were fed with the isolated *V. alginolyticus* developed the symptoms of WFS in 3–4 days from challenge while only 33 % of individuals fed with *V. fluvialis* developed the symptoms; *V. alginolyticus* and *V. fluvialis* were re-isolated from haemolymph of moribund shrimp from respective groups of shrimp (Koch's postulate test).

Antagonism assay of B. subtilis on V. alginolyticus and V. fluvialis

The cell-free extract of *B. subtilis* showed profound inhibitory activity against *V. alginolyticus* and *V. fluvialis* isolated from haemolymph of infected shrimp with WFS. The mean diameters of the inhibitory zones against the growth of *V. alginolyticus* and *V. fluvialis* were 3.5±0.3 cm and 3±0.3 cm respectively (Fig. 6).

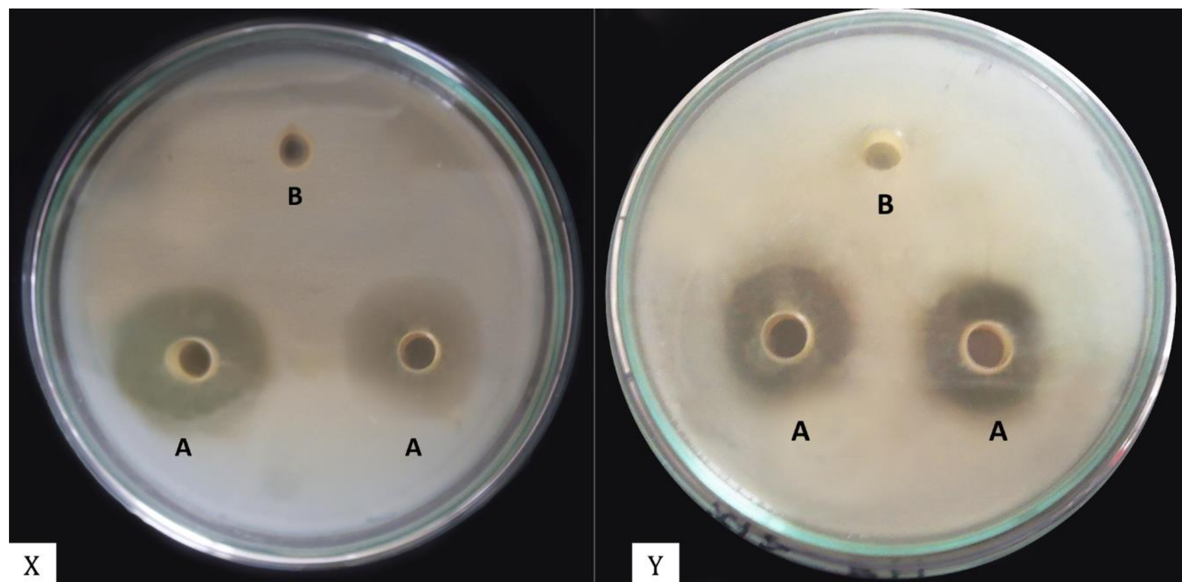

Fig. 6. Inhibitory zones (A) produced by cell-free extract of *B. subtilis* on a lawn of *V. alginolyticus* (X) and *V. fluvialis* (Y); control (B; sterilised culture medium of *B. subtilis*)

Multimodal strategy to control WFS

Shrimp in experimental grow-out ponds did not develop WFS over the production cycle while mean percentage occurrence of the disease in positive and negative control ponds were 87.5±4.02 % and 37.6±3.71 % respectively (Table 3). The major *Vibrio* species isolated from random samples of haemolymph, hepatopancreas and gut of infected shrimp from positive and negative control ponds was the same strain of *V. alginolyticus* (KU 891054); *V. fluvialis* (KX 361118) was also present in some of the infected shrimp. Table 3 provides the mean total bacterial count (TBC) and mean total *Vibrio* count (TVC) in haemolymph, hepatopancreas and gut of shrimp and culture water of experimental, positive and negative control grow-out ponds over the production cycle. Mean TVC in culture water and shrimp tissues (haemolymph, hepatopancreas and gut) of positive and negative control ponds was significantly higher ($p < 0.05$) than that of culture water and shrimp tissues of experimental grow-out ponds respectively (Table 3). Differences between mean TBC and mean TVC of shrimp tissues and culture water of experimental grow-out ponds were significantly higher ($p < 0.05$) than those respective values recorded for positive and negative control ponds.

Water pH, DO, alkalinity, unionised ammonia and transparency of culture water in experimental ponds were within the optimum ranges for shrimp over the production cycle while those parameters of culture water in positive and negative control ponds fluctuated greatly over the production cycle (Table 4). With the regular application of *B. subtilis*, mean concentration of unionised ammonia was significantly lower in experimental grow-out ponds ($p < 0.05$) which ranged from 0 to 0.006 mg.L^{-1} with the mean value of 0.004±0.001 mg.L^{-1} compared to both control groups of ponds.

The unionised ammonia concentrations fluctuated largely throughout the culture period in both groups of control ponds and the highest unionised ammonia values were recorded towards the end of the culture period. Shrimp in experimental ponds were active with a healthy appearance and had a significantly higher mean weekly growth rate throughout the production cycle ($p < 0.05$) compared to shrimp in positive and negative control ponds. At harvest, mean body weight of shrimp in experimental ponds was 27.93±0.69 g, which is significantly higher ($p < 0.05$) than those that were recorded for shrimp in positive and negative control ponds (18.71±1.56 g and 12.42±1.08 g respectively).

Table 3. Mean total bacterial count and mean total *Vibrio* count in haemolymph, hepatopancreas and gut of shrimp and culture water of experimental grow-out ponds compared to positive and negative control ponds (data are provided as mean ± SE) with mean percentage occurrence of WFS

Pg	Mean total bacterial count (TBC)				Mean total *Vibrio* count (TVC)				MPWFS
	hm (CFU. mL^{-1})	hp(CFU .g^{-1})	g (CFU. g^{-1})	cw (CFU. mL^{-1})	Hm (CFU. mL^{-1})	hp (CFU. g^{-1})	g (CFU. g^{-1})	cw (CFU. mL^{-1})	
E	$1.48 \pm 0.35 \times 10^{2\,c}$	$5.34 \pm 0.24 \times 10^{5\,c}$	$2.17 \pm 0.12 \times 10^{6\,c}$	$3.72 \pm 0.18 \times 10^{3\,a}$	$1.37 \pm 0.06 \times 10^{2\,c}$	$1.96 \pm 0.12 \times 10^{4\,c}$	$1.28 \pm 0.45 \times 10^{4\,c}$	$4.19 \pm 0.36 \times 10^{2\,c}$	Not observed
P	$4.96 \pm 0.17 \times 10^{4\,a}$	$7.52 \pm 0.28 \times 10^{6\,a}$	$4.78 \pm 0.08 \times 10^{7\,a}$	$3.44 \pm 0.35 \times 10^{3\,a}$	$4.38 \pm 0.92 \times 10^{4\,a}$	$6.22 \pm 0.71 \times 10^{6\,a}$	$4.63 \pm 0.25 \times 10^{7\,a}$	$2.86 \pm 0.34 \times 10^{3\,b}$	87.5 ± 4.02^{a}
N	$2.73 \pm 0.41 \times 10^{4\,b}$	$5.55 \pm 0.27 \times 10^{6\,b}$	$1.78 \pm 0.11 \times 10^{7\,b}$	$3.93 \pm 0.12 \times 10^{3\,a}$	$2.36 \pm 0.52 \times 10^{4\,b}$	$4.73 \pm 0.29 \times 10^{6\,b}$	$1.28 \pm 0.11 \times 10^{7\,b}$	$3.75 \pm 0.22 \times 10^{3\,a}$	37.6 ± 3.71^{b}

Pg: Pond group; E: experimental grow-out ponds; P: positive control grow-out ponds; N: negative control grow-out ponds; hm: haemolymph; hp: hepatopancreas; g: gut; cw: culture water; MPWFS: mean percentage occurrence of WFS (means with different superscripts in a column are significantly different from each other; $p < 0.05$, ANOVA & Tukey's Pair-wise Test)

Table 4. Water quality parameters in experimental, positive and negative control grow-out ponds (data are provided as mean ± standard error for the mean; ranges are given within parenthesis)

Water quality parameter	Experimental	Positive control	Negative control
pH	7.89 ± 0.06^b (7.21- 8.25)	8.87 ± 0.53^a (8.57- 9.01)	9.01 ± 0.46^a (8.87 - 9.17)
DO (mg L^{-1})	5.28 ± 0.22^a (4.83 - 6.88)	4.48 ± 0.12^a (3.54 -5.05)	3.50 ± 0.06^b (3.02 - 3.81)
Salinity (gL^{-1})	12.39 ± 0.33^b (11.2 – 14)	15.13 ± 0.89^a (11.3 - 17.12)	17.43 ± 0.21^a (11.32 – 19.23)
Alkalinity (mg L^{-1})	85.91 ± 2.26^c (75.56 – 126.89)	153.67 ± 5.94^b (90.52 – 220.21)	182.65 ± 9.82^a (135.25 - 270)
Unionised ammonia (mg L^{-1})	0.004 ± 0.001^c (0.00 - 0.006)	0.171 ± 0.029^b (0.041 - 0.329)	0.28 ± 0.042^a (0.06 - 0.54)
Transparency (cm)	36.23 ± 5.3^a (32.58 – 39.22)	54.79 ± 16.3^b (23. 5 – 74.5)	47.26 ± 13.4^b (28.5 – 60.5)

Discussion

The first external symptom of WFS observed during the present study was the white gut that was visible through the transparent cuticle of juvenile *P. monodon* as a white streak. Shrimp reduced the feed consumption and released faecal matter in the form of white, unsolidified material; severely affected individuals died while others showed growth retardation with loose shell.

Occurrence of WFS with similar symptoms was reported in black tiger shrimp (*P. monodon*) in India in 2000–2001 (Jayasree et al. 2006) and in both black tiger shrimp and Pacific white shrimp (*P. vannamei*) in Thailand in 2010 (Somboon et al. 2012). Results of the present study confirmed that gregarines are not involved in WFS; Flegel (2012) and Somboon et al. (2012) also have reported that gregarines were not the major cause of WFS. In this study, shrimp that were randomly collected from ponds affected with WFS had a very high mean TVC in haemolymph, hepatopancreas and gut ($3.2\pm0.53 \times 10^5$ CFU.mL^{-1}, $9.32\pm0.41 \times 10^6$ CFU.mL^{-1} and $7.54\pm0.17 \times 10^7$ CFU.mL^{-1} respectively); Jayasree et al. (2006) and Somboon et al. (2012) also have recorded significantly higher TVC in haemolymph and gut of shrimp infected with WFS compared to healthy shrimp. According to Rouse et al. (1999) and Intaraprasong et al. (2009), predominant *Vibrio* species in haemolymph that produce septicaemia in crustaceans include *Vibrio parahaemolyticus*, *V. alginolyticus* and *Vibrio anguillarum*. Present study isolated *V. alginolyticus* and *V. fluvialis* as the predominant species from haemolymph, hepatopancreas, and gut of shrimp affected with WFS. Inthusai (2006, cited by Somboon et al. 2012) reported on three species of *Vibrio,* viz. *V. fluvialis*, *V. alginolyticus* and *V. parahaemolyticus* from *P. monodon* infected with WFS in Thailand while *Vibrio harveyi* (Baumann et al., 1981), *V. alginolyticus* and *V. anguillarum* were identified from the same *Penaeus* species with similar disease symptoms in India by Jayasree et al. (2006).

Experimental infection carried out during the present study confirmed that the strain of *V. alginolyticus* (KU891054) is the major pathogen that causes WFS in juvenile *P. monodon* cultured in the North Western province, Sri Lanka though the strain of *Vibrio fluvialis* (KX 361118) also contributes to the development of the syndrome. Results of the antagonism assay in the present work showed that growth of *V. alginolyticus* and *V. fluvialis* could be controlled by the locally isolated, non-pathogenic strain of *B. subtilis*. The use of non-pathogenic bacterial strains to control pathogenic *Vibrio* species had been receiving much attention for many years (Rengpipat et al. 1998). *Bacillus subtilis* had exhibited profound antagonism effect against *V. harveyi* (Vaseeharan and Ramasamy 2003; Decamp et al. 2008). *Bacillus* bacteria are able to out-compete other bacteria for nutrients and space and can exclude other bacteria through the production of antibiotics (Farzanfar 2006; Rodríguez et al. 2007). Vaseeharan & Ramasamy (2003) and Decamp et al. (2008) have pointed out that *Bacillus* used as probiotic/ bioremediator to control *Vibrio* spp. in culture water could replace *Vibrio* species in gut of shrimp and could increase the disease resistance, survival and growth rate of shrimp.

The present study recorded significantly higher mean total bacterial count in the culture water of the experimental grow-out ponds ($p < 0.05$) in which the water was pre-disinfected, maintained under zero water exchange with regular application of *B. subtilis* than mean total *Vibrio* count of culture water; shrimp in those ponds received the feed with *B. subtilis* and the mean total bacterial count recorded in the gut of the shrimp also was significantly higher ($p < 0.05$) than mean total *Vibrio* count in their gut. Far et al. (2009) reported that *Bacillus* used as a bioremediator/ probiotic was able to colonise and replace the *Vibrio* species in culture water and in the gut of shrimp, which is in agreement with the present results.

During the present study, significant differences were not recorded between mean total bacterial count and mean total *Vibrio* count in rearing water as well as between mean total bacterial count and mean total *Vibrio* count in the gut of the shrimp in positive and negative control grow-out ponds ($p > 0.05$). White faeces syndrome was observed in shrimp grown in positive and negative control grow-out ponds where significantly higher total *Vibrio* counts were recorded in culture water and shrimp tissues; greater populations of pathogenic *V. alginolyticus* and *V. fluvialis* might have invaded and multiplied in shrimp tissues (haemolymph, hepatopancreas and gut) causing the syndrome. Mean percentage occurrence of WFS recorded in shrimp reared under the conditions of positive and negative control grow-out ponds during this study were 87.5 ± 4.02 % and 37.6 ± 3.71 % respectively with low survival and low growth rate. Mean total *Vibrio* counts recorded in haemolymph, hepatopancreas and gut of shrimp and culture water of infected ponds with WFS was significantly higher ($p < 0.05$) than those of uninfected ponds. Pathogenic *Vibrio* species that got into the positive control ponds must have developed resistance/ tolerance to different chemical treatments and antibiotics (approved antibiotics in recommended dosages by the National Aquaculture Development Authority, Sri Lanka) used, multiplied and increased their population and then attacked shrimp contributing to a greater percentage occurrence of WFS.

Pathogenic and non-pathogenic *Vibrio* species as well as other species of bacteria that got into negative control ponds with frequent water exchange (from open water sources) must have had a balancing effect on pathogenic bacterial populations (under no treatment) causing lower occurrence of the syndrome in negative control ponds compared to positive control ponds. The WFS was not observed in experimental ponds over the culture period and significantly lower mean total *Vibrio* counts ($p < 0.05$) were recorded in haemolymph, hepatopancreas and gut of shrimp and in culture water compared with those respective values of positive and negative control grow-out ponds indicating that colonised *B. subtilis* have had greater antibacterial activity against *V. alginolyticus* and *V. fluvialis* as well as against other *Vibrio* species. Balcázar and Rojas-Luna (2007) and Decampet al. (2008) pointed out that *B. subtilis* produce broad spectrum antibiotics such as difficidin, oxydifficidin, bacitracin, bacillin and bacillomycin B that could act against aerobic and anaerobic bacteria. According to Stein (2005), broad spectrum antibiotic compounds are produced naturally by *Bacillus* species while pathogenic bacteria are unable to develop resistance genes to all the antibiotics produced by the non-pathogenic *Bacillus* species.

The physiochemical parameters of culture water recorded during this study were within the optimum ranges for shrimp in experimental grow-out ponds over the culture period, while those parameters greatly fluctuated in positive and negative control grow-out ponds. Water in shrimp grow-out ponds is polluted over the culture period due to accumulation of metabolic waste of shrimp, decomposition of unutilised feed and decaying biotic materials (Farzanfar 2006). Bacterial species belonging to the genus *Bacillus* have the ability to mineralise organic matter and reduce the accumulation of organic sediment in the pond bottom, maintaining the water quality parameters under optimum condition over the culture period (Kautsky et al. 2000; Walker et al. 2011); water quality parameters of experimental ponds during the present study also must have been maintained within the optimum ranges for shrimp by *B. subtilis* used as a bioremediator and a probiotic. The results showed that the multimodal strategy tested during the present study could control WFS, an infectious gastro-intestinal disease of cultured shrimp caused by *V. alginolyticus* and *V. fluvialis* successfully. It is important to organise awareness programmes for shrimp grow-out farmers and disseminate the knowledge on the strategy; the use of locally isolated beneficial microorganisms to suppress pathogenic *Vibrio* species that cause WFS should be encouraged as an environmentally friendly method that complies with food safety regulations.

Conclusion

Vibrio species isolated from random samples of haemolymph, hepatopancreas and gut of moribund shrimp (with WFS) of positive and negative control ponds of the present study were of the same strains of *V. alginolyticus* and *V. fluvialis* (KU 891054 and KX 361118 respectively) and the former was the predominant species. The combination of disinfection of water with correct concentration of chlorine, zero water exchange and the regular application of locally isolated strain of *B. subtilis* as a bioremediator and as a probiotic could control the occurrence of WFS in juvenile *P. monodon* in grow-out ponds of the North Western Province of Sri Lanka.

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References

Balcázar, J.L. and T. Rojas-Luna. 2007. Inhibitory activity of probiotic *Bacillus subtilis* UTM 126 against *Vibrio* species confers protection against vibriosis in juvenile shrimp (*Litopenaeus vannamei*). 2007. Current Microbiology. 55: 409–412.

Baucer, A.W., W.M.M. Kirby, J.C. Sherrris and M. Turk. 1966. Antibiotic susceptibility testing by standardised single disk method. American Journal of Clinical Pathology 45: 493–496.

Decamp, O., D.J.W. Moriarty and P. Lavens. 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. Aquaculture Research 39: 334–338.

Far, H.Z., C.R.B. Saad, H.M. Daud, S.A. Harmin and S. Shakibazadeh. 2009. Effect of *Bacillus subtilis* on the growth and survival rate of shrimp (*Litopenaeus vannamei*). African Journal of Biotechnology 8: 3369–3376.

Farzanfar, A. 2006. The use of probiotics in shrimp aquaculture. FEMS Immunology and Medical Microbiology 48: 149–158.

Flegel, T.W. 2012. Historic emergence, impact and current status of shrimp pathogens in Asia. Journal of Invertebrate Pathology 110: 166–73.

Intaraprasong, A., K. Khemayan, T. Pasharawipas and T.W. Flegel. 2009. Species-specific virulence of *Vibrio* harveyi for black tiger shrimp is associated with bacteriophage-mediated hemocyte agglutination. Aquaculture 296: 185–192.

Jayasree, L., P. Janakiram and R. Madhavi. 2006. Characterization of *Vibrio* sp. associated with diseased shrimp from culture ponds of Andhra Pradesh (India). Journal of World Aquaculture Society 37: 523–532.

Kautsky, N., P. Ronnback, M.Tedengren and M. Troell. 2000. Ecosystem perspectives on management of disease in shrimp pond farming. Aquaculture 191: 145–161.

Lavilla-Pitogo, C., E. Leaño and M. Paner. 1998. Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent vibrios in the rearing environment. Aquaculture 164: 337–349.

Rengpipat, S., S. Rukpratanporn and S. Piyatiratitivorakul and P. Menasveta. 1998. Probiotics in aquaculture: a case study of probiotics for larvae of the black tiger shrimp (*Penaeus monodon*). In Advances in shrimp biotechnology. (ed. T.W. Flegel), pp. 177-181. National Center for Genetic Engineering and Biotechnology, Bangkok.

Rodríguez, J., Y. Espinosa, F. Echeverría, G. Cárdenas, R. Román and S. Stern. 2007. Exposure to probiotics and β-1,3/1,6-glucans in larviculture modifies the immune response of *Penaeus vannamei* juveniles and both the survival to White Spot Syndrome Virus challenge and pond culture. Aquaculture 273: 405–415.

Rouse, D.B., A. Potts and C.E. Boyd. 1999. Treatment of harvest discharge from intensive shrimp ponds by settling. Aquacultural Engineering 19: 147–161.

Somboon, M., W. Purivirojkul, C. Limsuwan and N. Chuchird. 2012. Effect of *Vibrio* sp. in white faeces infected shrimp in Chantaburi, Thailand. Kasetsart University, Fisheries Research Bulletin 36: 7–15.

Stein, T. 2005. *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Molecular Microbiology 56: 845–857.

Vaseeharan, B. and P. Ramasamy. 2003. Control of pathogenic *Vibrio* sp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Applied Microbiology 36: 83–87.

Walker, P.J., N. Gudkovs and P.A. Padiyar. 2011. Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) farms in Andhra Pradesh, India. Aquaculture 318: 277–282.

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