Validating the efficacy of a phytobiotic-based feed additive against white faeces syndrome

Efficacy of preventive strategies was tested in shrimp farms in Malaysia, Indonesia and India.

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White faeces syndrome (WFS) is considered a major cause of significant crop failures of *Penaeus vannamei* shrimp in Indonesia, Thailand, Vietnam and India. Early disease indications are evidenced by the presence of abundant white faecal strands in feed trays and water surface. The affected shrimp begin to eat less and show loose exoskeleton, distended midgut, pale midgut and hepatopancreas, and dark-coloured gills (Sriurairatana et al. 2014).

Microscopically, the hepatopancreas is characterised by the stripping of microvilli and the midgut appears to be filled with vermiform gregarine-like bodies (Sriurairatana et al. 2014). Production losses due to WFS are generally due to smaller harvest size and decreased survival, with the latter generally reduced by 20-30% but with cumulative mortalities as high as 50% during the summer period (Hou et al. 2018).

Outbreaks are usually associated with increased accumulation of organic matter and plankton blooms in ponds, a result of increasing stocking densities and feeding rates, as well as with increasing pond water temperatures (Mastan 2015; Raveendra et al. 2018). Since shrimp are highly exposed to exchanges of microflora between the pond environment and the digestive system, adverse environmental conditions favouring development of pathogenic organisms in the pond and destabilising the digestive bacterial community can potentially lead to WFS outbreaks (Hou et al. 2018).

Role of *Vibrio* spp and EHP

Pathogenic *Vibrio* bacteria along with the microsporidian *Enterocytozoon hepatopenaei* (EHP) have been reported to be associated with WFS. Five species of bacteria namely *Vibrio parahaemolyticus*, *V. fluvialis*, *V. mimicus*, *V. alginolyticus* and *Vibrio* sp. were isolated from the faeces of WFS affected shrimp (Mastan 2015). Among these species *V. parahaemolyticus* and *V. alginolyticus* were dominant in all diseased shrimp samples. *V. parahaemolyticus* is also associated with early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND). Progression of EMS/AHPND is regulated via bacterial cell-to-cell communication, the so-called quorum sensing, and driven by the production of a toxin that causes the sloughing of the hepatopancreas microvilli (Wang et al. 2011; Lee et al. 2015). Given the coincidental prevalence of WFS and EMS/AHPND, it has been suggested that the toxin produced by *V. parahaemolyticus* can contribute to the development of WFS (Sriurairatana et al. 2014). EHP spores were also identified in white faeces, with the prevalence of EHP in WFS infected ponds ranging from 16 to 96% (Rajendran et al. 2016; Tang et el. 2016; Ravendraa et al. 2018). EHP does not appear to cause mortality or to be the only agent causing WFS; however, EHP spores ingested via water or faeces will infect the hepatopancreatic microvilli, and will contribute to growth retardation in shrimp and increases the severity of the problem (Ravendraa et al. 2018).

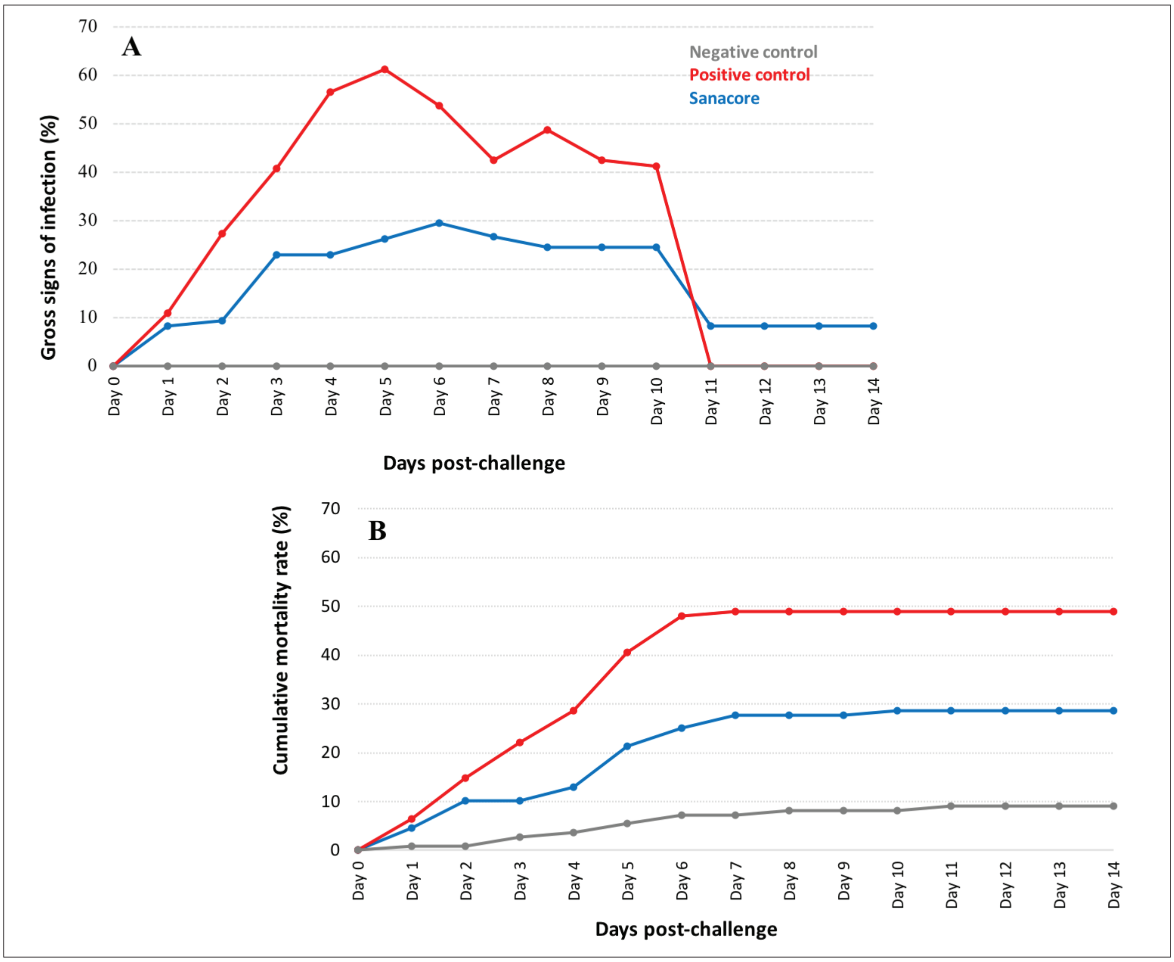


Figure 1. Sanacore supplementation on infection prevalence (A) and cumulative mortality (B) during 14 days post-challenge (n=4). Infection challenge was performed by feeding feed coated with the inoculums of *Vibrio* sp. (WFD5) to *Peneus vannamei*. A) At 5-6 days post-challenge, prevalence was reduced by 50-55% by the functional additive. B) At 14 days post-challenge, cumulative mortality was significantly reduced by 41% in relation to positive control. Statistical analysis by one-way ANOVA followed by Duncan test.

Control approaches

A first approach to control WFS is focused on preventive pond management measures such as application of chemical treatments before stocking to kill EHP spores (Sritunyalucksana et al. 2015; Thitamadee et al. 2016; Aldama-Cano et al. 2018), use of filtered water, efficient disposal of organic waste (e.g. shrimp toilets), maintenance of oxygen saturation and alkalinity within optimal levels, water application of probiotics to reduce *Vibrio* spp. populations, reduction of feeding immediately after outbreak detection, and removal of white faecal matter from ponds to avoid re-infection (Tang et al. 2016).

A second approach is to reinforce the prevention strategy via functional nutrition aimed at reducing pathogenic organisms in the digestive tract of shrimp. Indeed, recent evidence suggests a close association between decreased diversity and increased heterogeneity of the digestive bacterial community and WFS (Hou et al. 2018). SANACORE® is a functional feed additive based on a synergetic blend of phytobiotic extracts with a broad antimicrobial spectrum and with capacity to interrupt the quorum sensing communication system of pathogenic bacteria (Coutteau and Goossens, 2014). It can be incorporated into feed during feed manufacturing or via top-coating at the farm; both ways are aimed to deliver an adequate supply of natural antimicrobial activities that has been proven to promote a more diverse and robust microbial community in the digestive tract and therefore to reinforce prevention mechanisms (Robles et al. 2017).

The present article demonstrates the efficacy of this health promoting feed additive under infection challenge conducted under both controlled experimental and field conditions. Results support its use in preventive and combined preventive/corrective strategies against WFS.

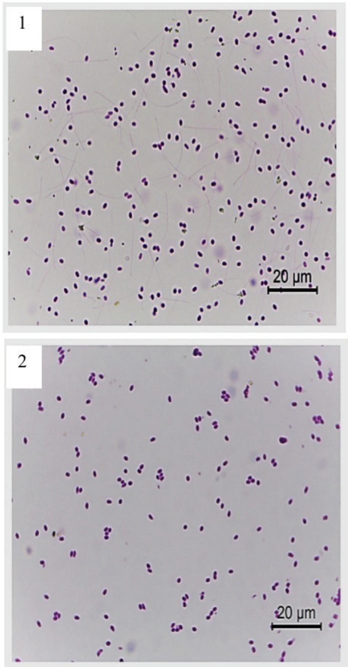
Pre-evaluation of preventive strategies

In recent years, *in vivo* and *in vitro* infection models have been developed to evaluate potential preventive strategies against WFS. ShrimpVet Lab (Vietnam) has developed and standardised an *in vivo* infection model that simulates the natural route of infection of WFS-associated *Vibrio* spp. The model consisted of shrimp *P. vannamei* (~1g) fed with control feed and feed supplemented with Sanacore at 3kg/tonne of feed over a duration of 14 days before an infection challenge. During challenge, control feed (positive control) and treated feed (Sanacore) were coated with an inoculum of WFD5 (i.e. a virulent strain of *Vibrio* sp. isolated from WFS-infected animals) and fed for 2 days. A non-infected control group (negative control) was also created.

Infection was confirmed when disruption of moulting and increased mortality at 24 hours post-challenge were observed, and gross signs of infection (e.g. white to yellow faecal strands, distended midgut and pale midgut and hepatopancreas) and mortality were monitored for 14 days. The highest prevalence was detected at 5- and 6-days post-challenge, during which the functional feed additive led to 50-55% reduction relative to the positive control group (Figure 1). Over the 14 days post-challenge, the prevalence and cumulative mortality were reduced by 40% and 41%, respectively (Figure 1).

These results are in line with similar improvements in survival after *in vivo* infection with the EMS/AHPND strain of *V. parahaemolyticus* (Tran et al. 2015). In both cases, the positive effects of this feed additive with a *Vibrio* challenge can be attributed to the combination of the direct bactericide/bacteriostatic properties and quorum sensing inhibition. The minimum inhibitory concentration against *Vibrio harveyi* and *V. parahaemolyticus* has been reported to be between 0.1-0.3%, suggesting *in vivo* inhibitory activity

A



B

Batch of spores	spores/µl	Germination rate (%)
1	1.05×10^6	61.0 ± 7.6
2	3.25×10^6	80 ± 5.0

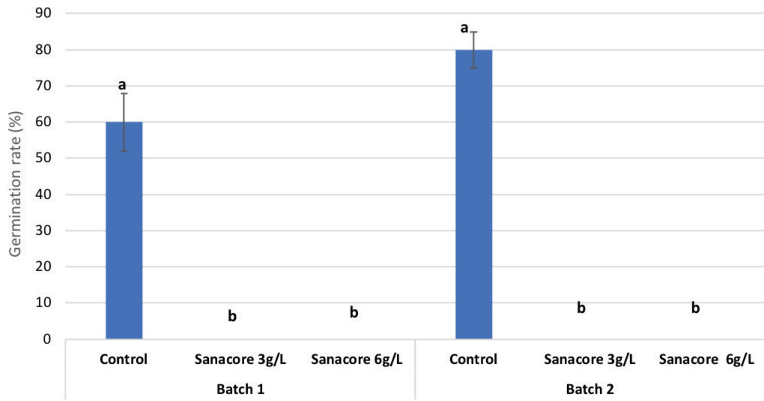


Figure 2. A) Representative views of germinated and non-germinated EHP spores: 1) Germination of the polar tubes from the spores purified from WFS-infected shrimp. 2) Spores that did not germinate. Phloxin B was used as a method to trigger germination as well as to stain the spores and assess germination rates. B) Number of spores in each batch and average percentage of spore germination after 120 min of incubation with Sanacore at two concentrations. Statistical analysis by one-way ANOVA followed by LDS PostHoc. Error bars represent standard deviation.

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of the functional additive at low concentrations in the digestive tract. At similar concentrations, interruption of the quorum sensing regulation of *V. parahaemolyticus* has been confirmed *in vitro* through the depressive effect of the functional additive on the signaling pathways determining bacterial density and toxin production (unpublished data).

Activity of potential inhibitors

Centex Shrimp (Mahidol University, Thailand) offers an *in vitro* bioassay to evaluate the activity of potential inhibitors of EHP (Aldama-Cano et al. 2018). Briefly, spores from hepatopancreas from two batches of infected shrimp (*P. vannamei*) were purified and incubated for 120 min with Sanacore at two different concentrations, 3 and 6g/L, equivalent to the dietary inclusion of the additive at 3 and 6kg/tonne of feed. Germination or spore viability was triggered by Phloxin B and assessed under light microscope by the discharge of the polar tube from the interior of the microsporidian (Figure 2A). This discharge seems to be the mechanism to invade host cells and complete the life cycle.

Results showed that while germination rate of EHP spores averaged 70% in the control groups for each batch, the feed additive at both concentrations completely inhibited germination (Figure 2B). Although the *in vitro* model may not accurately reflect EHP pathogenesis in the gastrointestinal (GI) tract of shrimp, this result certainly suggests a potential reduction of the viability of EHP spores in the GI tract and therefore of the severity of WFS. It must be emphasised that the effect of the phytobiotics on preventing germination and reducing spore infection rates of microsporidian parasites with similar polar extrusion mechanism to EHP has been demonstrated and thus some of these botanicals have been proposed for disease control (Maistrello et al. 2008).

Validation at farm level

Further to this, the efficacy of Sanacore against WFS under controlled laboratory conditions has been further validated under field conditions, with focus on its preventive, as well as its combined preventive and corrective applications.

The first trial was conducted in a shrimp farm in Penang, Malaysia where the objective was to evaluate the preventive effects of the functional additive. This farm was historically affected by WFS, EMS and white spot syndrome virus (WSSV). Two ponds were used as control and six ponds were fed feed supplemented with a preventive top-coated dose (1-3kg/tonne of feed) from 1 day of culture (DOC 1) to harvest. All ponds had the same density of 110 post larvae(PL)/m^2. Emergency harvest was required in control ponds at DOC 42 due to the high mortality and growth retardation. In treatment ponds, gross signs of infection were detected at DOC 60, but the outbreak was categorised as mild and production was continued until DOC 129. Overall, application of this feed additive as preventive strategy reduced mortality from 62% to 34% and recovered growth to acceptable levels after 14 days post-outbreak.

A second trial was conducted in a farm located in Subang-Karawang, Indonesia, an area previously affected by WFS and WSSV. This trial evaluated the preventive and corrective effects of Sanacore against WFS under two different supplementation strategies. Each strategy was applied to three ponds, each with a density of 100 PL/m^2. In the first strategy, the control feed was supplemented with a corrective top-coated dose (5kg/tonne of feed) and shrimp were fed only during the first 7 days following the detection of an outbreak. In the second strategy, the feed was formulated with a preventive dose (3kg/tonne of feed) and shrimp were fed from DOC 20 until harvest. The same treated feed was

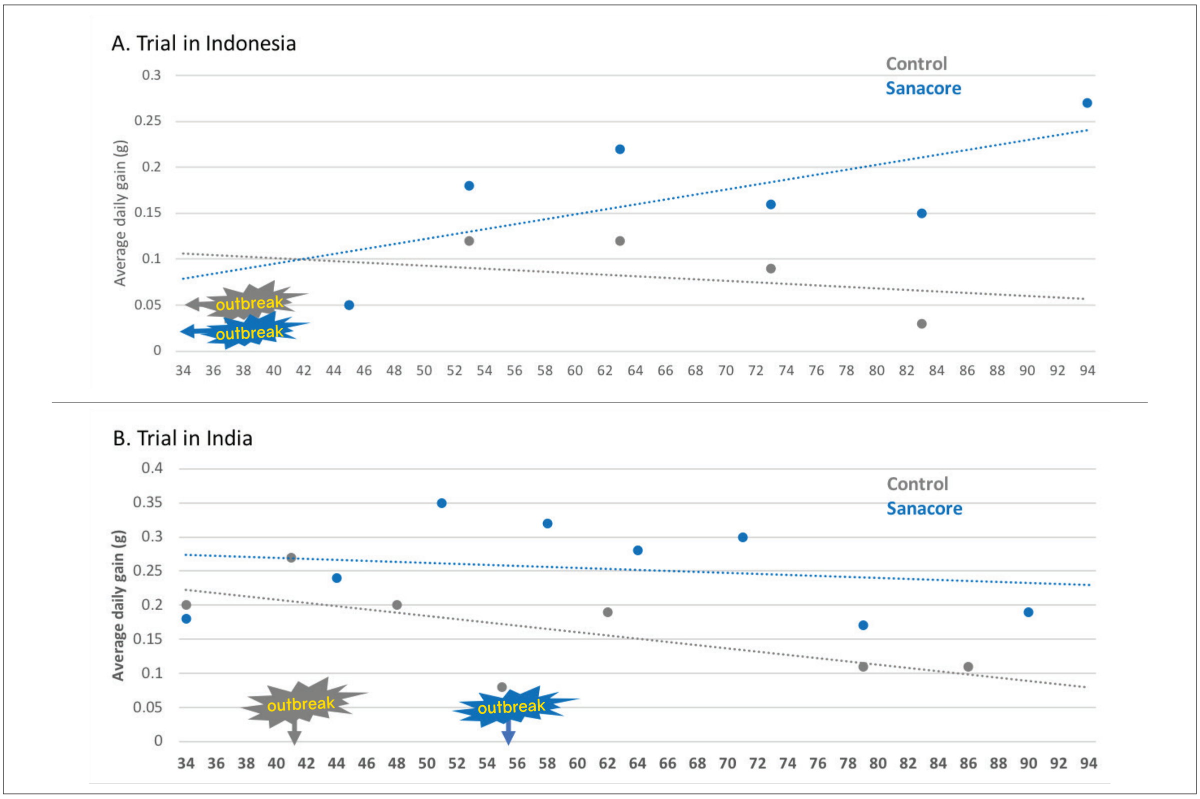

Figure 3. Average daily gain of shrimp in field trials conducted in Indonesia (n=3) (A) and India (n=1) (B), evaluating the effect of supplementing Sanacore. In both trials, the preventive dose of 3-4kg/tonne of feed was boosted with a curative dose (top-coated) of 4-5kg/tonne of feed during approximately 7 days. In Indonesia (A), outbreaks occurred at DOC 23-26. In India (B), outbreaks occurred at DOC 41 and 51 in control and treatment ponds, respectively. The curative dose in the treatment group was applied immediately after outbreak detection in both trials. Trendlines show that daily gain was recovered to acceptable levels or maintained under feed additive supplementation.

reinforced with an additional corrective top-coated dose (5kg/tonne of feed) and fed to shrimp exclusively during the first 7 days following outbreak detection (i.e. overall 8kg/tonne of feed).

All ponds received 7 days of the corrective dose after detection of an outbreak at DOC 23-26. Although the corrective strategy eliminated gross signs of infection in all ponds, only the combined preventive/corrective strategy recovered the average daily growth (ADG) to acceptable levels 20 days after the elimination of gross signs of infections (Figure 3A). While the corrective strategy allowed a full harvest at DOC 85, the combined strategy completed harvest at DOC 100 and achieved 25% higher average body weight and over three times more biomass.

A third evaluation was conducted in a farm in Odisha, near Balasore, a region with frequent outbreaks of WFS, WSSV, as well as other diseases (Laxpmappa 2017). In line with the trial in Indonesia, this trial aimed to validate the combined preventive/curative supplementation strategy of a WFS outbreak. Two ponds were stocked with post larvae (PL 10-11) at a density of 65 PL/m^2 at the beginning of September. This time of the year is typically more challenging in this region due to variable climate conditions with high variations in temperature and consequently in salinity.

During the trial, outbreaks occurred at DOC 41 and DOC 51 in control and treatment ponds, respectively. For the treatment pond, Sanacore was top-coated with a preventive dose (4kg/tonne of feed) in three out of four meals before an outbreak (DOC 1-51). After an outbreak and until gross signs of infection disappeared (DOC 52-58), shrimp were fed feeds reinforced with a top-coated corrective dose (4kg/tonne of feed) for two out of four meals. Then the dose went back to the preventive (5kg/tonne of feed) in three out of four meals until harvest (DOC 59-90). Besides a delay of about 10 days in the treatment pond, growth depression was observed in the control pond but not in the treatment pond (Figure 3B). The functional feed additive improved survival by 31%, final weight by 35%, and final biomass by 49%. Feed conversion ratio was also improved by 34% and overall production cost by 18%.

Conclusion

WFS is a concurrent problem requiring urgent control. The health prevention strategy must aim to reduce the growth of pathogens in the pond environment while promoting the health status of the shrimp stock. Pond management measures must go hand in hand with nutritional strategies aimed at stabilising the digestive bacterial community. Sanacore delivers in every meal a concentration of phytobiotics proven to inhibit *Vibrio* spp. and EHP isolated from WFS infected shrimp. This inhibition is consistent with the results obtained under different field conditions and can be attributed to the combination of bactericide/bacteriostatic properties, quorum sensing inhibition and antiparasitic properties. Field data indicate that the application of the corrective dose once the outbreak is detected helps to mitigate gross signs of infection. Field data also show that the combined preventive and corrective strategy can maintain or bring up growth rates and survival to pre-WFS levels.


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