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Inhibitory capacity of a microbial enhanced protein against *Vibrio* spp. in Pacific white shrimp

A series of trials demonstrated that diet supplementation confers a protective effect against the *Vibrio* species associated with AHPND.

By Darwin Zambrano and Sergio F. Nates

Acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS) has been associated with *Vibrio parahaemolyticus*. AHPND has spread to the Western Hemisphere and emerged in Mexico in early 2013. Since the first reported case in southern China in 2009, AHPND has also been observed in shrimp farms in other parts of Asia and the Americas. AHPND outbreaks have inflicted severe damage to the global shrimp farming industry with annual losses of more than USD 1 billion.

In 2017, the first cases of AHPND were reported in Ecuador with levels of *V. parahaemolyticus* and *V. vulnificus* of $3x10^4$ CFU/g and mortalities up to 100% in larval culture tanks. The disease affects larvae in early stages with mortalities up to 100% while in juvenile shrimp, mortalities of 30-40% have been reported during the first 20-30 days after stocking.

To offset high mortalities from AHPND, supplements have been incorporated into diets. These include a wide variety of probiotics and products such as organic acids, essential oils, biomolecules and in some cases, antibiotics. On the other hand, molecular diagnostics developed in recent years have made it possible to determine the presence and quantification of *Vibrio* loads by PCR techniques, using specific primers to detect the AP4 toxin. In addition, a more specific media (ChromAgar) was also developed to differentiate the growth of various strains of *Vibrio*.

A promising mitigation step

Recent studies on the development of practical diets for shrimp production systems using a microbial enhanced protein, ME-PRO® (Prairie Aquatech, South Dakota, USA), have shown to be a promising solution to produce eco-friendly aquafeeds. The protein is processed at a state-of-the-art plant using non-GMO (non-genetically modified) soybean meal and a naturally occurring, non-toxigenic fungi, *Aureobasidium pullulans*. The fermented co-product also offers significant amounts of short-chain peptides and free amino acids that confer excellent attractability and palatability properties. Results from numerous feeding trials have demonstrated that this product can sustain shrimp health, high-performance growth, and feed efficiency with inclusion levels as high as 50% of the total amount of ingredients in the diet.

The objective of this study was to evaluate the inhibitory capacity of ME-PRO at different doses (0.5%, 1% and 2%) on *Vibrio* spp. extracted from the midgut of Pacific white shrimp *Litopenaeus vannamei*. This study was carried out at the Microbiology and Molecular Biology Laboratory, QSBIOTECH, located in La Libertad, Santa Elena province, Ecuador.

Agar medium for analysis

For the analysis of total *Vibrio*, thiosulfate citrate bile sucrose agar (TCBS, Difco™) was prepared, supplemented with 2% sodium chloride and incubated at 35°C for 24 hours. The pH of a litre solution was adjusted to 8.6 and heated to boiling point with frequent stirring for 2 minutes. The solution was then placed on sterile plates and stored at 2°C-8°C until use.

ChromAgar *vibrio* medium was used for *V. parahaemolyticus* and *V. vulnificus*. Powdered agar (74.7g) was dissolved in 1L of distilled water, homogenised and heated to boiling at 100°C.

For isolation, purification and growth tests with probiotic strains, tryptic soy agar medium or TSA (Difco) was used. Powdered agar (40g) was dissolved in 1L distilled water. The solution was homogenised for 2 minutes and subsequently plated and autoclaved at 121°C for 15 minutes.

TSB (trypticase soy broth, Difco) was used for the isolation, purification and growth tests of strains of *V. parahaemolyticus, V. vulnificus* and several commercial probiotics. Powdered agar (30g) was dissolved in 1L distilled water. The solution was homogenised for 2 minutes and subsequently was plated and autoclaved at 121°C for 15 minutes.

Test doses

Sub-samples ME-PRO were resuspended in distilled water at the test doses (0.5%, 1% and 2%) and were added to all the agar and liquid media at three concentrations. All inclusions into the culture media at the indicated concentrations were used to measure the effect against *V. parahaemolyticus* and commercial probiotics from three companies.

Agar innoculations

Shrimp post larvae (0.1g) were weighed, macerated and dissolved in 200uL sterile distilled water. A 50uL sample was inoculated in TCBS agar and ChromAgar. All *Vibrio* and probiotic bacteria were resuspended in TSB medium. A 50uL sample was inoculated in TSA agar plates in the case of probiotics and on ChromAgar for the *Vibrio* species.

The results of bacterial growth count in agar were expressed as CFU/g for larvae macerates and mL for resuspensions. Growth curves for *Vibrio* species and probiotics were generated from readings of their optical density at 600nm using a Photometer 9300 and expressed as cell/mL.

PCR analysis

DNA extraction and evaluation protocols were completed using a lysis buffer and PCR techniques. The amplification conditions in the MiniAmp Plus thermal cycler for denaturation were 94°Cx0:30 sec; hybridisation 55°Cx0:30 sec; and polymerisation 72°Cx1:45 min. There were 25 cycles for both the first and second PCR tests.

Inhibitory capacity

Results showed that inhibitory capacity of ME-PRO was present at inclusion levels of 0.5%, 1% and 2% against *Vibrio* bacteria in macerates of shrimp larvae and inclusion in culture media ChromAgar Vibrio and TCBS.

Samples of shrimp larvae were evaluated to determine the presence of *V. parahaemolyticus* and *V. vulnificus*, as a routine protocol established to evaluate the health of the animals when shrimp larvae are being purchased from a hatchery. Daily, over seven consecutive days, five samples of live larvae from commercial hatcheries (suspected to be infected with AHPND) were collected and processed. When the microbial enhanced protein was incorporated in TCBS and ChromAgar culture media, results indicated inhibitory activity for *Vibrio* type 1 (yellow colonies) between 38%, 48% and 57% respectively. For type 2 *Vibrio*, the inhibition rate was 78% and 100% for the 1 and 2% doses and for *V. parahaemolyticus*, 38%, 45% and 62% respectively (Figure 1). The evaluation of shrimp larvae macerates using TCBS agar indicated an inhibitory activity of *Vibrios* in the presence of the protein (Figure 2). Similarly, in ChromAgar a reduction in *V. parahaemolyticus* occurred when the 0.5% dose was used (Figure 3).

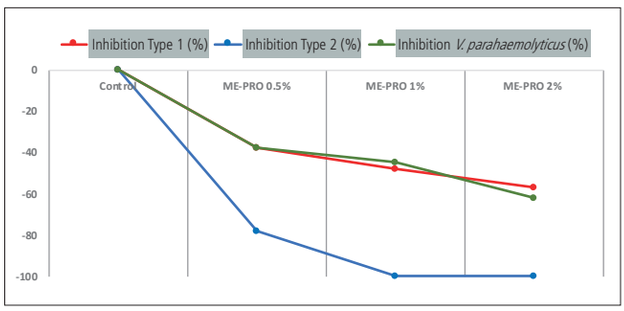

Figure 1. Inhibitory activity of ME-PRO® against *Vibrio parahaemolyticus* at three doses, 0.5%, 1% and 2%.

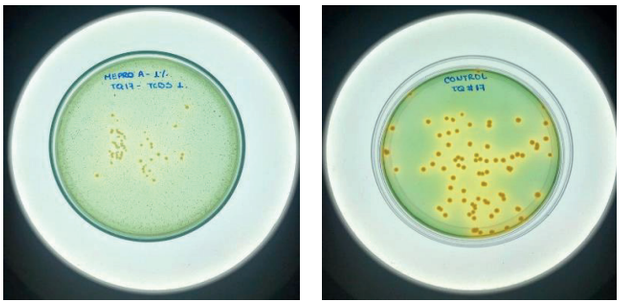

Figure 2. Photographs of ME-PRO® activity at 1% (left) versus control (right) using TCBS and shrimp larvae macerates.

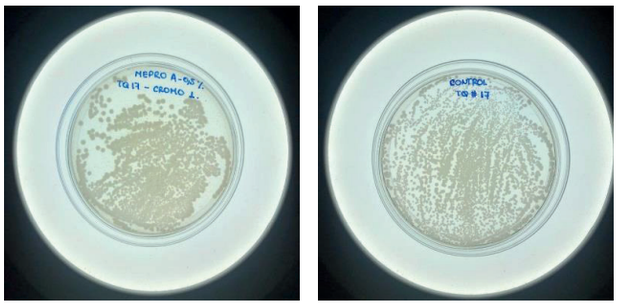

Figure 3. Photographs of ME-PRO® activity at 0.5% (left) versus control (right) using ChromAgar and shrimp larvae macerates.

VP AHPND inhibition dynamics with time

Three EMS-associated strains were isolated from larval samples, grown on ChromAgar and TCBS and tested using PCR. In addition, a strain of *V. parahaemolyticus* (*VP* AHPND) was cultured in liquid TSB medium with the inclusion of ME-PRO at 1 and 2%. Every 6 hours petri dishes were inoculated to verify the bacteria dynamics and the effect of the enhanced microbial protein on bacteria growth. The results showed that at 1 and 2% at time zero an inhibition effect of 27% was obtained. The growth of *V. parahaemolyticus* was compromised after the first 6 hours of culture for both dosages with a 40% reduction in growth. At 12 hours there was a slight recovery, and at 18 hours, the reactivation bacteria growth was back to normal (Figure 4).

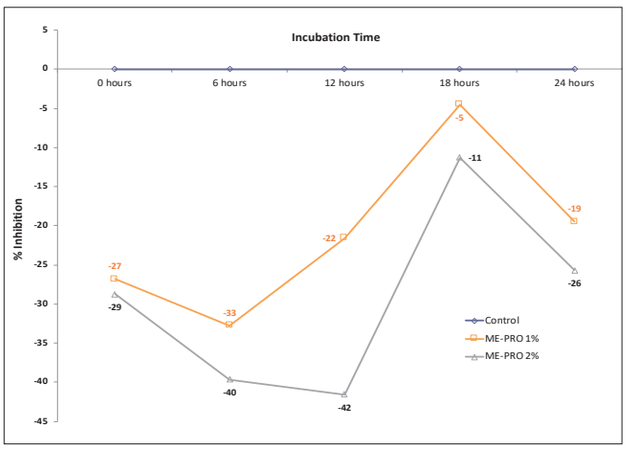

Figure 4. Inhibition (%) of *Vibrio parahaemolyticus* in the presence of ME-PRO® at 1 and 2% inclusion levels; incubation of 0hrs, 6hrs, 12hrs, 18hrs and 24hrs in ChromAgar Vibrio.

Inhibition of *V. vulnificus*

Strains of *V. vulnificus* were isolated from larval samples with disease signs and used in this test. In Ecuador, the association of *V. parahaemolyticus* and *V. vulnificus* at levels of 10^4 CFU/g has been determined to cause high mortalities.

Strains of *V. parahaemolyticus* and *V. vulnificus* were resuspended and incubated to reactivate for 1 hour at 32°C in TSB culture medium with the inclusion of ME-PRO® at 5%, 10% and 15%. Incubations were carried out immediately in ChromAgar *Vibrio* to determine the effect on bacteria growth. The results showed that ME-PRO at 10% could strongly affect *V. vulnificus* growth rate with a 72% reduction in bacteria populations at 18 hrs. At the 15% inclusion level the growth rate of *V. parahaemolyticus* was reduced significantly up to 63% (Figure 5).

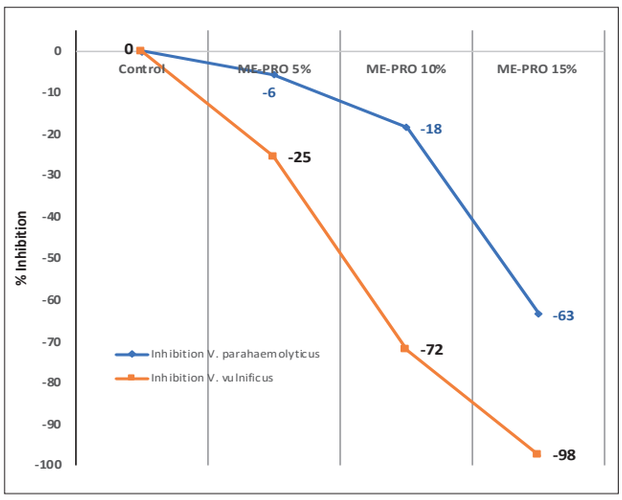

Figure 5. Inhibition (%) of *Vibrio parahaemolyticus* in the presence of ME-PRO® at 5%, 10% and 15% inclusion levels; incubation in ChromAgar *Vibrio*.

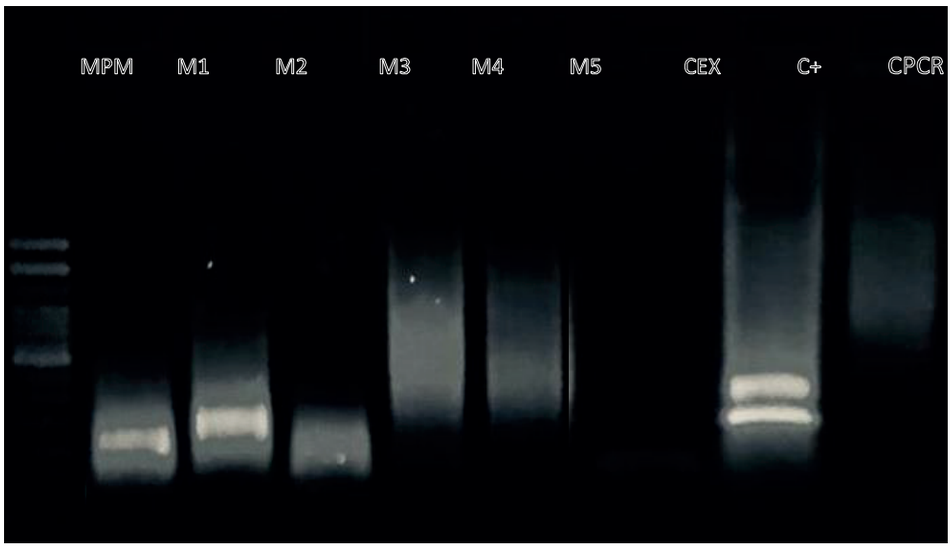

Figure 6. PCR amplification of *Vibrio parahaemolyticus*. Line 1: MPM; Line: 2,3 (PCR positive); line: 4-6 (PCR negative); line 7: Cex; line 8: C+ PCR. Line 9: CPCR.

Detection of VPQB020 with PCR

For this test, the strain VPQB020 (*V. parahaemolyticus*) previously identified by PCR was used. Bacteria were grown in ChromAgar *Vibrio* with ME-PRO inclusion levels of 1% and 2%. CFU were counted in plates and PCR of each of the bacteria that grew in the culture medium was carried out at the different inclusion levels.

The results of PCR amplification using the VP4 toxin primers indicated the detection of AHNPD in samples 1 and 2; samples 3-5 were negative. The extraction controls, PCR and positive PCR control were excellent (Figure 6).

The results of the bacterial CFU counts showed that a 2% inclusion level of ME-PRO inhibited the growth of *V. parahaemolyticus* load by up to 50% resulting also on a negative PCR for the AP4 toxin (Table 1).

Treatment	*Vibrio parahaemolyticus* (UFC)	% Inhibition	PCR EMS
Control	95,200	0	Positive
ME-PRO® inclusion			
1%	61,600	-35	Positive
2%	48,800	-49	Negative

Table 1. Growth of *Vibrio parahaemolyticus* in the presence of ME-PRO® at inclusion levels of 1%, 2%. Bacteria presence was detected using PCR.

Inhibitory activity with probiotics

An evaluation of the inhibitory activity of ME-PRO at inclusion levels of 1% and 2% against three strains of commercial probiotics was carried out. The strains were reactivated in TSB culture medium at these two doses and incubated at 30°C for 24 hrs. Subsequently, samples were incubated every 6 hours in TSA medium without salt. The results were expressed as CFU readings and bacteria levels counted in the agar plates versus CFU counts obtained in the controls. The ratio was used to find a relationship; commercial probiotics showed an inhibition of 71%, 38% and 6% with ME-PRO at 1%, while at 2%, it was 72%, 44% and 8%. (Figure 7).

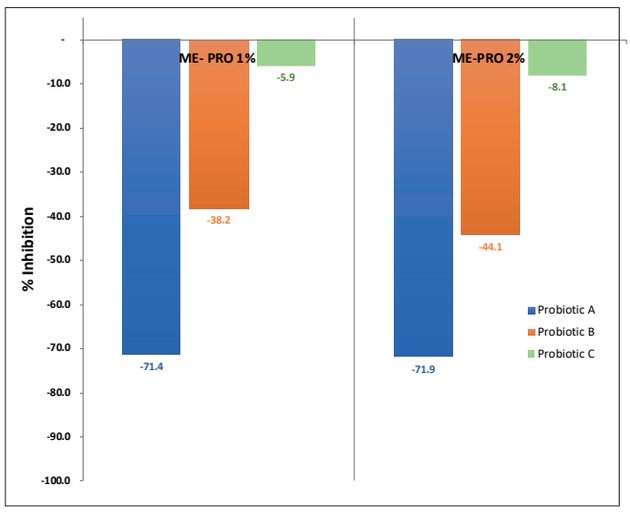

Figure 7. Inhibition (%) of three commercial probiotics in the presence of ME-PRO® at 1% and 2% inclusion levels; incubation in TSB.

Conclusion

The assessment in this study indicated that an inclusion level as low as 1% ME-PRO improved resistance to *V. vulnificus* and *V. parahaemolyticus* associated with high mortalities in shrimp aquaculture. Similar to previous studies, the results confirmed that supplementation into the diet of white shrimp, *L. vannamei*, will confer a protective effect against the *Vibrio* species associated with EMS.

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