Developing preventive feeding strategies to mitigate Penaeid shrimp sensitivity to EMS

By Stephane Ralite[1], Mathieu Castex[1], Dorothée Gotz[2], Lallemand Animal Nutrition

[1]Lallemand Animal Nutrition, [2]Lallemand Aquapharm

Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Diseases (AHPND) is one of the latest pathologies in shrimp that has caused some important economic losses in Asia and Latin America. Even though the causative agent and mechanisms are not fully understood yet, AHPND is likely to be due to the production of some toxins by bacteria. Different strains of *Vibrio parahaemolyticus* (VP) have been isolated from the field, which have shown the ability to induce EMS when ingested by the shrimp. Due to the high toxicity of the toxins and the important development rates of VP, preventive approaches appear as the most effective way to control this pathology.

In the past, the use of selected beneficial bacteria as dietary probiotics or bioremediation agents has shown effective against vibriosis in shrimps. Research presented at WAS 2015 by Lallemand Animal Nutrition described the search for effective probiotic bacteria strains and microbial fractions *for V. parahaemolyticus* prevention.

Looking for the best probiotic candidates

Hundreds of live beneficial microbes and microbial fractions issued from the Lallemand collections (including one of the largest private marine microorganisms collections fromLallemand Aquapharm, Oban, UK) were screened *in vitro* using a dual procedure for their ability to impact *V. parahaemolyticus's* growth and maintenance in solution. The most promising candidates were further assessed in vivo. Two different approaches were taken to screen the candidates. In the case of probiotics (live bacteria), candidates were screened for their antimicrobial properties against *V. parahaemolyticus* (antagonism assay). 331 different bacteria strains were selected based on their identification and their origin of isolation. They were screened *in vitro* using Lallemand Aquapharm unique screening platform: AquaSearch®.

Fourteen strains of interest were selected from this first round (Figure 1). The strain *P. acidilactici* MA 18/5M, already proven to reduce vibrios concentration in shrimp guts and registered as probiotic for use in shrimp for years, was used as a positive control (Figure 2). Classical microbiology co-culture experiments were then used to validate the specific antimicrobial activity of the probiotic strains against *V. parahaemolyticus*. From these *in vitro* testing, only four strains were retained as potential candidates for animal testing.

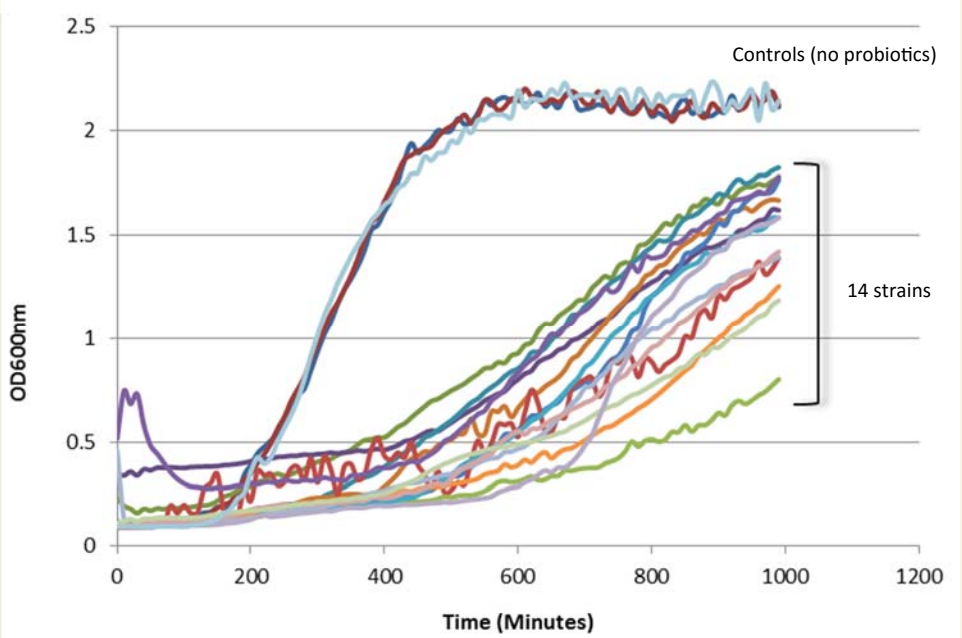

Fig 1 Growth of different *Vibrio Parahaemolyticus* strains (measured by the Optical Density at 600 nm) with or without candidate probiotic's metabolites issued from Lallemand Aquapharm bank.

Pathogen binding screening

In a separate screening approach (internal method), microbial fractions generated from several different strains of fungi were tested and selected for their ability to adhere to *V. parahaemolyticus* cells in vitro. Here, a proprietary *S. cerevisiae* yeast fraction was used as a positive control. The most promising fractions were selected for *in vivo* testing.

In vivo challenge in shrimp

The best candidates were tested on *Litopenaeus vannamei* juveniles

in an immersion challenge with 100ml of a culture of a virulent strain of *V. parahaemolyticus* able to induce AHPND at 1.1x 10^9 CFU/ml (grown in TSB + 2% NaCl (TSB+) at 28°C for 18h / testing done with Doctor Loc Tran at Minh Phu Aquamekong Shrimp Vet Lab in Vietnam).

Two trials were performed with *Litopenaeus vannamei* juveniles, of respectively 0.55±0.07g and 2.07±0.05g, that were fed for 21 days with diets supplemented or not with the tested candidates prior to the immersion challenge. Mortalities were monitored over 10 days and the cumulative survival for each diet was statistically compared to the control within each trial using a non parametric procedure and the Kaplan and Meier survival curves (Kaplan and Meier, 1958).

- **Probiotics:** Two of the potential probiotics selected and *P. acidilactici* MA18/5M (Bactocell®) were evaluated in triplicate. Bactocell® showed

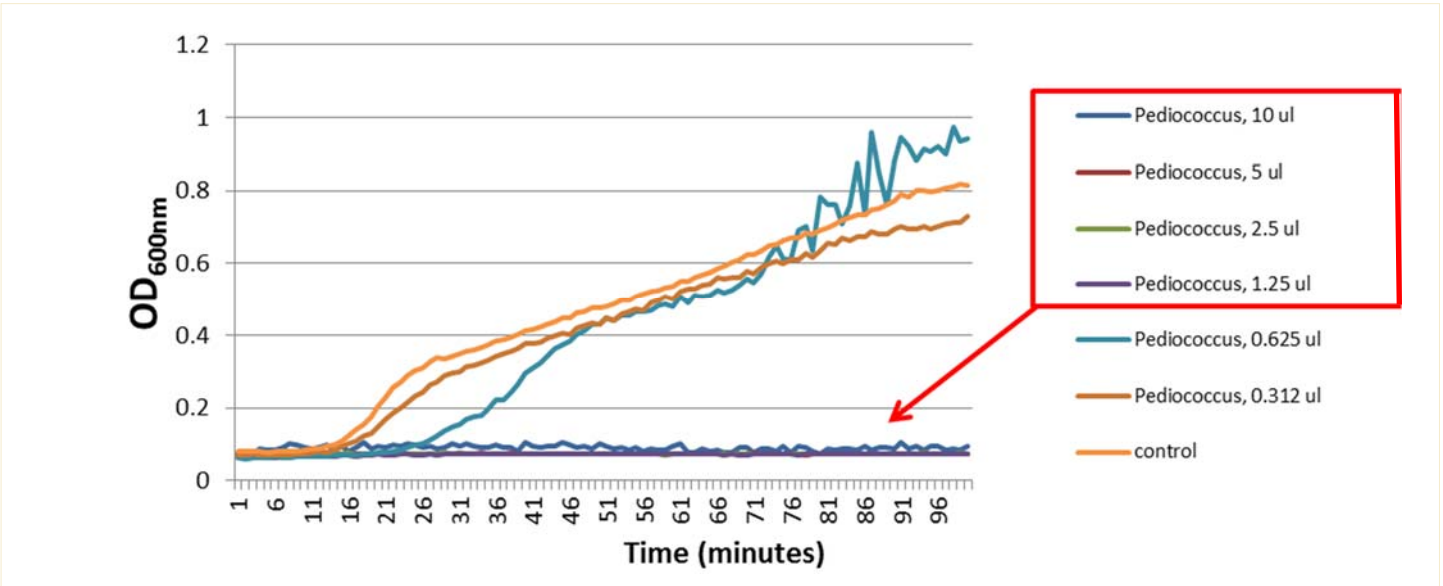

Fig 2. Growth of *Vibrio Parahaemolyticus* with or without *P. acidilactici* MA 18/5M (Bactocell®) metabolites.

protective effect against *V. parahaemolyticus* as the survival went from 40% in the infected control to nearly 70% with Bactocell®.

" ... these studies indicate that specific microbial based solutions are efficient tools for preventive strategies to mitigate the sensitivity of penaeid shrimp juveniles to vibriosis, and more specifically to AHPND. "

One of the candidates confirmed its potential to prevent mortality induced by *V. parahaemolyticus*, as the survival went slightly over 70% (no statistical difference was found between Bactocell® and this new candidates) (Figure 3). Kaplan and Meier survival curves were constructed for each diet and the curves were compared using the log-rank test to determine differences between curves and whether the trends in survival were different between treatments. The significance level was set at P = 0.05.These results confirm that Bactocell® can be considered as a valuable tool to limit vibriosis, as previously reported (Castex et al, 2009; 2010; Panigrahi et al, 2011). The second candidate only resulted in a slight numerical improvement compared to the control infected group, supporting the need to use *in vivo* studies to validate any *in vitro*

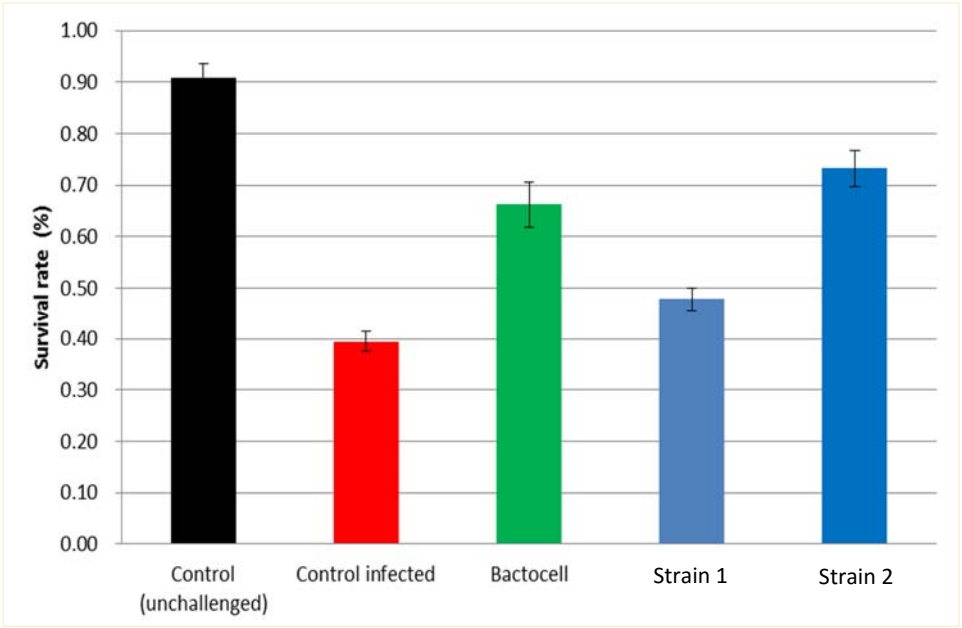

Fig 3. Survival of *L.vannamei* fed three different probiotics and exposed to an immersion challenge with a virulent *Vibrio Parahaemolyticus* strains .

screening process.

- **Microbial fraction:** Based on the *in vitro* assays, a product has been formulated with the most promising fungi fractions. An *in vivo* challenge was performed under the same conditions as the first challenge reported above, using in this case a dose response design.
- The formulated microbial fractions product showed a significant linear dose response effect on shrimp survival following the vibrio challenge with a final survival rate increase from 12.1 % in the control to nearly 60 % with the higher dose tested (Figures 4 and 5.) (Log Rank (Mantel-Cox), $p<0.01$).

In conclusion, these studies indicate that specific microbial based solutions are efficient tools for preventive strategies to mitigate the sensitivity of penaeid shrimp juveniles to vibriosis, and more specifically to AHPND. Prevention of EMS must likely be based on a multi-factorial program. We have demonstrated that two different types of specific microbial solutions (live probiotic bacteria and microbial fractions) can bring a contribution to such programs. Finally, the beneficial effect of *P. acidilactici* MA18/5M (Bactocell®) against vibriosis has been once again confirmed, and a new microbial fraction formula has shown some very good capacity to protect *L. vannamei* during an AHPND infection.

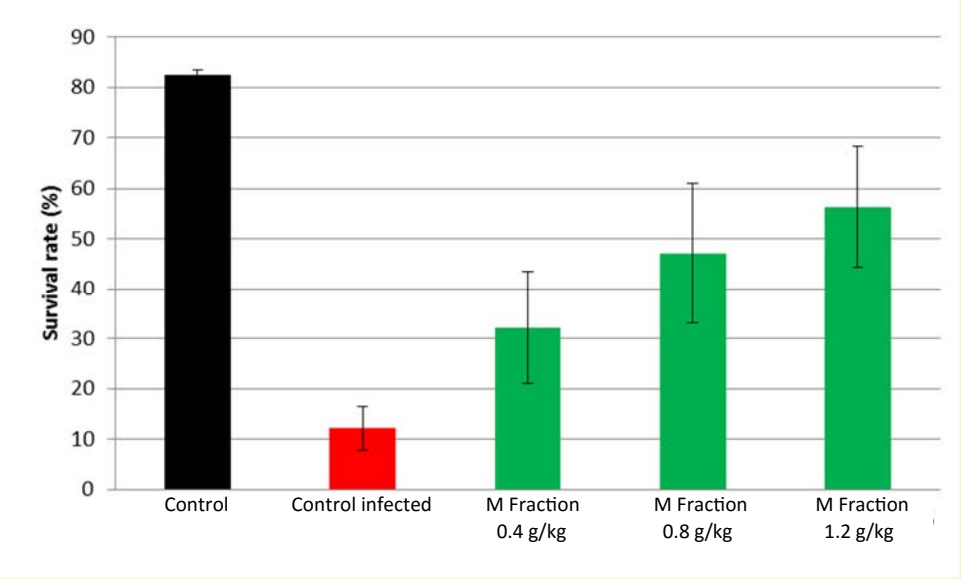

Fig 4. Survival of *L. vannamei* fed a new formulated microbial fraction product and exposed to an immersion challenge with a virulent *Vibrio Parahaemolyticus* strains.

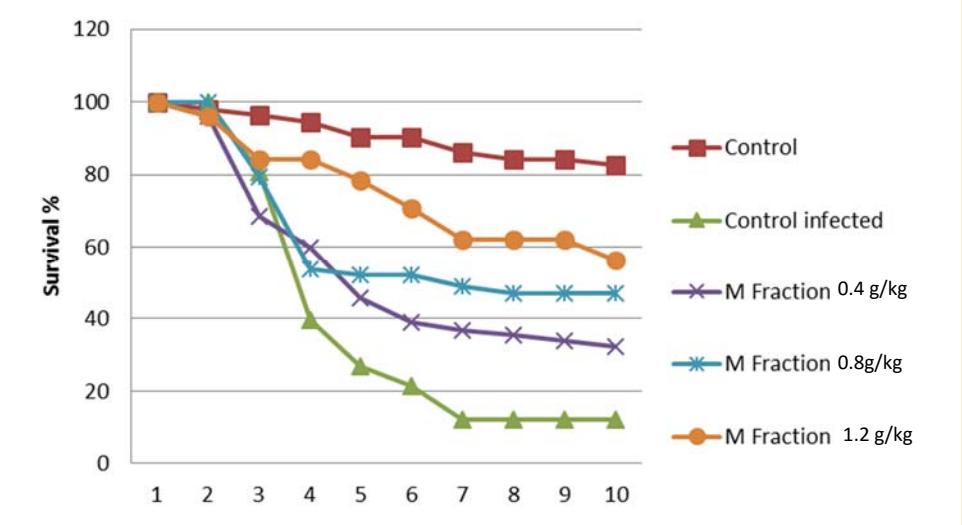

Fig 5. Survival rate during the course of the challenge experiment with the new formulated microbial fraction product.

The Authors



Mathieu Castex *Stephane Ralite* *Dorothée Gotz*

More information:

Stephane Ralite, Aquaculture Product Manager, Lallemand Animal Nutrition.

E: mail aqua@lallemand.com