BiOWiSH® TECHNOLOGIES

Research Study

BiOWiSH® MultiBio 3P

Effects of BiOWiSH® MultiBio Technology on Growth Performance, Gut Microbiota, and Pathogen Resistance in L. *vannamei* Shrimp

J. Barnes, J. P. Gorsuch, M. S. Showell, C. L. Kitts, A. L. Lawrence, R. S. Carpenter

Introduction

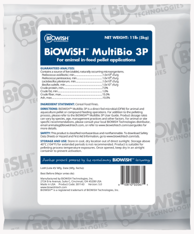
With the ever-increasing growth of the world's population, intensive farming practices in crop and animal production have received much interest and investment. In shrimp aquaculture, intensive farming practices have been in place since the 1990s. The shrimp industry employs millions of people, generating US$12–15 billion in global commerce.

The United Nations Food and Agriculture Organization (UN FAO) estimates that half of the world's seafood demand will be met by aquaculture in 2020, as wild-capture fisheries are overexploited and in decline [Moriarty, 1999]. Due to intensive farming practices in shrimp aquaculture (high stocking densities in hatchery tanks and ponds), the industry is beset with increasing disease issues, mostly due to pathogenic bacteria (especially Vibrio spp.) and viruses. Antibiotics have been used to treat these issues primarily from a clinical therapeutic perspective. That is, the disease was treated, rather than its underlying cause. Sub-therapeutic levels of prophylactic antibiotics are commonly used in large quantities, even when the pathogens are not evident. This is leading to a growing concern on the overuse of these technologies with the risk of increasing antibiotic resistance. In turn, this has increasingly led to the introduction of tighter controls on the use of antibiotics during production and particularly to meet export standards for developed markets.

The solution to disease management therefore lies in the field of farm management practices and microbial ecology, not pharmacology. The use of beneficial bacteria (probiotics) to displace the impact of pathogenic bacteria by competitive processes is a better remedy than utilizing sub-therapeutic administration of antibiotics. BiOWiSH® MultiBio is just such a probiotic product. It is a combination of beneficial probiotic bacteria of the genera Bacillus and Lactobacillus produced by a unique, proprietary process. These BiOWiSH® proprietary bacteria have been shown to improve shrimp growth, digestive tract bacterial microbiota, and survival [Moriarty, 1999; 1997].

The results from the following studies show the beneficial effects of BiOWiSH® MultiBio on improving overall growth performance, combined with a positive impact on shifting intestinal microbiota to improve gut health, and increasing Vibrio pathogen resistance in L. vannamei shrimp.

BiOWiSH® MultiBio 3P



- All-natural direct-fed microbial (DFM) for pelleted and extruded feed manufacturing

Available Sizes

- 1kg/2.2lbs
- 5kg/11lbs
- 10kg/22lbs
- 25kg/55lbs

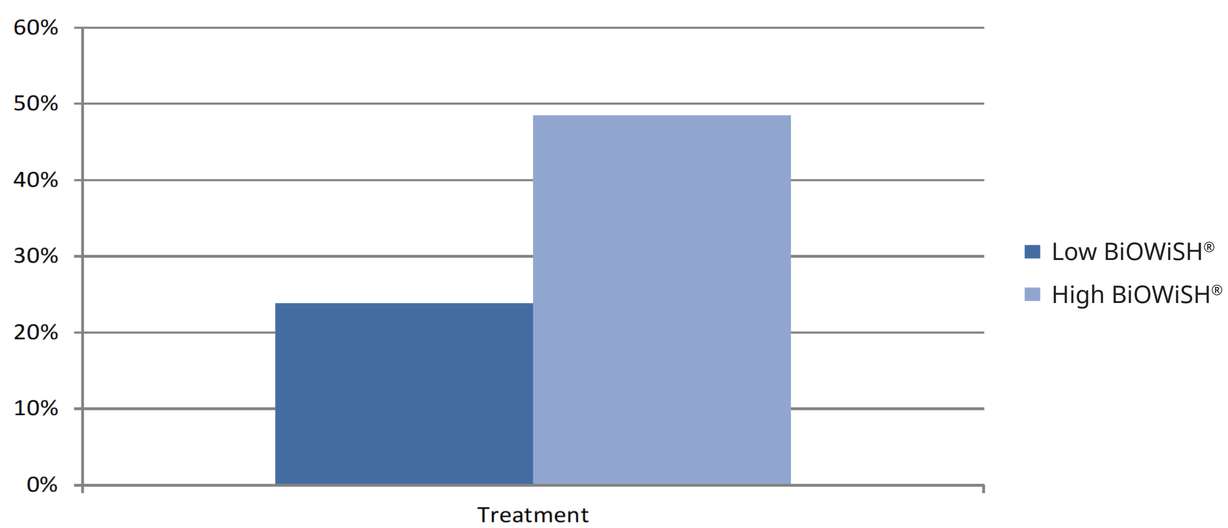
I. Growth Performance of L. vannamei Treated with BiOWiSH® MultiBio (In vivo)

BiOWiSH Technologies conducted a university study in 2014 to determine the effects of BiOWiSH® MultiBio on shrimp growth in a raceway-based system. A 53-day feeding study was conducted under the following conditions:

- Shrimp (PL10s) were placed in a zero water exchange raceway system. Stocking density was 3,384 shrimp/m2/tank.
- Treatments
    1. Control (no BiOWiSH® MultiBio)
    2. BiOWiSH® MultiBio (0.25 ppm—low dose)
    3. BiOWiSH® MultiBio (2.5 ppm—high dose)
- BiOWiSH® MultiBio was dissolved in water and added daily as a solution during the morning feeding.
- All tanks were fed 40% protein diets based on a diminishing Feed Conversion Ratio (FCR) through days 0–5. During days 0–3, 9g of 45% protein standard reference diet (SRD) were also added daily.
- Feed protein rate and FCR were evaluated.
- Samples of L. vannemai were taken every 3 days and frozen for future analysis.

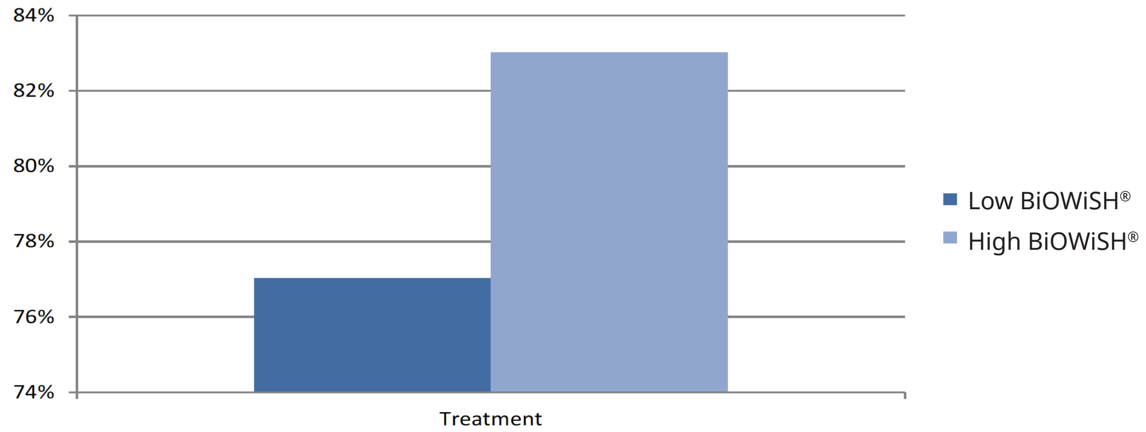
After 53 days, the study was terminated and average shrimp weight, total biomass, and percent survivability in each tank was determined. As shown in Figures 1, 2, and 3 for the 53-day study, addition of BiOWiSH® MultiBio at the higher dosage increased the average size of shrimp by almost 50%, nearly an 83% increase in shrimp biomass, and a 60% improvement in survivability versus best feeding practices over the control (no BiOWiSH® MultiBio). Results with BiOWiSH® MultiBio at the lower dosage gave proportionately similar results.

Figure 1: Percent increase in average size of individaul shrimp at 53 days



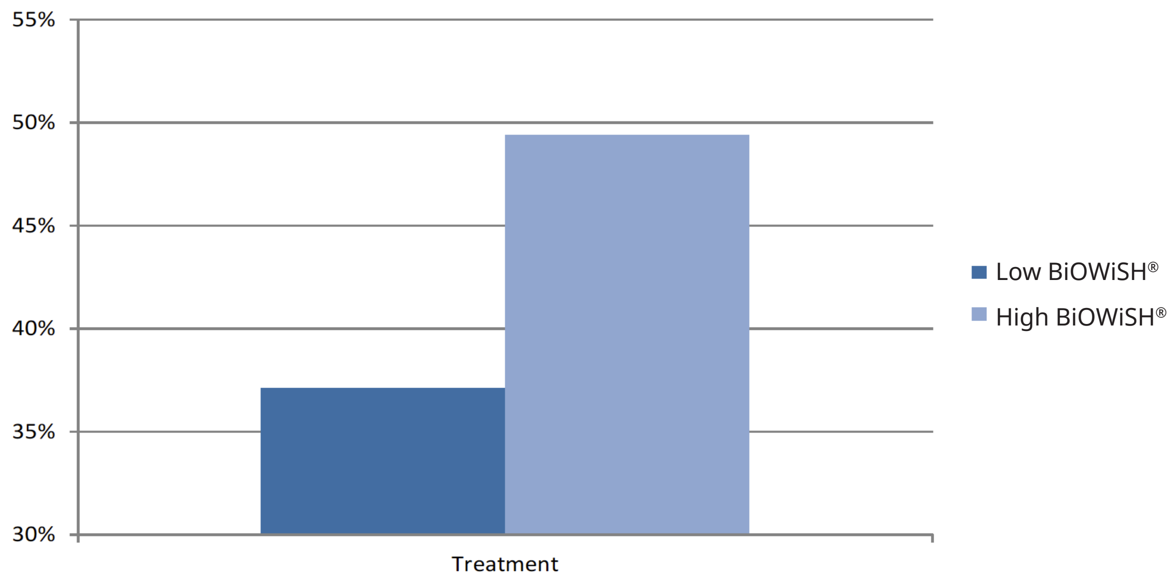
Control (no BiOWiSH® MultiBio) is baseline and therefore not shown. BiOWiSH® MultiBio treatments illustrate percent increases in addition to Control shrimp production size gains.

Figure 2: Percent Increase in Total Biomass per Treatment at 53 Days



Biomass percent increase is a function of additional effects of the individual shrimp size and percent survivability. Control (no BiOWiSH® MultiBio) is baseline and therefore not shown. BiOWiSH® MultiBio treatments illustrate percent increases in addition to Control shrimp production biomass gains.

Figure 3: Percent Improvement in Survivability at 53 Days



Control (no BiOWiSH® MultiBio) is baseline and therefore not shown. BiOWiSH® MultiBio treatments illustrate percent increases in addition to Control shrimp production survivability.

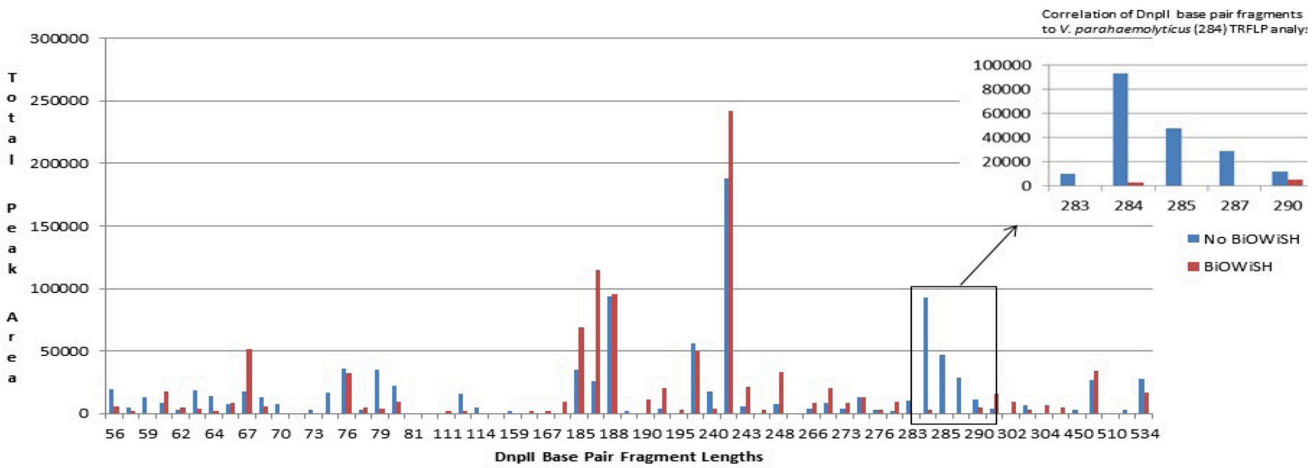
II. Microbiota Shifts in the Intestines of L. vannamei Treated with BiOWiSH® MultiBio

Frozen samples of stock shrimp and shrimp from each treatment tank at the termination of the growth study above were shipped on dry ice to another U.S. university for bacterial taxonomic profiling. Intestines of the shrimp were extracted and pooled together for each treatment. DNA was extracted from the samples, and taxonomic profiles of bacteria in the samples were created using a Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis [Kitts, 2001]. Bacterial profiles were compared between treatments to look for significant differences.

The results showed that BiOWiSH® MultiBio significantly shifted the microbial community present in shrimp intestines. Microbial analysis of shrimp from the BiOWiSH® MultiBio treatment group showed a significant difference ($p = 0.026$) in their microbial profile versus shrimp that did not receive BiOWiSH® MultiBio. Furthermore, one of the key differences between those shrimp treated with BiOWiSH® MultiBio versus those that were not treated can largely be correlated to the reduction in the DnpII restriction fragment length of V. parahaemolyticus from a prior study using BiOWiSH® technology (Figure 4).

And while additional research is ongoing, these results show that routine use of BiOWiSH® as a feed supplement additive from nursery stage through grow out positively impacts the gut microbiota of shrimp.

Figure 4: Bacterial Taxonomic Profile Comparison of L. *vannamei* Gut Flora treated with BiOWiSH® MultiBio versus Control (no BiOWiSH® MultiBio)



III. Inhibition of Pathogenic Bacteria (Vibrio spp.) Using BiOWiSH® Technology (In vitro)

To investigate more directly the Vibrio inhibitive properties of BiOWiSH® MultiBio, University and BiOWiSH® researchers conducted (in vitro) screening studies on BiOWiSH® MultiBio probiotics to better understand its inhibitory effects in vivo.

Methods: Growth inhibition zone testing on agar plates

BiOWiSH® probiotic organisms and Vibrio spp. were grown and cultured with their appropriate growth media: either MRS or TSB broth for BiOWiSH®, or Photobacterium Agar for Vibrio spp., or Marine Broth for V. parahaemolyticus. BiOWiSH® probiotics and Vibrio spp. were then inoculated onto agar plates using proprietary screening methods. Inoculated plates were allowed to then incubate and develop at room temperature (~21°C) for 48 hours prior to reading.

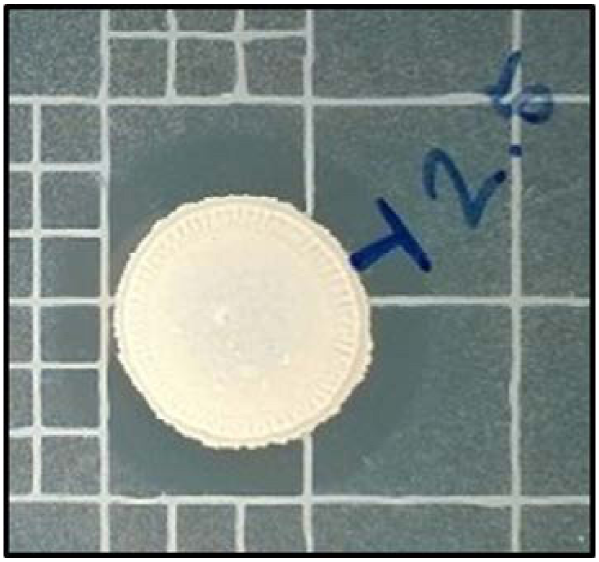
Inhibition of Vibrio growth was noted by recording and quantifying defined inhibition zones for each of the species tested against BiOWiSH® probiotics.

Results

Zones of inhibition were measured using the radius of inhibition for each plate, and then using the median of values for each of 3 replicates (Figure 5). The 3 replicates were then averaged to obtain an average value of inhibition for each Vibrio spp. The graphs below show the magnitude of inhibition for 3 key BiOWiSH® organisms (Figures 6 & 7).

These results show solid evidence that BiOWiSH® can inhibit growth of a number of Vibrio species associated with pathogenic effects in L. vannamei shrimp.

Figure 5: Zone of Vibrio Inhibition



Typical zone clearing assay showing the BiOWiSH® organism (large creamy-white circle) and the inhibition (clearing) zone generated in a field of Vibrio (hazy background).

Figure 6: Average Zone of V. parahaemolyticus Inhibition

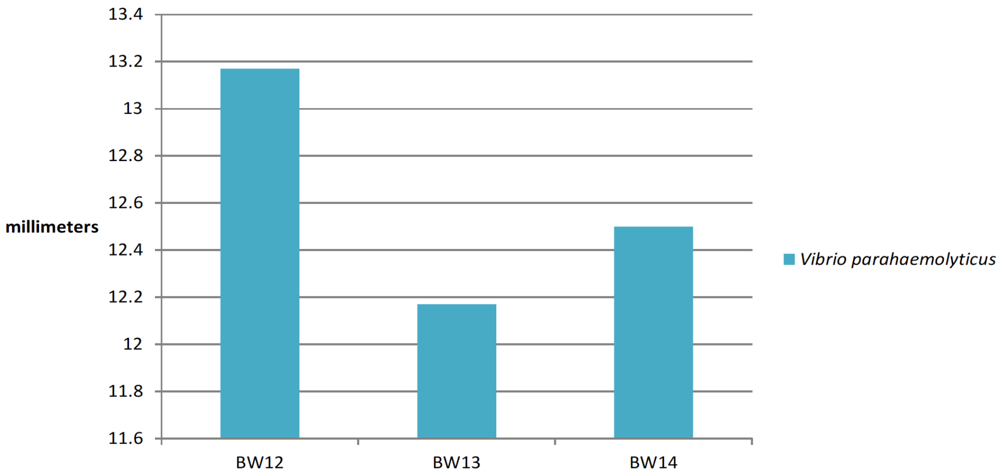
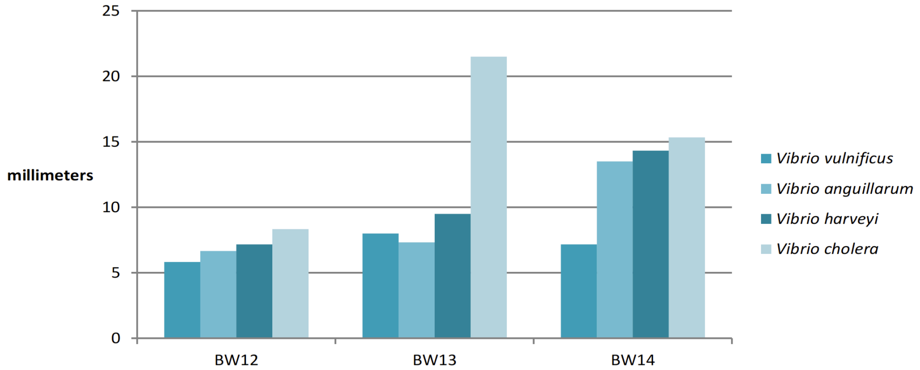


Figure 7: Average Zones of Inhibition for Vibrio spp.

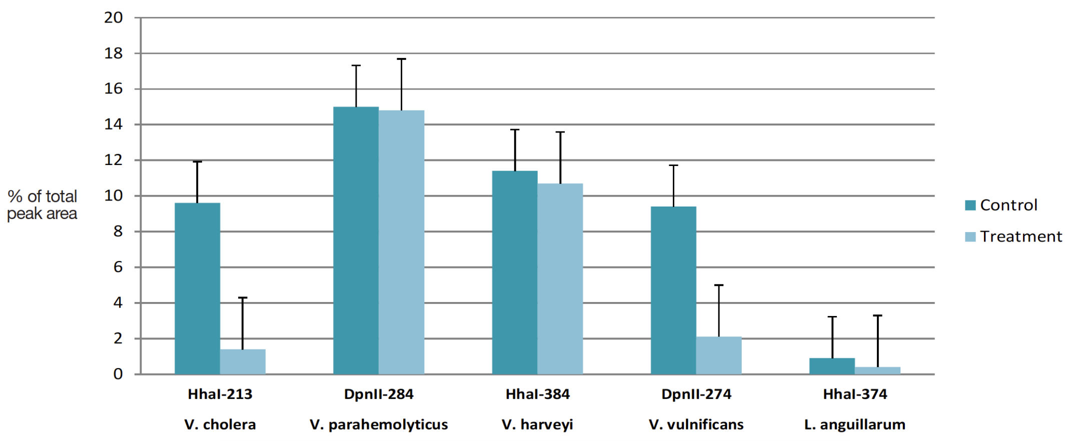


IV. Effects of BiOWiSH® Technology on the Bacterial Gut Flora of L. vannamei in a Zero Water Exchange System (In vivo)

Lastly, shrimp fed the BiOWiSH® Technology as a supplemental feed additive in a similar intensive, zero water-exchange growth study were sent to a U.S. university for bacterial profiling of their intestinal microflora. Intestinal DNA was extracted and taxonomic profiles of the bacteria were created using the TRFLP protocol [Kitts, 2001]. A double profile (using two different restriction enzymes) was generated to maximize differentiation. Bacterial profiles were compared between treatments to look for significant differences. In addition, several species (Vibrio spp.) of bacteria were analyzed and compared to determine the effects of BiOWiSH® Technology addition on the prevalence of these shrimp pathogens.

Though difficult to fully quantify, the in vivo results showed a clear decreasing trend in the relative levels of Vibrio spp. in the BiOWiSH® treated shrimp (Figure 8).

Figure 8: Bacterial Profiles of Shrimp Intestines BiOWiSH® versus Control (no BiOWiSH®)



V. Early Mortality Syndrome (EMS) Challenge Study in L. vannamei Treated with BiOWiSH® (In vivo)

Shrimp from all exposure groups in the aforementioned BiOWiSH® MultiBio growth study were sent to a leading U.S. research university for testing in an EMS challenge study. EMS is caused by Vibrio parahaemolyticus strains [agent causing Acute Hepatopancreatic Necrosis Disease (APHND)]. Five 90L tanks were stocked with shrimp from the BiOWiSH® MultiBio high dose rate. Four 90L tanks were stocked with shrimp from the BiOWiSH® MultiBio low dose rate, and three 90L tanks were stocked with shrimp from the control or with no BiOWiSH® MultiBio added. Two additional tanks were stocked with SPF [specific pathogen (AHPND-causing V. parahaemolyticus) free] shrimp which had been reared at this university as an additional control group. The negative control tanks were kept isolated in a separate building and fed before the V. parahaemolyticus challenge tanks to ensure biosecurity.

BiOWiSH® MultiBio was provided in powdered form and was added directly to the tank water. The low-dose group was exposed to 2.5 ppm daily and the high-dose group was exposed to 25 ppm daily. The feed was a 23% protein shrimp pellet.

BiOWiSH® MultiBio was applied daily to each tank water treatment during the morning feeding period. On the day that Vibrio parahaemolyticus was introduced into each tank (day 0 post-infection), BiOWiSH® MultiBio was added to each tank approximately 2 hours before the Vibrio challenge.

On day 0 of the Vibrio challenge, all aquaria were fed a commercially pelleted diet (Rangen 40% protein) which had been soaked in a broth containing the AHPND-causing V. parahaemolyticus in two doses.

All tanks were again re-challenged a second time (on day 2 post the initial infection), in the same manner as day 0. All aquaria were then checked once a day for moribund and dead animals. Moribund animals were preserved in fixative to confirm AHPND infection by histopathology. The study was terminated after 10 days with live animals counted as survivors (Table 1).

Results in Table 1 show that BiOWiSH® MultiBio-fed shrimp increased their resistance to V. parahaemolyticus by 37% to 49% indicating a significant increase in V. parahaemolyticus resistance. Also, increasing the dosage by tenfold did not proportionately increase resistance, indicating that lower dosages could be equally effective.

Yield Increase	Control Program + BiOWiSH® Crop	80% Control Program + BiOWiSH® Crop
Red Fruit	15.6%	4.1%
Green Fruit	30.1 %	26.2%

Discussion and Conclusion

In the 53-day growth performance study, BiOWiSH® MultiBio showed an overall improvement of almost 50% in the average size of shrimp, an almost 83% increase in total biomass, and a 60% improvement in survivability over the control (no BiOWiSH® MultiBio). These findings corroborate other studies that have shown dietary supplementation of a Lactobacillus probiotic for increased growth rates in shrimp [Venkat et al., 2004]; and separately that a Bacillus probiotic can increase survivability of shrimp [Rengpipat et al., 1998].

As regards the mode of action from probiotic-supplemented diets, improved survivability has been associated with increased stress resistance from both direct and indirect immuno-modulatory effects [Marteau et al., 2002; Gill, 2003].

In aquatic organisms, gut bacterial microbiota are mainly comprised of Gram-negative bacteria [Vine et al., 2006]. Studies have shown that such microbiota can be modified by the addition of Gram-positive bacteria in the diet [Ziaei-Nejad et al., 2006; Vieira et al., 2008]. Results from BiOWiSH® MultiBio testing also show a significant shift ($p = 0.026$) in the microbiota of shrimp fed BiOWiSH® MultiBio versus those not fed BiOWiSH® MultiBio. Shifts or modifications of gut microbiota such as these can be an important tool in the prevention of disease due to beneficial bacteria (probiotics) displacing pathogenic bacteria by competitive processes [Gomez-Gil et al., 2000].

With the increasing intensive practices in aquaculture production, disease problems caused by bacterial pathogens of the genus Vibrio have emerged. Widespread use of antibiotics have given rise to antibiotic resistant strains of these pathogenic bacteria, causing concerns of antibiotic resistance transfer to human pathogens and gut bacteria. Beneficial bacteria

(probiotics) have been shown to have an inhibitory effect on these pathogenic bacteria [Moriarty, 1999; Aquafeed.com, 2011; Gatesoupe, 1999; Verschuere et al., 2000; Irianto and Austin, 2002; Balcazar et al., 2006; Gatesoupe, 2008].

In research studies, BiOWiSH® MultiBio probiotics show a particular inhibitory effect on several Vibrio spp. known to cause pathogenic effects in L. vannamei. BiOWiSH® MultiBio, supplied as a feed additive, further showed a trend of decreasing Vibrio spp. in the gut of L. vannamei shrimp.

Combined results of both BiOWiSH® MultiBio low and high doses showed a significant 81% increase in survivability over the positive control when challenged with pathogenic V. parahaemolyticus.

These results have shown clear evidence of the beneficial effects of BiOWiSH® MultiBio on shrimp production and improved disease resistance, particularly when administered as part of an overall feed regimen starting at the nursery stage through grow out.

Further research and field trials continue on the positive effects of BiOWiSH® MultiBio feed additive technologies in aquaculture.

Acknowledgements

BiOWiSH Technologies Inc. would like to gratefully acknowledge the following for their contributions to this work:

Dr. Donald Lightner and staff at University of Arizona for providing his equipment and facilities in the EMS Challenge Study.

Ms. Alice Hamrick at California Polytechnic State University for completing the in vitro data set on Vibrio spp. inhibition

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Contact us:
aquaculture@biowishtech.com
+1 312 572 6700
biowishtech.com

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