Organic acids - synergy at work to prevent vibriosis and promote growth in shrimp

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Disease imposes major yield-limiting effects on production and represents the most significant constraint on the development and sustainability of the shrimp industry. Infections by bacterial diseases such as the acute hepatopancreatic necrosis disease (AHPND) associated to *Vibrio parahaemolyticus*, or other vibriosis, are generally triggered by stressful environment (i.e. poor water quality, low oxygen, high temperatures, etc.) and can cause up to 100% mortality in post-larvae shrimp (Dash *et al.* 2016).

The increasing levels of disease are associated with the intensification in culture practices, thus there is an ever-growing importance on the containment of pathogens. In this aspect, shrimp farming brings some unique challenges as compared with fish production. As an invertebrate, shrimp do not possess an acquired immune system and rely solely on their innate immune responses that are generally characterised as rapid (i.e. hours to days). This translates into higher susceptibility to pathogens than fish and in greater need of antimicrobial treatments, hygiene, and biosecurity measures.

The use of antimicrobial agents such as disinfectants and fungicides has become an integral part of many shrimp farming operations. Antibiotics are still being used, often as immediate resort to control infections. However, antibiotic usage without adequate protocols is inefficient due to the risks associated to antimicrobial resistance dissemination (Thornber *et al.* 2019). Indiscriminate usage also poses a significant economic risk since positive testing for prohibited antibiotics or residues above maximum permitted levels can result in the rejection of importations from a whole country.

The use of alternative antimicrobial options such as phytobiotics, probiotics or organic acids is common across production species. Specifically, organic acids are characterised by their antimicrobial and growth-promoting actions as well as cost-efficient application. Maximum antimicrobial efficacy is achieved by specific combinations of organic acids that act synergistically to damage the integrity of the bacterial cell wall and alter cytoplasm pH. This synergistic action occurs under a species-specific digestive environment, particularly pH, that must be considered when formulating organic acid blends. This also explains why single organic acids or blends that have proven efficacy in poultry or swine do not show any effect in tilapia or shrimp (Hosein *et al.* 2017).

Bacti-nil®Aqua is a cost-effective blend of organic acids, specifically formulated for aquatic species and with long-established use in shrimp feed. The additive can be applied during feed manufacturing in the feed mill as well as top-coated on the feed in the farm. In this article, we present two separate studies that contribute to the proven efficacy of this additive, firstly as preventive strategy to reduce the impact of *Vibrio* spp. infection, and secondly as growth promoter.

A preventive tool to reduce the impact of *V. parahaemolyticus*

The first study focused on the bactericidal efficacy of Bacti-nil® Aqua against *V. parahaemolyticus*, both under *in vitro* and *in vivo* conditions. This study was conducted at the Centro de Investigación en Alimentación y Desarrollo (CIAD) in Sinaloa (Mexico).

The minimum inhibitory concentration (MIC), the lowest concentration at which a substance inhibits bacterial growth after incubation is a commonly used *in vitro* method to assess the potential susceptibility of a bacterial pathogen to a feed additive. Here, the tube dilution method according to McDermott *et al.* 2005 was used to test the additive efficacy against *V. parahaemolyticus* (M0904). The test was carried out under a pH range of 7-8 to mimic the digestive pH of shrimp. The additive was compared against two other commercial products. Product A was a blend based on different short-chain organic acids at overall concentration similar to Bacti-nil®Aqua, while product B was a monoacid solution with double overall concentration than the other two treatments. Table 1 shows that the additive generally resulted in the lowest MIC in relation to the other two commercial products. Given the similar and lower acid concentrations of the additive in relation to products A and B, respectively, the more positive results can be likely attributed to the superior synergistic action of its active components under the tested pH range.

In vitro antimicrobial effects by the additive were further validated under *in vivo* conditions. *Penaeus vannamei* of 4-5g were stocked in nine aquariums of 10L capacity at a density of 10 shrimp/aquarium, with saline water (35ppt) maintained at 29°C during the 24-hour experimental period. Infection by *V. parahaemolyticus* (M0904) was via immersion with a 50mL inoculum per aquarium (1 x 10^8 CFU/mL). A commercial shrimp feed formula was top coated with the additive. A total of three groups were evaluated: 1) negative control (non-supplemented and non-infected, n = 3), 2) positive control (non-supplemented and infected), and 3) Bacti-nil® Aqua at 0.3% (supplemented at 3kg/tonne feed and infected, n =3). Shrimp were fed 15 minutes after infection and then every 3 hours. Symptoms of infection such as reddish colouration of antenna, muscular opacity, discolouration of hepatopancreas and decubitus position, were detected 1-hour post-infection.

Shrimp consumed feed during the experimental period and intake by additive supplemented groups did not differ from that by positive control. After 24 hours, survival of the positive control group was reduced to 13%, while it was almost five times significantly higher (60%) in the groups supplemented with the additive (Figure 1A).

pH 7.0			pH 7.5			pH 8.0		
Bacti-nil® Aqua	Product A	Product B	Bacti-nil® Aqua	Product A	Product B	Bacti-nil® Aqua	Product A	Product B
3000	9000	5000	3000	9000	3000	3000	5000	5000

Table 1. Minimum inhibitory concentration (mg/L) of Bacti-nil® Aqua and other commercial products based on organic acids under pH 7-8.

The shrimp's defense mechanisms against external agents such as *Vibrio* spp. include the production of haemocytes, which are defense cells present in the haemolymph (Aguirre-Guzmán *et al.* 2009). The main two types of haemocytes are hyaline, responsible for absorbing pathogens or foreign particles through phagocytosis and for the process of coagulation, and granular, responsible for destroying invading elements through encapsulation and enzyme secretion. A third type is semi-granular, a transitional state between granular and hyaline states. Under infection, the haemocyte defense mechanisms stimulate hyaline haemocytes to become granular as a mechanism to increase the rate of pathogen elimination. This pattern was confirmed in the non-supplemented groups, with the infected group (positive control) showing a four-times higher ratio of granular:hyaline haemocytes (4.8 vs. 1.2) in relation to the non-infected group (negative control) (Figure 1B). However, in the infected and additive supplemented group, the ratio (1.8) was maintained more in line with that of the non-infected group (negative control). This compensation can be attributed to the bactericidal efficacy of Bacti-nil® Aqua against *V. parahaemolyticus* that likely reduced pathogen presence in haemolymph and therefore the need of an immunological reaction via haemocyte transformation.

Histopathological symptoms by *V. parahaemolyticus* include sloughing of the hepatopancreatic tubule epithelial cells and haemolytic infiltration as a result of toxin secretion (Li *et al.* 2017). This is consistent with the observations found in surviving animals at the end of the infection challenge. The infected but non-supplemented group (positive control) was characterised by cellular detachment of hepatopancreatic tubules and infiltration of haemolymph and haemocytes in proximal intestine (Figure 2). Conversely, these signs were partly mitigated by the additive, since despite haemolymph and haemocyte infiltration in the hepatopancreas, the structure of hepatopancreatic tubules was maintained. The reduced hepatopancreatic damage can also be explained by the bactericidal activity of the additive and the overall lessening of the *V. parahaemolyticus* virulence in the digestive tract. Additionally, less damage also suggests better tissue recovery.

A tool to support growth under increasing stressful culture conditions

The second study aimed to evaluate the growth promoting effect of Bacti-nil® Aqua in shrimp cultured under stressful conditions. The study was conducted at the Government Research Centre for Marine Aquaculture (BBPBAP) in Jepara, Indonesia.

A total of ten tanks with 100L capacity each were arranged in a recirculating seawater system. After acclimation, shrimp were individually weighed and averaged 2.47g. Stress was induced by increasing the stocking density (40 vs. 10 shrimp/tank). The mash of a basal feed formula based on fish meal, soybean meal, tapioca, wheat flour and vegetable oil, was supplemented with the additive. A total of three groups were tested: 1) negative control (10 shrimp/tank and non-supplemented, n=2), 2) positive control (40 shrimp/tank and non-supplemented, n = 4), and 3) Bacti-nil® Aqua at 0.2% (40 shrimp/tank and supplemented, n = 4). Bulk weight of each tank was measured every 2 weeks during the eight-week trial duration, while weights were measured individually at the end of the trial. Water temperature, salinity and quality were monitored daily and remained within the standard conditions for shrimp.

Shrimp cultured at low densities achieved better weight gain starting from the second week (Figure 3A). Weight gain was negatively affected by high densities, while Bacti-nil® Aqua at 0.2% compensated for this negative effect. The positive growth effect by the additive was consistent along trial duration and statistically significant at the sixth week. At high densities, the additive supplementation improved average weekly gain by 13% (Figure 3B). In line with the conclusion reached in the infection trial, the growth promotion action of the additive can be explained by the antimicrobial effects against pathogenic bacteria in the digestive tract, leading to a more stable gut microbiota, a more efficient nutrient digestion and absorption, and overall to a healthier digestive system.

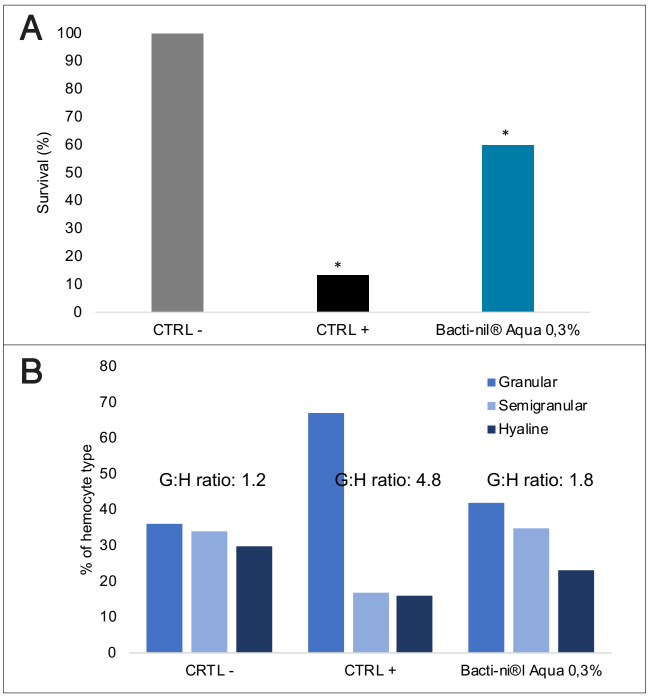

Figure 1. Infection challenge of *Penaeus vannamei* with *V. parahaemolyticus* (M0904) to evaluate the impact of Bacti-nil® Aqua. A) Survival after 24-hour infection. B) Haemocyte proportions after 24-hour infection. Negative control (CTRL -), positive control (CTRL +) and Bacti-nil® Aqua 0.3% (n=3). G.H ratio: ratio of granular to hyaline hemocytes. (*) indicates statistically significant difference.

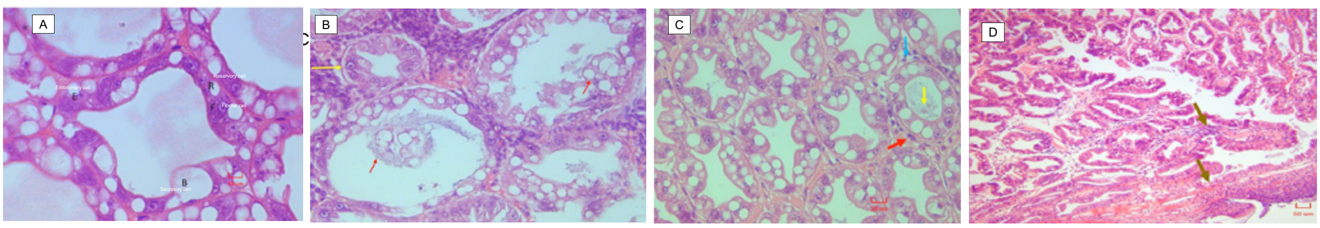

Figure 2. Histopathological analysis of hepatopancreas and proximal intestine in *Penaeus vannamei* after 24-hour infection with *V. parahaemolyticus* (M0904). A) Negative control (CTRL -): Normal hepatopancreatic tubules without infiltration of haemolymph. B) Positive control (CTRL +): Cellular detachment of hepatopancreatic tubules. C) Positive control (CTRL +): Infiltration of haemolymph and haemocytes in proximal intestine. D) Bacti-nil® Aqua 0.3%: Normal hepatopancreatic tubules with infiltration of haemolymph and haemocytes.

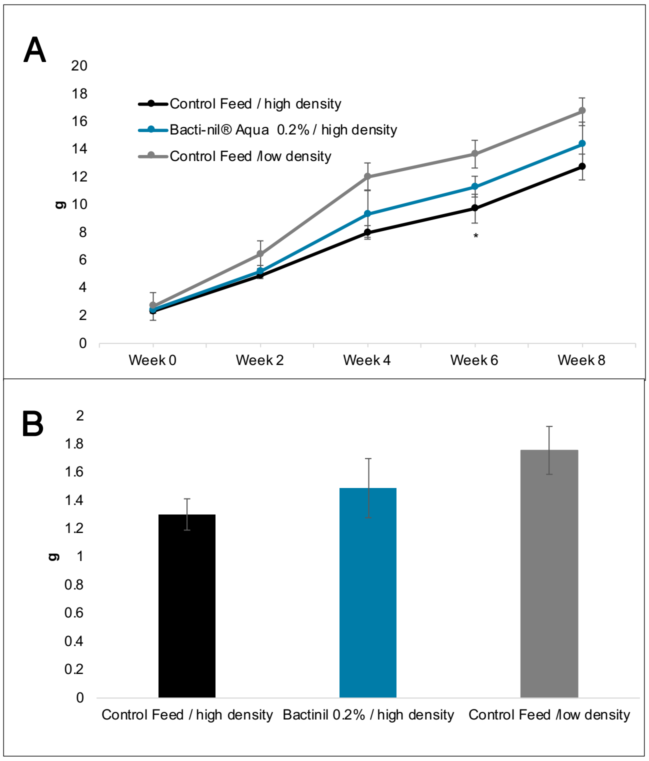

Figure 3. Feeding trial of *Penaeus vannamei* evaluating the impact of Bacti-nil® Aqua on growth. A) Average weight during the eight-week trial duration. B) Average weekly gain. Control feed and Bacti-nil® Aqua 0.2% at high densities (40 shrimp/tank) (n=4). Control feed at low densities (10 shrimp/tank) (n=2). (*) indicates statistically significant difference.

Conclusion

The use of dietary organic acids to prevent disease in shrimp production must undoubtedly go in hand with additional strategies such as improved water quality, better hygiene, and stricter biosecurity measures. With that in mind, Bacti-nil®Aqua has proved to be an efficient antimicrobial to reduce the impact of vibriosis, specifically against *V. parahaemolyticus* infection. Such direct antimicrobial efficacy is attributed to the synergetic combination of specific short- and medium-chain organic acids. Indirect benefits of supplementation include a better preservation of the immune response, reduced hepatopancreatic damage, and growth promoting action. Overall, this is an effective tool to prevent disease and promote growth.



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