Immune Stimulants and Antibiotics for Shrimp Culture
By Stephen G. Newman Ph.D.

Abstract

Disease prevention strategies fall into two broad categories. These are reactive and proactive strategies. There are various tools available for these and various management philosophies incorporate some or all of them. The most common tools for reactive management of diseases are antibiotics. In the US there are very few antibiotics that are specifically approved for use in aquaculture, though recent legislation does allow for veterinarians to prescribe, at their own risk, a much wider variety of compounds. The most common tools for proactive or disease prevention are compounds that stimulate the immune system. Immune stimulants fall into three categories, nutrients that act indirectly on cell physiology, those that work in a specific manner such as vaccines and those that are non-specific in nature such the beta 1-3 glucans, alginates and LPS based materials. This paper briefly reviews the use of antibiotics as reactive and proactive tools and the role of non-specific immune stimulants in shrimp culture.

Introduction

The farming of shrimp for profit has become a commercially successful global industry during the last twenty years. Global production in 1995 was estimated at 712,000 metric tons with peaks and troughs around this figure during the last few years (1). Some predictions are that the commercial cultivation of shrimp may grow to as much as 1.6 million metric tons within the next decade (2). This, forecast, in light of the problems of the last three years should be regarded as overly optimistic. Viral outbreaks have decimated the industries in Taiwan, the Philippines, China and India. They are seriously affecting productivity in Thailand (3) and Latin America (4) at this time. Vibriosis is probably the major cause of mortality wherever shrimp are cultured. There is little doubt that infectious disease is the number one problem affecting the economic viability of the industry.

Disease management strategies can be placed into two general groupings. These are:

1) Strategies to prevent disease, defined as **proactive** disease management strategies and
2) Strategies to treat the disease once it occurs defined as **reactive** strategies

From an economic, environmental and overall management approach the prevention of disease (proactive) is a wiser approach to controlling disease then the reactive approach. However, there are many occasions when this is not feasible and reactive strategies are all that are available to deal with problems.

Antibiotics

Antibiotics are the tools of choice for the reactive management of bacterial diseases. Antibiotics were first isolated by accident and it has since been found that this huge

group of compounds are ubiquitous in microorganisms and are used by them to ensure access to food sources by preventing other organisms from doing so. Since their initial discovery, many have been isolated, synthesized and are in use. Recently it has been found that many animals, including invertebrates, produce antibiotics as well (5). There is little doubt man made antibiotics have had a very significant impact on the overall health of humanity, both directly by their impact on human disease and indirectly by their use in agriculture to cure diseases and to improve growth and feed conversions.

The use of and reliance upon antibiotics in global aquaculture is a common practice and for the most part, is essentially unrestricted. The United States and Europe are both highly regulated environments and access to antibiotics, unlike most of Asia and Latin America, is severely restricted. In the US only three antibiotics are approved for use in food fish (Table 1). No antibiotics are approved for use in shrimp. Approvals are granted to specific companies for specific formulations and a narrow range of dosages for certain diseases and can entail the expenditure of many tens of millions of dollars.

Table 1. Approved Antibiotics for use in Aquaculture in the US

Antibiotic	**Trade Name and Company Approved**
Oxytetracycline monoalkyl trimethyl ammonium	Terramycin, Pfizer, Inc.
Sulfadimethoxine, ormetoprim	Romet 30, Hoffman La Roche
Sulfamerazine	Not currently available

The US is a minor producer of farmed shrimp though it is a major consumer. Since the FDA allows veterinarians to prescribe antibiotics for their clients, in theory shrimp farmers in the US have access to the same antibiotics that are used to treat similar conditions in animals. However, this is risky with out knowledge of how each specific antibiotic is metabolized. The few studies that have been done on shrimp however have shown them to be quite capable of metabolizing a variety of antibiotics (6,7). Through the use of HACCP guidelines, stringent criteria that ensure that shrimp that enter the US are free of contaminating materials specifically drugs, pesticides, antibiotics and filth, there is a potential path for impacting the global use of antibiotics. At this time though, the monitoring of shipments into the US for antibiotic residues is adequate to discourage abuse of antibiotics late in the growth cycle or under conditions that are conducive to residues being present.

Use of antibiotics is a necessary management tool in aquaculture. Table 2 lists some of the advantages and disadvantages of the use of antibiotics. If they are properly used, they are very valuable tools. They can stop serious disease problems very quickly. They also have the potential of modifying the flora of the guts of the animals potentially improving feed conversion ratios, though the author is unaware of any such efforts in shrimp. They are widely used prophylactically often at levels that are more likely to lead to resistance rather then eliminate bacteria that might pose problems. The development of resistance is undesirable and can result in serious negative consequences, ranging from human disease problems with strains of bacteria that are resistant to common antibiotics to the failure of the most efficient and cost effective antibiotics to cure diseased shrimp.

Feeding late in the cycle can lead to residues in animals at harvest, which may result in product being refused for import with disastrous financial consequences.

Table 2. Contrasting the Advantages and Disadvantages of Antibiotics

Advantages	**Disadvantages**
Powerful tools for stopping disease	Development of resistant organisms
Useful in impacting feed conversions	Environmental residues
Prophylactic use can prevent disease	Residues in animals
	Inadequate safeguards to ensure optimum dosages
	Can not be used late in the growth cycle

Outside of the US and Europe, antibiotics are freely available and widely used. Table 3 lists some of these and some applications. The author is making no statement about the suitability of any these compounds for use in the manner and dosage described.

Table 3. Antibiotics Used in Shrimp Culture

Antibiotic	**Usage**	**Dosage**	**References**
Erythromycin phosphate	Indefinite bath against bacterial necrosis and vibriosis in hatchery	0.5 to 2 ppm	(8,9)
Tetracyclines	Indefinite bath to prevent bacterial necrosis and vibriosis Prophylactic treatment for broodstock Oral application in feeds for treatment of NHP	2 ppm 1 ppm 1.5 to 5 kg/MT	(8) (10) (11)
Furazolidone Nitrofurazone	Indefinite bath to prevent bacterial necrosis and to reduce bacterial loads in hatchery waters, rearing tanks and Artemia culture	1-2 ppm	(10)
Chloramphenicol	Indefinite bath to prevent bacterial necrosis and reduce bacteria loads	2 ppm	(10)
Oxolinic Acid	Oral for 30 days for vibriosis	35 mg/kg	(12)
Sarafin - sarafloxacin	Oral for 5 days for vibriosis		(7)
Flumequin	24 hour bath	10 ppm	(13)
Baytril- Enrofloxacin	24 hour bath	8-10 ml/cu3	(13,14)

This is only a partial list as it is beyond the scope of this paper to review all of the various ways in which antibiotics have been used in shrimp culture. The use of these compounds is widespread with little regard to their correct application.

Antibiotics can be used responsibly. The following is a list of guidelines for their responsible use.

- Develop management philosophies and strategies designed to minimize the potential of bacterial disease outbreaks. These include using water disinfection systems in hatcheries such as ozone or UV in combination with sand, charcoal and bag filters to minimize bacterial loads entering into hatcheries. Run routine quality control on all water systems to ensure that the filters are having the desired affect.
- Use responsible techniques for disinfection of brood females, nauplii and PL's.
- In ponds, use techniques designed to maintain as high of a quality pond environment as possible.
- Only use antibiotics when they are needed. Never use them as a routine tool for preventing potential problems, though occasional use in this manner may be necessary.
- Using antibiotics at lower than therapeutic levels is an excellent way to generate antibiotic resistant bacteria. This makes it harder to treat subsequent infections, encourages the spread of resistance and can potentially pose a threat to other animals and humans.
- Use antibiotics only when you have bacterial or ricketsial infections. Do not use them to treat fungal, viral or protozoan infections unless you know that there are secondary bacterial pathogens involved in the disease process.
- Isolate the bacteria that are the cause of the problems and determine what the correct antibiotic and dosage of antibiotic is that will kill the bacteria. This is not always practical but even if done as the treatment is being performed can provide useful information for future treatments.
- Treat for the entire time period recommended. Do not be tempted to stop simply because animals look healthier.
- Use antibiotics that are from a reliable source. Many companies sell antibiotics that have been mixed with inert materials that can dramatically reduce the potency of the product.
- Wherever possible, mill the antibiotic into the feed instead of top dressing. This provides the assurance that the antibiotics will not leach out immediately after the feed is added to the pond.
- Use an adequate withdrawal period (varies with the antibiotics though for many this is not known-21 days is considered in most cases to be adequate).
- Don't store feed containing antibiotics for long periods of time.
- If after the administration the problem reoccurs consider determining the underlying cause, changing antibiotics, changing dosages and checking with staff to ensure that they fed the material as directed.

There is little doubt that the responsible use of antibiotics is a valuable tool that can substantially positively impact shrimp culture. On the other hand there is also little doubt that their irresponsible use can negatively impact shrimp culture. Any and all efforts that the farmers expend to prevent disease problems in the first place are going to provide a greater benefit in the long run than relying on the use of antibiotics to stop outbreaks once they occur. Prevention of disease is usually much more desirable than treating once outbreaks have occurred. The non-specific immune stimulators are a class of compounds that may provide the farmer with a tool that can be helpful.

Nonspecific Immune stimulators

Shrimp have immune systems that though quite complex are not as sophisticated as vertebrate immune systems and are relatively poorly characterized. Considering that farmed shrimp represent an 800,000 MT a year industry it is surprising that more studies have not been done. What is known however is that crustacean immune systems are substantially different from those of the more complex vertebrates (15). They do not produce specific antibodies, proteins that vertebrates manufacture in response to the structural components of a particular pathogen. They do not appear to have any memory of being exposed to a particular pathogen and seem to react to the same pathogen each time as if it was the first exposure. It should be noted however, that there is evidence that the response that they do have can last for some period of time, though the exact nature of this is not known. Recent studies have suggested that shrimp hemocytes may undergo a proliferation in response to an immune stimulus, which raises interesting questions, about what is involved in their development of a memory (16). There is however little doubt that shrimp immune systems function in a non-specific manner.

In recent years there has been considerable discussion on the use of cell wall fragments and components from various types of microorganisms as "non-specific immune-stimulants" in shrimp. Some formulations have been tested in the lab and the field with good though not always universal success. It is necessary to make a distinction between nutrients that impact the immune system by supplying limiting nutrients at high enough levels to ensure optimum functioning of the immune system, an example of which would be Vitamin C, and substances that actually directly impact the protective mechanisms present in hemocytes. Many compounds that are being marketed as having immune stimulating properties are actually nutrients that impact the physiology of the shrimp hemocyte in a general manner. None of these compounds are considered in this discussion. However, it always possible that some of the compounds that apparently possess some type of non-specific immune stimulating activity may be acting as nutrients as well.

There has been a tendency to refer to the exposure of shrimp to dead suspensions of organisms as vaccination. There is no evidence to support the use of this term. There are at least three important aspects of vaccination that evidently do not occur in shrimp. These are specificity, duration and memory.

White blood cells react to important components of vaccines giving an animal the ability to resist disease caused by the **specific** organism in the vaccine. In higher animals this is partly due to the production of antibodies, some of which, in concert with the cellular immune response, protect against the organism that the vaccine has been developed for. Shrimp do not produce antibodies of any type and there is no evidence that any component of their immune response has the degree of specificity that occurs with a vaccine (15). Shrimp apparently produce proteins that are lectin (proteins that bind to carbohydrates-structural components of the cell walls of microorganisms) like. They may function in a capacity that is similar to antibodies, though largely non-specific in

nature. It is quite likely that shrimp also produce a wide array of other compounds with anti-microbial activity, which have yet to be characterized.

A second hallmark of vaccination is that the response is relatively **long lasting.** This ties in with the memory component below. With shrimp, there is conflicting evidence regarding the length of time of their reaction to an immune stimulus. A number of papers have demonstrated that the initial response, at least in-vitro, is relatively short-lived (17-19). Some published data suggests that the protective effect is measurable in days or a few weeks (19,20) though some field observations suggest that a benefit can persist for at least eight weeks (20,21).

A third very important component is **memory**. Animals that are immunized remember being exposed to the material to which they have been vaccinated. This is a critical characteristic of vaccination as it ensures that there can be a life long ability to remember the initial exposure. The strength of the response after the initial exposure wanes but the immune system remembers the exposure and responds rapidly to the presence of the pathogen in some cases many years after the initial exposure. There is scant published evidence that shrimp have a memory component to their immune response, though there are anecdotal and unpublished reports that suggest that shrimp that survive epizootics may be less susceptible to repeat infection. The mechanisms involved are not known.

None of the classic features of vaccination appear to exist in shrimp. Therefore the use of the term in connection with shrimp is not only wrong but also misleading. Due to inherent limitations in the complexity of shrimp immune systems, it seems unlikely that compounds that stimulate the immune systems of shrimp will be able to provide as great of a level of sustained protection as vaccines do for higher animals.

There are many dozens of compounds that have been found to have the ability to directly affect hemocytes. Table 4 lists the three most studied of these. Each is discussed separately. The results of lab and field data are discussed with the emphasis on field observations where they exist so that the aquaculturist can relate to the potential ability of these products to help their operations.

Table 4. Compounds with Possible Non-specific Immune Stimulation Properties**.**

Compound	**Sources**	**Structure**
Peptidoglycan (PG)	Gram positive bacteria	Proteins, lipids and sugars
Glucan (G)	Yeast, fungi, algae, bacteria	polyglucose
Lipopolysaccharide (LPS)	Gram negative bacteria, algae	lipids and sugars

Peptidoglycan (PG)

These are protein, lipid and sugar combinations that are primarily found in the cell walls of gram positive bacteria. These bacteria rarely cause disease in commercially reared shrimp species though a few, such as *Aerococcus viridens* cause severe disease problems

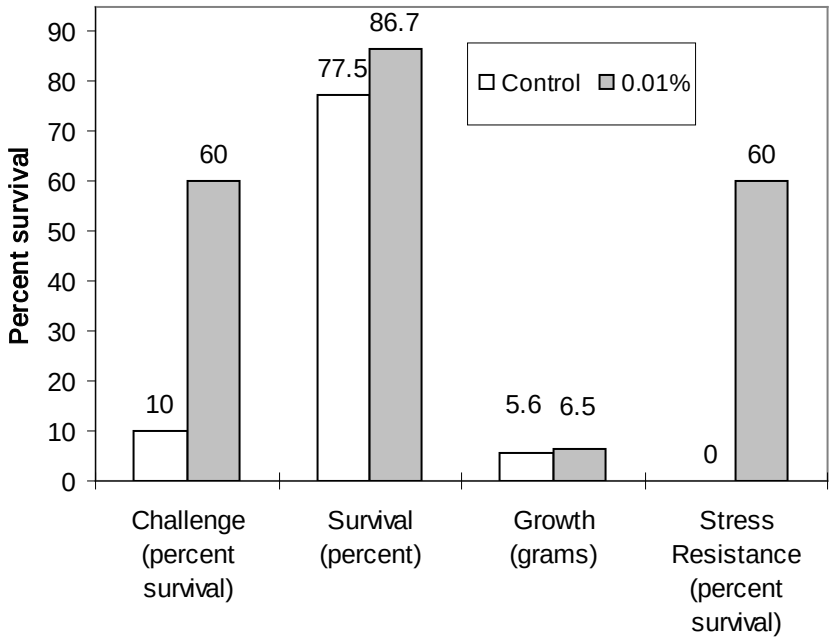
in lobsters. The gram-positive bacteria appear to be a fairly innocuous group of bacteria for shrimp.

Itami et al. in 1992 (22) described the results of feeding trials with PG derived from *Bifidobacterium thermophilum*. Kumura prawns were fed 0.2mg/kg body weight for 7 days on and 7 off for a 95-day period. Animals were removed at day 65 and 95, and exposed to a virulent vibrio. They noted that the treated animals were better able to withstand challenge than non-treated animals though no data was provided regarding sample size or the specifics of the challenge.

Boonyaratpalin et al. (23) in 1994 reported the effects of PG from *Brevibacteriun lactofermentum* (Ajinomoto Co. Inc., Tokyo, Japan), on the growth, survival, the immune response and tolerance to stress in *Penaeus monodon*. Post larval shrimp were fed three levels of PG, 0.005, 0.01 and 0.1%, four times a day for 8 weeks. Twenty shrimp were tested per diet, each with six replicates. At two-week intervals all of the shrimp were weighed and growth, survival and feed conversion recorded. Only one level, 0.01%, provided a consistent statistically beneficial effect (Figure 1).

Shrimp fed 0.01% showed the highest increase in their phagocytic index (a measure of immune function). There were no differences seen in feed conversions during the course of the eight week study, though the animals fed 0.01% did show a statistically significant increase in growth ($p<.01$). Only the survival and growth experiments were performed

Figure 1. Peptidoglycan evaluation (Boonyaratpalin et al.)



on enough animals for statistical evaluation. In the challenge, ten shrimp were injected with the Yellowhead Baculovirus (YBV) and survivals compared with non-fed animals.

This sample was too small to make any significant conclusions from, but it appeared that was a benefit in terms of increased disease resistance. Interestingly this effect did not occur at both higher and lower dosages, making these results somewhat suspect. The authors also noted that animals fed at the 0.005 and 0.01% levels showed an increase in survival, compared with controls, prior to being challenged. Those animals receiving 0.01% showing the highest survival rates after 5 days of a daily salinity stress. As with the injection challenge this was only done on one group of ten animals making it impossible to draw any statistically valid conclusions.

This compound is being sold in SE Asia as an immune stimulant for use as a top dress or in feed for both shrimp and fish. Several different feeding regimes have been proposed. Among these are feeding for 5 days on and 5 off for the first two months or every second day for two months or until harvest (Newman-personal observations). There have been no published field trail results to date. The observation that the effect may be dosage dependent and that too much material can impact the observed effect is disconcerting as it is difficult to ensure that shrimp consume what one intends them to consume. The validity of their laboratory tests remains to be borne out by field observations.

Some of these preparations are very high in lysine, an important amino acid and as indicated previously this raises the possibility that there may be some nutritional aspect to the observed effect.

Beta 1-3 Glucan

The use of glucans in shrimp has been the subject of considerable recent interest. Glucans are a structural component of the cell walls of fungi. They are also found in a variety of other organisms including many plants. They are all polyglucose molecules consisting of chains of glucose molecules with different types of linkages between them. The most common linkage associated with immune stimulating properties is the Beta (ß) 1-3 with ß1-6 side chains.

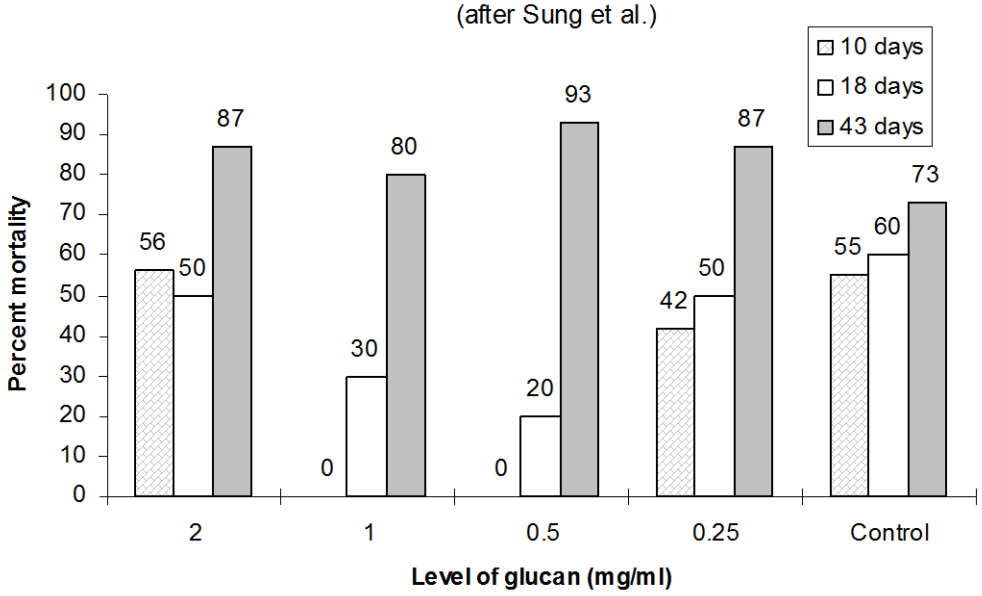
The ability of glucans to impact the mammalian immune system has been studied for some time and they have been found to have anti-cancer, anti-viral and antibacterial activity (24). Much remains to be learned about their mechanisms of action and there have been occasional adverse reports associated with their use, this despite the fact that one of the most common sources is from a yeast that is consumed widely by humans, *Saccharomyces cerevisiae.*

The first published reports regarding the use of glucans in shrimp is from 1992. Itami et al. (22) noted that Kumura prawns fed 50 or 100 mg/day of dried *Shizophyllan commune* (VitaStim™-Taito, Tokyo, Japan) cells demonstrated an increase in cellular immune function. No challenges were reported.

In 1994, Sung et al. (25) exposed 4 groups of 30 post-larval *P. monodon* to an insoluble ß 1-3/ß 1-6 glucan derived from the cell walls of *S. cerevisiae* (Macrogard™, Mackzymal AS, Tromso, Norway) for 3 hours. Fifteen to twenty shrimp from each of the four levels

of exposure were removed for weight and body length determination. The same numbers of animals were challenged by exposure to *Vibrio vulnificus* at 10, 18 and 43 days post treatment. The weights and size of the animals were the same at the lowest level of exposure (0.25 mg/ml) as the controls. At 0.5, 1.0 and 2.0 mg/ml there was an increase in weight and size when contrasted with controls over the course of the 39 days of testing. However at the 2 mg/ml level of exposure the gills were noticeably shrunken following exposure. The significance of this is uncertain though it suggests that these materials may have a negative impact if animals under any type of respiratory distress are exposed to them. The results from the challenged animals are depicted in figure 2.

Figure 2. Effects of beta glucan on vibrio challenged *P. monodon* (after Sung et al.)



A single immersion treatment in the glucan suspension conferred at least 18 days of protection against a laboratory challenge. At the two intermediate dosage levels the effect was the strongest. Note that a phenomenon similar to that reported with PG occurred, at both the higher and lower dosages tested, no protection was noted. This could have an impact on the oral use of this product as well. How this work can be transferred to a practical application in the field remains to be determined. Immersion applications are possible in the hatchery or during acclimation and occasionally post stocking of animals in nursery ponds.

Oral applications of glucans have been evaluated in the field though the reported observations, in corporate promotional literature, are limited in scope (26). In 1994 in commercial ponds, *P. monodon* were fed at 1 kg of glucan per MT (0.1%w/w) of feed for every other week for 4.5 months. In the two ponds reported on, compared with two control ponds, there were no noticeable differences in the days of culture, the average weight at harvest, survival in the ponds, or harvest tonnage. There was however evidence that the animals were more resistant to handling stresses. Animals treated with the glucan seemed better able to tolerate transport stresses with fewer animals dying from transport than non treated animals. This has been reported as well with both

peptidoglycan and lipopolysaccharides (Newman-personal observations). When a group of animals were challenged with *Vibrio vulnificus* or the etiologic agent of the white spot disease (SEMBV) there appeared to be a protective effect. Unfortunately no information is available on the conditions of the challenge such as the sample sizes tested, preparation of the challenge material, the length of time of the challenge, etc. It appears that the glucans exert a beneficial effect of some type on shrimp but the exact nature of this impact and degree of the effect is inconclusive.

In the promotional literature of Immudyne, another company selling a glucan (Immustim™) challenge studies were performed against the Taura Syndrome Virus, a virus implicated in epizootics in the US, Central and South America. The results were inconclusive. Juvenile *P. vannamei* were fed three different levels of Immustim™ for 6 days and then fed infected tissues for two days and then fed diets containing Immustim™ again until day 9 and day 12 respectively in each of the two groups reported on. Survivals in control animals were reported to be in 7-35% range for the first experiment and 3 to 20% for the second with the survival rates in glucan fed groups ranging from 5 to 92%. This variability in the results made statistical inferences impossible. Nonetheless, coupled with the previous reports on Macrogard™, there is an indication that the glucans may exert some anti-viral activity in shrimp.

The glucans have only a very short history of use in the shrimp industry and there are only a few published non-corporate promotional literature studies. However, there is little doubt that they can confer limited short-term protection upon shrimp by immersion and possibly when administered in the feed. What remains to be determined is what the optimum delivery and dosage rates are as well as what the range of the benefit is in terms of pathogens that it might work against, and whether or not there is a cost benefit.

Lipopolysaccharide (LPS)

This group of compounds has the greatest amount of published data on their use in shrimp. LPS is an important structural component of the cell walls of gram negative bacteria, perhaps the single most important group of pathogens, specifically the vibrios, affecting commercially reared shrimp species (27). Composed of lipids and carbohydrates, these cell wall components are often the first structures that invading bacteria present to the hosts' immune system. Classically referred to as endotoxins, LPS's have been the subject of thousands of papers and are known to exert both specific and non-specific effects on the immune system of animals, and potent non-specific effects in crustaceans.

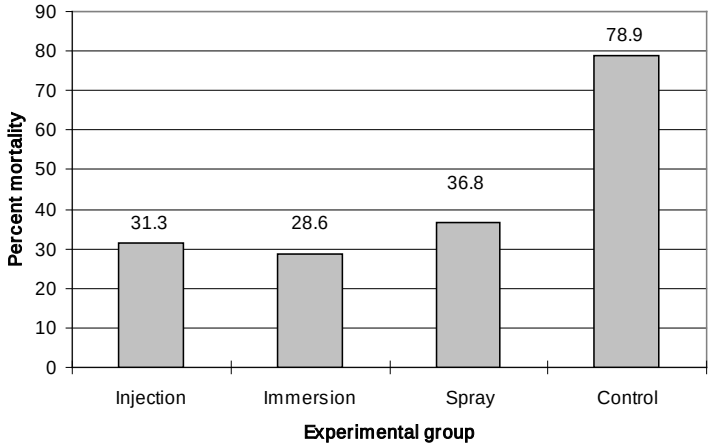
The first published observations on the impact of LPS on shrimp date back to the early 1980's. Crowder (28) reported on the work of Lewis and Lawrence at Texas A&M in April of 1981. Post larval *P. stylirostrus* were exposed to a dead suspension of a vibrio bacteria for ten to 15 minutes in a hyperosmotic solution (high salt concentration). Sixteen total ponds were stocked, two with treated animals. Four months later the ponds were harvested. The ponds that were treated with the dead vibrio suspension had an 8-10% increase in production. One hundred treated and 100 non-treated animals were

brought back to the lab and were temperature stressed in the lab. Mortality from the temperature stress was much less in the treated shrimp. Groups of 100 animals were also exposed to a living pathogenic vibrio. It took almost 500,000 bacteria to kill treated shrimp compared with 5000 for non-treated shrimp. Though critical experimental details were not provided, this article marks the beginning of a series of experiments conducted with dead suspensions of vibrio over the ensuing fifteen years that have shown that LPS based material can exert a potent productivity increasing impact on the culture of shrimp.

In 1983, Lewis and Lawrence (29) reported some additional observations. *P. setiferus*, at the PL6-7 stage, were exposed to a dead suspension of vibrio for 30 minutes in a hypotonic solution (contrasted with the first reports using hyperosmotic infiltration). The shrimp were stocked into 0.2 acre ponds at a density of 12,000 animals per pond. Six weeks post-stocking animals were harvested and weighed. Samples were removed for challenge with virulent vibrio. The mean weights for the three groups of treated shrimp were 7.9, 11.5 and 11.7 grams, contrasted with 4.1 and 7.2 grams for the two non-treated groups. The lethal dose of injected bacteria required to kill 50% of the animals was more than 10,000 bacteria per animal for the treated group compared with a little more than 10 per animal for the non-treated group or almost a 1000 fold difference. There was little doubt that the treatment conveyed a benefit on the animals in terms of substantially increased weights (presumably due to increased growth rates) and considerably increased disease resistance measured six weeks after the treatment. These first two studies showed that a single immersion exposure to a suspension of dead vibrio bacteria provided a six week to a four month benefit under field conditions of use.

In the late 1980's, Itami et al. (30) exposed Kumura prawns to a dead suspension of a vibrio. Groups of 8-11 adult Kumura prawns (average weight 20 grams) were immersed in a 1% suspension for 1 hour, sprayed for 10 seconds or injected with 0.1 ml per animal. Thirty days post exposure they were challenged by injection with a virulent strain of vibrio. The results of the replicate studies are depicted in Figure 3. Statistically significant levels of protection were noted in all three of the groups, demonstrating that several routes of exposure could induce a protective effect.

Figure 3. Comparative survival of kumura prawns exposed to virulent vibrio species 30 days post exposure to a dead vibrio suspension (after Itami et al.)

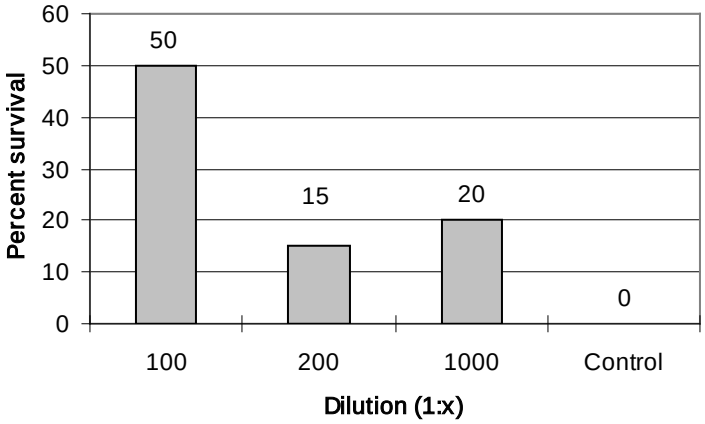


In 1990 Song et al. (31) reported their observations on the feeding of a dead suspension of *V. vulnificus*, milled into an artificial feed at 0.1% (w/w), to *P. monodon.* When fed to three times daily starting at PL30's, for an extended period of time, an increase in the rate of growth, contrasted with a single exposure by immersion at PL15, was noted. Their analysis of protection failed. In 1991, Sung et al. (32) reported on the repetition of the immersion portion of the trial and noted a stimulatory effect on growth from a single treatment at PL13, though the animals were exposed to a 1:10 dilution of the bacterial suspension, an impractical dilution for most hatcheries. Their analysis of protection failed for technical reasons. Challenges can fail for many reasons besides the lack of a protective effect (Newman-personal observations). These include the route of exposure to the pathogen, the virulence of the pathogen and the overall condition of the animals being challenged. Over and under challenges can also mask any protective effects.

Itami and Takahashi (33) in 1991 tested the ability of an orally delivered dead vibrio cell suspension to impact survival of *P. monodon*. Cells were fed at 0.05, 0.5 and 5% of the weight of a diet to zoeal stage larvae for four consecutive days. Those groups fed the material all showed higher survival and molt rates to mysis. Since no analysis was made of the presence of disease, the authors speculate that the vibrio cells may have contributed to the enhanced survival in some undefined nutritional manner. Since vibrio strains produce a variety of hydrolytic enzymes, it is possible that some factor did contribute to an increase in the availability of critical or critical nutrients.

Itami et al. (34) continued their studies and published additional observations in 1992. Kumura prawns were exposed to three different concentrations of a dead vibrio suspension, 0.1, 0.5 and 1%, for five hours and challenged by exposure to a virulent vibrio strain. The results are in Figure 4. This protective effect persisted for at least 50 days. No statistics were provided.

Figure 4. Results of challenges after three levels of exposure.



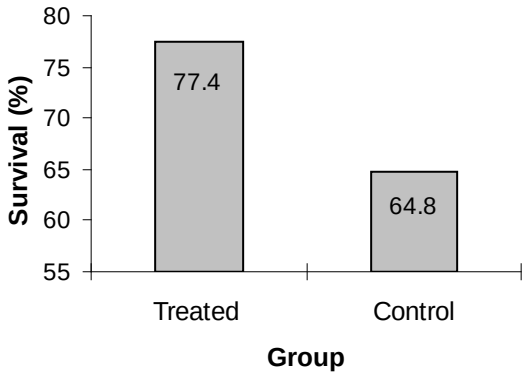
In a second article (35), the authors reported on their observations with several vibrio preparations including ultrasonicated, heat-killed, cell-free and whole cell. These

preparations were administered as 10% solutions for ten minutes. They noted that all of the preparations protected shrimp against artificial challenge. They observed that the active component was a heat stable material located in the cells and in the culture broth. This is the LPS. They were unsuccessful with a challenge on animals that had been fed material and also noted that there were some differences between bacterial strains as to the degree of protection from challenge. Since no statistics or experimental details were provided, no firm conclusions can be reached regarding the significance of their results.

The papers discussed so far dealt with lab based studies, with the first few integrating both lab and field based. The following focus more on field studies. There is little doubt that exposure to LPS does have a significant beneficial impact both in the lab and the field on cultured shrimp.

In 1992 Laramore (36) reported on the results of field based studies in which post larval *P. vannamei* were exposed to a killed suspension of a vibrio species. Figure 5 shows the differential survival rates. Survival was followed from the nursery phase to harvest. The average survival in four replicates was 77.4% for treated animals and 64.8% for controls. This is a 12.6% difference or an almost 20% increase in survival. They also noted a 23% increase in yields (lbs/acre) in the treated groups. The differences noted in survival were no longer apparent at harvest (116 days post treatment) with survivals in both groups being in the mid 80 percentile. If the benefit of the treatment could be attributed to an impact on disease, then high survivals would tend not to lend themselves towards seeing

Figure 5. Panama-nursery pond studies (after Laramore 1992)

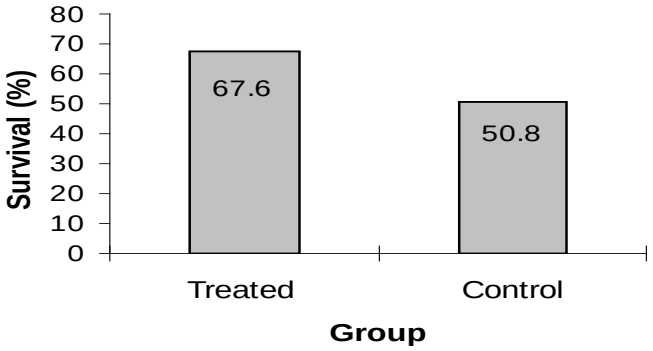


a benefit; i.e., there were no disease problems that the treatment could have protected the animals against. However the increase in yields persisted with the ponds containing the treated animals displaying a 17% greater yield, suggesting that there may have been an impact on the animals other than that of disease.

Dr. Laramore (36) also reported on the results of testing performed in cooperation with the Ministry of Agricultural Development (MIDA), Panama, in which treated PL's were directly stocked. These results are depicted in Figure 6. There was a significant difference between the treated and non-treated groups with the difference in average

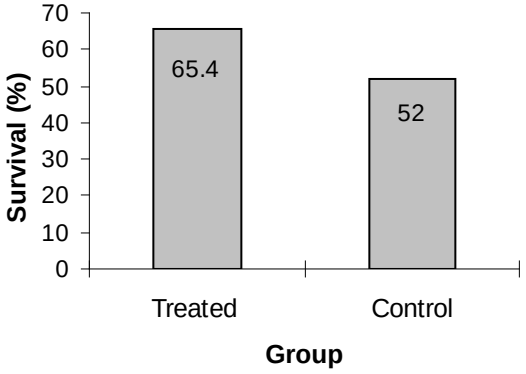
survival being almost 17%. These studies demonstrated that exposing shrimp to dead suspensions of bacteria under field conditions resulted in a benefit in terms of increased survivals and/or yields that persisted to harvest. In all of the field studies reviewed, the impact of these treatments on the actual incidence of disease can only be speculated upon as no diagnostic work ups were done to determine if any of the observed differences could be related to disease incidence. However, there is little doubt that the increases in survival and yields seen were statistically significant. The consistent increase in yields could be attributable to a general overall increased resistance to disease or to other unknown factors. The observation that when survivals were high, increases in survival did not occur and that when survivals were lower there was a significant increase in survival is similar to those noted in subsequent experiments reported by Newman et al. (37).

Figure 6. Growout evaluation. MIDA study: four replicates: 35000 animals per acre (after Laramore 1992)



In the technical literature of International Aquaculture Biotechnologies Ltd. (PenStim™, Kirkland, WA., USA), the results of both a combination immersion treatment and an oral

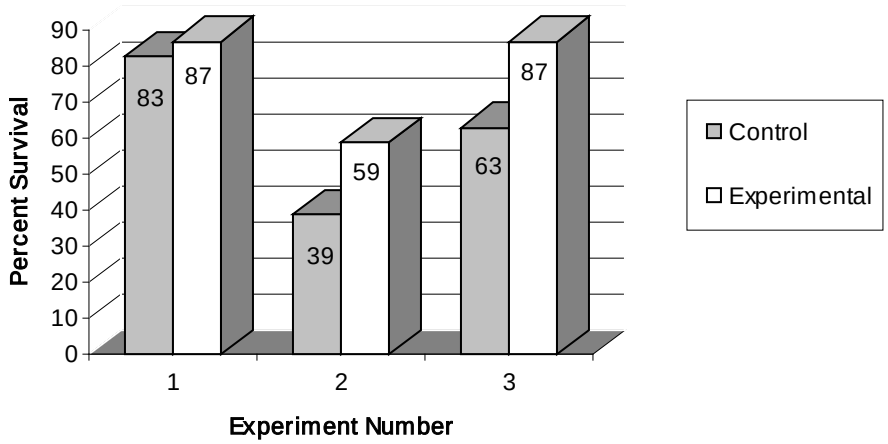
Figure 7. Panama Field Trials-1993



evaluation of a commercially available suspension of vibrio were described in 1993 (Figure 7). *P. vannamei* PL's were immersed in a 1:1000 dilution of the suspension for 90 minutes and stocked. Nine groups were treated and six were left untreated. They were harvested around 110 days later. Approximately 1.5 million animals were in each group. In the treated group survivals averaged 65.1% compared with 51.8% in untreated controls. At this time oral evaluations were also done (unpublished observations). Though the results were somewhat disparate, there was an indication that three oral treatments (one in the nursery for three days) spaced 30 days apart for 6 days impacted the presence of vibriosis.

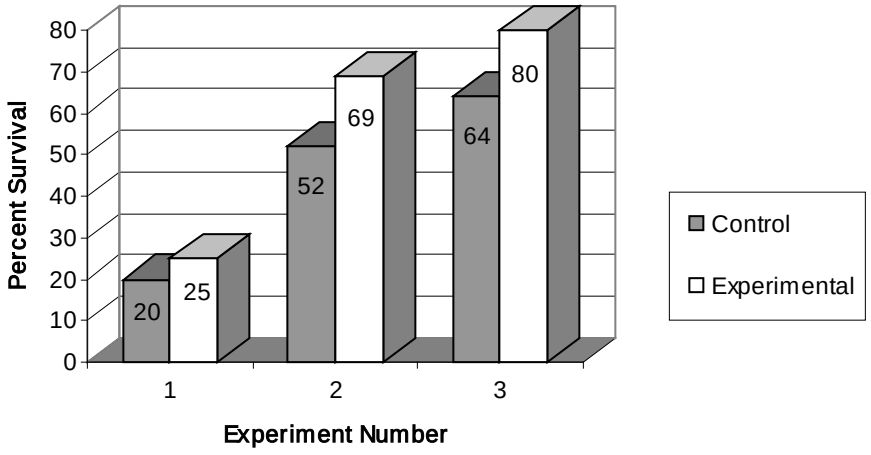
Newman et al. (37) reported similar observations to those of Laramore using a commercial product. The results are depicted in figures 8 and 9.

Figure 8. Honduras Field Trial Results (i)-GMSB; immersion; 59 days post treatment



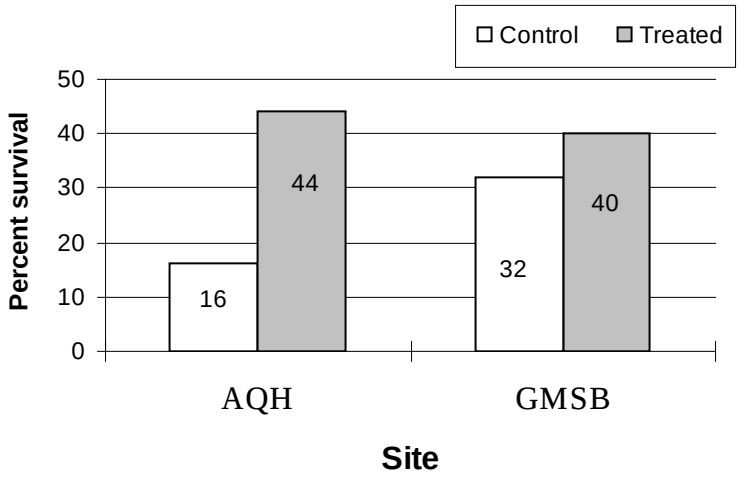
The experiments depicted in figure 8 took place in 2-hectare nursery ponds stocked at high densities, 200-300 PL/m2. These observations were made at about 50 days post stocking after an immersion treatment of three hours at a 1:1000 dilution. The average difference in survival noted in the treated groups was 14.7% with an increase of 21%. Figure 9 depicts the results of another study in nursery ponds. The results in figure 9 were from nurseries located on a dead end reservoir and were made 28 days post stocking. Discounting the group with the low survival (though there was still an increase in survival) there was an average difference of 18.5% and an increase in survival of 42%. Note that in those experiments when the survival of untreated animals was at its highest, Figure 8, experiments 1 and 3, in one of the groups, the differences in survival between treated and non-treated groups was relatively small. This could be accounted for by the lack of a problem that the product could have had a benefit against. In experiment 2, this was not the case. It was evident from these experiments that a single immersion exposure could enhance survival for at least 60 days post exposure in the field.

Figure 9. Honduras Field Trial Results



These experiments have been repeated using cages in grow-out ponds and the results are depicted in Figure 10 (unpublished observations). Two sites were examined, with differences in survival at one site of 28% and 8% at another. The studies differed from the others in that the shrimp were held in cages. At Aquacultivos de Honduras (AQH), the cages were placed into a single pond at 40 animals per cage with 4 experimental cages and 4 controls. At Granjas Marina San Bernardo (GMSB), a single cage was placed into a single pond. There were three controls and three experimental cages each containing 60 animals. A single immersion exposure to this material resulted in a substantial benefit 56 days later.

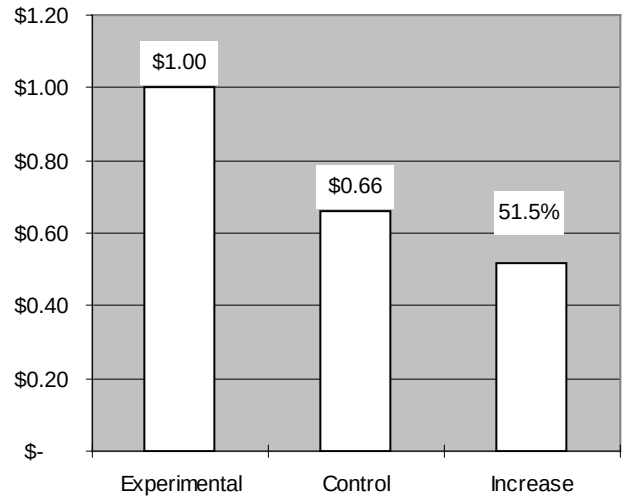
Figure 10. Honduras Cage Studies 1995



In late 1994 and early 1995, at a farm in the outskirts of Guayaquil, Ecuador a much larger field experiment was performed in which animals were followed to harvest (Figure

11). Wild larval *P. vannamei* were treated at a 1:1000 dilution for 3 hours with PenStim™ prior to being directly stocked. Nine groups with controls were tested for a total of more than 20 million animals. One of the best measures of success is increased profits. The average pond size was 10 hectares with survivals being about the same, in the 50-60 percentile ranges. The treated animals weighed, on an average, almost 1 gram more at harvest, a 7% increase. The margins increased by 52% with a cost benefit of more than 90 fold (for every dollar spent on the use of the product they saw a 90 fold

Figure 11. Revenue Increased by 51.5%



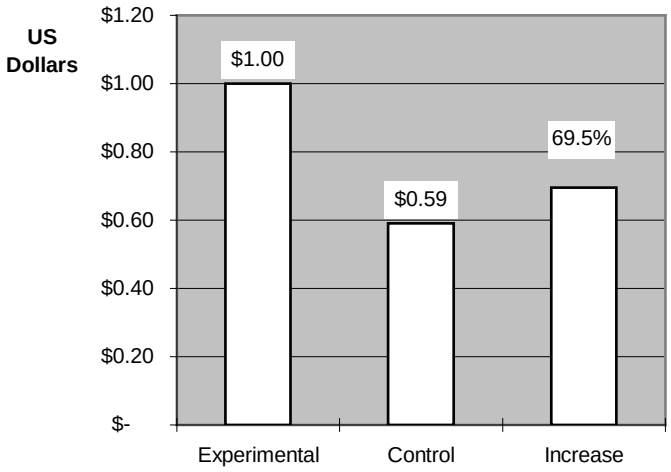
increase in their net margin).

These experiments were repeated later with another similar product. Thirty two ponds, comprising more than 300 has were used. Almost 15 million PL's were fed during acclimation prior to stocking. Survivals increased by 11.3% in the groups fed the material. These animals were 7% larger and the yields per hectare increased by almost 18%. This resulted in a 69.5% increase in net profits (Figure 12), a more than 80 fold return on investment. Many other farms in Ecuador have reported similar results, though the cost benefits are usually in the $10 range.

Horne et al. (38), in 1995, published the results of an extensive evaluation on the use of dead suspensions of vibrio for the control of vibriosis in *P. monodon*. Their observations were consistent with those reported by previous authors. Their testing was extensive and they reported significant protection by injection, immersion or by the oral route. When animals were immersed in a 1:100 diluted suspension of their test material for 6 hours, held in the laboratory for 60 days and challenged by injection, they reported a 38% percent survival compared with 21% for controls. They also noted that oral administration of their material, when tested 14 days post administration, conferred a protective benefit (20% survival in controls and 30-70% in fed groups). When animals were held in the field and challenged at 50 days post treatment they also noted a difference between controls and treated groups. They concluded that a single immersion

treatment conferred a benefit that lasted 4-6 weeks and that repeat oral treatments at 4-6 week intervals were required to maintain the protective effect. They also concluded that

Figure 12. Revenue Increased by 69.5%



LPS based treatments provided much higher levels of protection than did beta glucans in a lab based challenge.

In 1994, International Aquaculture Biotechnologies Ltd., in conjunction with a large farm in Honduras evaluated the ability of PenStim™ to protect shrimp against the Taura Virus as determined by LC50. The results are depicted in Figure 12. Groups of *P. vannamei* PL's were fed at 0.05% (w/w) for 5 days, held for ten days and exposed to a waterborne suspension of virulent TSV at 4 different concentrations. Three replicates of ten animals each were run at each level of challenge. The animals were held in a tank that prevented cannibalism from occurring. The challenge was conducted for a week. At the end of the week mortalities were tabulated and the lethal challenge dose killing 50% of the animals calculated. The glucan tested was from a Lactobacillus species. No LC50 could be calculated for the animals fed the vibrio cells as there was insufficient mortality occurring at even the highest challenge dose. The data suggests that the animals fed the vibrio cells were better able to resist the Taura challenge, although the difference noted was small (7-10%). Since then these results have been replicated several times. On a commercial scale, this difference could translate into significant revenues.

Overstreet and Newman (unpublished observations-1996) tested a killed vibrio cell suspension for its ability to impact shrimp challenged with the Taura Virus using a different challenge methodology from that used in Honduras. These results are in Figure 14. Juvenile *P. vannamei* were immersed in a diluted suspension for three hours at a 1:1000 dilution and held for 72 hours prior to being exposed to the Taura Virus. Each experiment was run in duplicate with 50 animals per group in tanks where cannibalism could occur. Both negative and positive control groups were run. As is evident the treated animals fared much better then the control. Two out of the 100 control animals

survived compared with 62 of the treated animals. To date these results represent the strongest evidence that dead vibrio cells can protect against viral diseases.

Figure 13. LC50 with PenStim and glucan

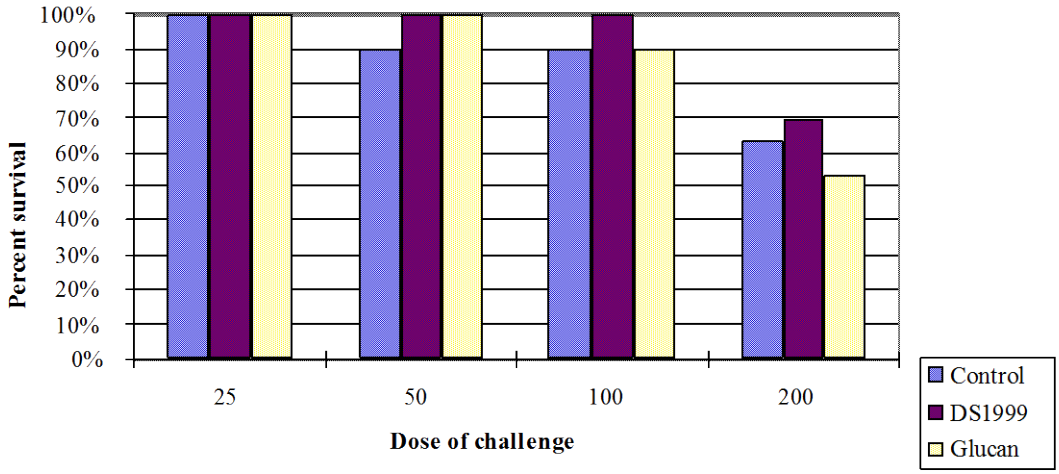
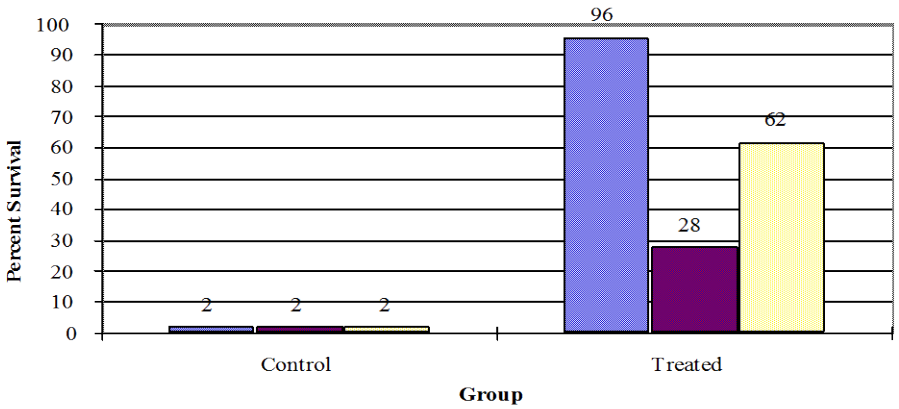


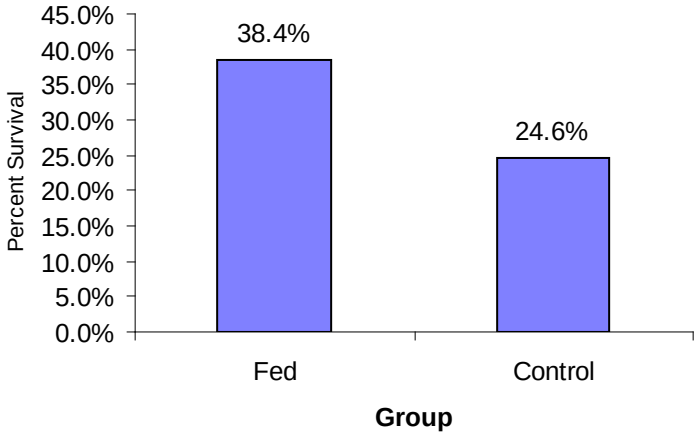
Figure 14. TSV survivals-1996



During 1996, a set of experiments was performed in Panama in which *P. vannamei* were fed a combination of a marine bacterial extract (IABL) and a beta 1-3 glucan (Aqua In Tech Inc, Lynnwood, WA). Approximately 30 days post stocking, the animals were fed a diet containing both of these materials for 6 days on and seven off. This was done for the entire life cycle until harvest. Six ten-acre ponds in all were evaluated, with the controls and fed ponds matched as to history and location. The animals were all from the same production lots. The differences were substantial. The fed animals showed an average almost 14% difference in survival, a more than 50% increase. Fed animals weighed 9% more than controls. The fed ponds realized a 97% increase in return as contrasted with the controls. The cost benefit was relatively small in the 3 to 1 range, unlike the experiments in Ecuador. However, the survivals in these instances were low

and the environment was deteriorated. The differences noted between groups was statistically significant (P<.05)

Figure 15. Oral field trial results



CONCLUSIONS

Antibiotics are very useful tools that are currently being widely abused by the shrimp farming community. Simple adherence to a few common sense guidelines will go a long way towards minimizing the negative impact that this abuse is sure to create. When they are used properly they can have broad reaching substantial benefits.

The use of immune stimulants to prevent disease in shrimp is in its infancy. Upon reviewing the available literature, it is apparent that a range of cell wall materials can impact the ability of shrimp to resist disease as well as providing other benefits. The exact degree and nature of this effect remains to be elucidated as many of the experimental protocols reviewed suffer from some type of experimental flaws (i.e. inadequate replicates, no challenges, etc.). Furthermore since the pond environment is such a highly variable and complex environment, conclusions that are reached from lab experiments are of limited value. Studies with PG and glucans are just beginning to suggest that their use may be beneficial, though much more work remains to be done to determine how to use them cost effectively and to optimize their use. The most widely studied compounds (LPS) in shrimp are those that are derived from gram negative bacteria, specifically the vibrios. The data that has been generated on these materials, while still suffering from some shortcomings, is nonetheless compelling. There is no doubt that the use of these materials can benefit shrimp. The preponderance of evidence at this time suggests that the use of LPS is a better approach to take than the use of either PG or glucan. Data has been generated that shows that LPS provides a benefit when used by immersion, orally and by injection. This benefit can manifest itself in a variety of ways ranging from increased resistance to disease to higher growth rates, better yields

and increased profits. They can easily be viewed as promoting increased productivity. Cost benefit analyses show that the costs run between $10 and $250 or so per hectare depending upon the regime that is used (Newman-personal observations). It is quite likely that the use of LPS and other products such as glucans and PG and the others that are on the horizon will become an integral part of routine management practices in shrimp farming.

The immune system of shrimp is fairly complex though it is not on the order of complexity of the vertebrates and it is unlikely that the compounds described above will be able to fulfill the expectations in terms of protecting animals against disease that vaccines have in the vertebrates. This is very important. The immune system of crustaceans is sufficiently different from mammals that a great deal of caution must be exercised in extrapolating between them. There is little reason to believe that the use of compounds that may be immune stimulants in shrimp will ever provide very high levels of broad protection for extended periods of time. They must be used wisely and at those times when they can benefit. They must also not be viewed as magic bullets that are going to prevent diseases that are a result of poor quality environment, large numbers of pathogens, etc. In many cases there is no clear cut evidence that these materials are acting by minimizing disease, but instead they may be acting by some other mechanism, possibly nutritional.

These compounds, when used as components of an enlightened management strategy will provide a cost beneficial means of fighting the onslaught of disease but they will not provide a panacea.

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