

The Positive Effects of Proprietary Udenatured β -Glucans on Stressed Marine Life

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Abstract

The safety of beta-glucans, including undenatured beta-glucan (UDBG), has been exhaustively studied over the recent past and has shown to be safe and efficacious in rodents as well as in humans with less than optimal immune systems. As such, we sought to find the effect of UDBG on certainly stressed crustaceans and fish. The survival of shrimp larvae fed the UDBG and other diets was measured on day 16. The diet containing 11.7% UDBG reduced larvae mortality by 18%; which is almost twice as much as the mortality reduced by the MacroGard diet, and up to 19.5% less than a standard Commercial Diet. In an effort to understand how UDBG protects against stressed marine life, UDBG was given to LPS-challenged trout. The UDBG positively influenced the level of transcription of several genes relevant to the immune system and overall health of the trout. The experimental findings indicated that UDBG: 1) modifies inflammatory responses, 2) makes responses more flexible and versatile, 3) protects from cellular stress, and 4) enhances “effector” mechanisms. Importantly, there was no evidence of immune system over-activity.

Keywords

Undenatured Beta-Glucan, Pig Growth, Shrimp Survival, Trout Gene Transcription

1. Introduction

Research conducted by different research institutions proved that UNBG is safe [1] [2] [3], and sought to determine if beta-glucan maintained in its undenatured tertiary state would be more efficacious in immune system stimulation than the denatured lentinan. When the fruiting body of the shiitake mushroom is treated by a proprietary fermentation process [2] [3], it is shown that beta-glucan can be isolated in its undenatured tertiary structure, thus preserving its

biological efficacy [4] [5].

Beta-glucans from various sources, including mushrooms and yeast, have been shown to have some positive effects on the immune system in animals and humans. These beta-glucans have typically been extracted from the botanical source with solvents or other treatments that destroys the tertiary structure of the beta-glucans. Research conducted by us sought to determine if beta-glucans maintained in its tertiary structure would be more efficacious in immune stimulation than the denatured beta-glucans. UDBG from shiitake mushrooms are found as β -1,3 beta-glucan with β -1,6 branching and a molecular weight typically of $\leq 300,00$ to $\geq 1,000,000$ Da, with maximum immunostimulating activity $\geq 500,000$ Da. When the fruiting body of the shiitake mushroom is treated by a proprietary fermentation process, it was shown that beta-glucans can be isolated in their undenatured tertiary structure. Testing has supported the understanding that these UDBGs are far superior to any other denatured beta-glucan, from mushrooms or yeast, in their ability to enhance the immune system [6] [7].

UDBG is an experimental supplement that has been shown to have immuno-enhancing characteristics in animals and humans [8]. The product contains undenatured beta-(1,3)(1,6)-D-glucan, a proprietary bioactive compound. This polysaccharide is different from other commercial glucans in that it is not “extracted” from its botanical source, but is rather “exported” in its undenatured, tertiary triple helix state from the mycelium of Shiitake mushrooms, at the entire molecular weight spectrum. Most activity is seen in the fractions that exceed 10^5 Daltons. This undenatured state is believed to be responsible for the superior immuno-enhancing effects seen with UDBG when it is compared to other similar yeast-derived or mushroom-derived beta-glucans.

Three prior studies with humans who self-described themselves as fatigued, depressed, or otherwise devoid of optimal Quality-of-Life, were administered either UDBG or a placebo [8]. Both groups measured their perceived Quality-of-Life using a standardized Visual Analogue Scale periodically over a 28-day period. This stressed group administered the UDBG had significantly improved Quality-of-Life variables while the placebo group either did not report improvement or, in some cases reported a decline in Quality-of-Life.

As a result of the human studies, it was decided to pursue studies to see if this positive effect could be reproduced in fauna in which stress states are known to exist, or in which stress states could be induced.

It is universally accepted that a reduction in mortality and morbidity in the marine farming and animal husbandry industries are required to insure profitability. While there are products on the market that improve mortality and morbidity in certain species, shrimp and fish-farming are still seeking a product that will increase immunity, and reduce mortality and morbidity in these species, and thus bring an added advantage to business.

Commercial shrimp farming is haunted by high, varying, and unpredictable mortality in the early life stages. This high mortality leads to high and non-sustainable use of antibiotics in shrimp farming representing a possible challenge to con-

sumer health. Thus, there is a need for feed supplements to reduce larval mortality without the need for prophylactic use of antibiotics. Mortality in farmed shrimp larvae typically ranges above 30% and may reach 40% to 50% in some facilities. A supplement that can reduce mortality by 10% or more would provide an economic benefit to the farmers. A study was conducted to determine if an immune-enhancing product, UDBG, would be able to reduce mortality in farm-raised shrimp larvae.

Shrimp farming continues to grow at a rapid rate as natural ocean stock is depleted by over-harvesting, pollution, and other environmental stresses. The shrimp farming industry, while generally profitable, still suffers from mortality rates that range from 30% up to 50%. Most of this mortality typically occurs during the larvae growth stage in hatcheries. Reducing mortality by 10% or more would signify a substantial economic benefit to the shrimp farmer.

2. Shrimp Farming Studies

Methods and Materials—Shrimp Farming Studies

In the first phase of the trial 1000 shrimp larvae (*P. monodon*) were stocked in eight glass tanks containing 100 litres of purified seawater. The larvae were fed four experimental diets from day 5 (Zoea-stage 3) until after post-larvae stage 5 (16 days), with each experimental diet being added to two tanks. The larvae in the tanks were fed either a standard diet containing either MacroGuard®; or one of two doses of UDBG (2.5 gm/Kg dose/11.7% of Feed; or 0.25 gm/Kg dose/1.2% of Feed).

UDBG is an immune enhancing product containing a proprietary un-denatured beta-(1,3)-(1,6)-D-glucan. A “Commercial Diet” that did not contain either MacroGuard or UDBG was fed to the shrimp in the two remaining tanks.

MacroGard^{®1} was added to the feed according to the manufacturer’s recommendations. MacroGard is reported to be an environmentally sound alternative to feed antibiotics and chemotherapeutics for livestock, pets and cultured aquatic organisms. MacroGard, which has been in use world-wide for almost 15 years as an immune modulating agent in animal husbandry and aquaculture, contains 60% yeast-sourced beta-(1,3)(1,6)-glucan. Thus MacroGard contributed 0.6% yeast-sourced beta-(1,3)(1,6)-glucan to the larvae’s diet. UDBG was added to the diet at percentages (11.7% and 1.2%), which would provide 0.01% and 0.001% beta-(1,3)(1,6)-glucan to the larvae’s diet (**Table 1**).

Results and Discussion—Shrimp Farming Studies [9]

The data generated from the first study indicated that supplementation of the diet with 0.25% UDBG resulted in a significant reduction in mortality of the shrimp larvae when compared to the diets.

Survival of the larvae was measured at day 16. The mortality rates for the Commercial Diet, the 0.025% UDBG Diet, the MacroGuard Diet, and the 0.25% UDBG Diet were approximately 32%, 25%, 21.5% and 13.5% respectively (**Figure 1**). Greater than 10% survival increase was therefore obtained by diets containing

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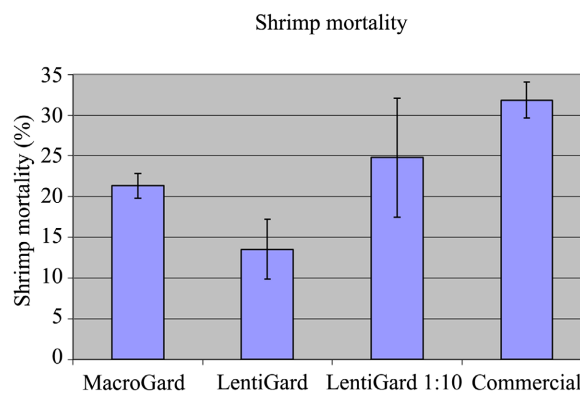


Figure 1. Reduction of shrimp mortality LentiGuard is a Tradename for UDBG.

Table 1. Diets fed to shrimp larvae.

Tanks	Basic Diet	Additive	% of Feed	% β -Glucan
A1 & A2	Standard Feed	MacroGard	1.0	0.6
B1 & B2	Standard Feed	LentiGuard	11.7	0.01
C1 & C2	Standard Feed	LentiGuard 1:10	1.2	0.001
D1 & D2	Standard Feed	None	-	-

the 1% MacroGard and the 11.7% UDBG supplementation. Further, the diet containing 11.7% UDBG reduced larvae mortality by 18%; which is almost twice as much as the mortality reduced by the MacroGard.

Following the survival phase of the study, a subset of surviving larvae were subjected to stresses caused by the addition of formalin to the water, changes in water temperature, or changes in water salinity. Three tanks per diet treatment were used for these tests. This phase of the trial was designed to study the effects of UDBG supplementation on the survival of farmed shrimp larvae, and to determine if any differences could be observed in the larvae fed UDBG when exposed to environmental stresses. A subset of surviving larvae was subjected to stress caused by the addition of formalin to the water, changes in water temperature, and changes in water salinity. Eight glass tanks each containing 100 liters of purified sea water were assigned to the study. There were four different dietary regimens so two tanks were assigned to each dietary regimen. 1000 larvae were placed in each tank and the feeding procedures and amounts fed were carried out based on existing protocols for the hatchery. The larvae were fed only algae during the first 4 days and during this period only; probiotics were added to the tanks.

On day five (Zoea-stage 3), the experimental feeds were introduced, with amounts increasing during the following days. Both algae and the experimental diets were fed together until day 7, and starting on day eight the algae were eliminated and the larvae were only fed the experimental diets for the remainder of the study. *Artemia* was added to the tanks from day 10 onward. The survival phase of the study ended on day 16.

Post-larvae shrimp that survived the Phase 1 Survival test were again divided according to the experimental diet they had previously consumed to see if any of the diets affected survival when the shrimp were exposed to environmental stresses. The number of tanks was increased from two to three for each diet therapy.

In the first stress test, 200 ppm of formalin was added to the twelve tanks. Each three-tank group each contained 100 post-larvae shrimp fed the respective experimental diets from the Phase 1 Survival test. The larvae were subjected to this stress for one hr and survival was observed. All larvae survived at approximately the same rate, regardless of their prior diet regimen. The survival rate narrowly ranged from 66% to 72%.

The second stress test involved twelve tanks that each contained 100 post-larvae shrimp from the Phase 1 Survival test as described above. The shrimp were subjected to a sudden decrease of salinity by 60%. Survival was observed after 24 hours, and again all survival rates were similar with no significant differences, although the spread was broader, ranging from 76% survival to 96% survival.

The final stress test involved 12 tanks, with each tank again containing 100 post-larvae shrimp per tank as before. These shrimp were subjected to sudden change in temperature over time, and survival was recorded. These shrimp seemed somewhat impervious to the temperature changes with all shrimp groups demonstrating greater than 96% survival.

Conclusions—Shrimp Farming Studies

The Phase 1 Survival feeding experiment showed clear differences in larval mortality between the different feeds. The larvae fed the commercial feed experienced the highest mortality (31.9%), while the larvae fed the diet containing 11.7% UDBG experienced the lowest mortality (13.5%). Further, the results show that these larvae experienced a substantially lower mortality than the larvae fed the diet containing the MacroGard (21.5%).

The difference between the diets containing MacroGard or UDBG becomes even more evident if the concentration of active ingredient (beta-glucan) is taken into account. The MacroGard feed was mixed according to the instructions from the MacroGard manufacturer and contained 1% MacroGard. MacroGard contains 60% yeast glucan, which give glucan content in the feed of 0.6%. The UDBG feed, however, contained 11.7% UDBG, and as this diet contains just 0.1% beta-glucan, the beta-glucan concentration in the UDBG feed was just 0.01% or 1/60 of the beta-glucan content in the MacroGard feed. The results also show a clear dose dependant response to addition of UDBG in the feed, shown by the fact that the larvae fed the diets that contained the 1/10 dilution of UDBG (0.001%) experienced a substantially higher mortality (25%) than those larvae that were fed the diet containing 11.7% UDBG.

In the second phase of the trial, a subset of surviving larvae was subjected to stress caused by the addition of formalin to the water, changes in water temperature, or changes in water salinity. The stress tests revealed no systematic differences between larvae from the different feeding regimes.

With a scientific certainty of 95% ($p < 0.05$), there is no difference between any groups except on the salinity test where group F (commercial diet) gave higher survival than group B (MacroGard). As the survival after the growth experiment was significantly lower in group F, this has little practical significance [6].

3. Trout Studies

Methods and Materials—Trout Studies

Groups of rainbow trout were fed 0 g, 2 g or 4 g of UDBG per kilo of feed for four weeks. Each group of trout was then divided into two groups. Half the trout were challenged with an injection of lipopolysaccharide (LPS), an endotoxin known to evoke an inflammatory response in animals. The remaining trout were injected with a saline placebo. After isolation of mRNA from the animals, a subtractive hybridization was performed to obtain the transcripts that are specific for the animals treated with LPS, with and without UDBG.

The rainbow trout were divided in three groups. One group was fed the normal feed that did not contain any UDBG; one group was fed 2 grams of UDBG per kilo of feed (0.2%), and the third group was fed 4 grams of UDBG per kilo of feed (0.4%).

To determine the effect of UDBG on the level of transcription of a large number of genes, modern DNA “Microarray” technology was employed. Microarray is a analytical method of choice for studies of complex and poorly explored conditions. It provides high-throughput and accuracy; does not require prior knowledge or a hypothesis; and it has strong bio-information support for statistical methods and gene annotation evaluation. It allows diagnostics and classification of samples, (e.g. stressors, pathogens, toxicity); a search for biomarkers; an evaluation of the effects and interactions of immune factors (e.g. stressors); and provides mechanistic insights.

After 4 weeks of this feeding, half the fish were injected with LPS, and the other half were injected with a saline placebo. 3 days later the fish were sacrificed and RNA was extracted from splenocytes.

After isolation of RNA from the animals, a subtractive hybridization process was performed to obtain the transcripts that are specific for the animals treated with both LPS and UDBG. Target mRNAs were extracted and RT/PCR was used to label cDNA, using a red fluorescent dye for the UDBG + LPS group, and using a green fluorescent dye for the control group. The labelled cDNA from one group was allowed to hybridize with RNA from the other group (*i.e.* subtractive hybridisation) to obtain labelled cDNA unique for each group. This was finally allowed to hybridize to oligonucleotides corresponding to a large number of relevant genes on the slide. The slide is then scanned and the differences between the control group and the test (*i.e.* lentinan treated) group can be determined (see **Figure 2** and **Figure 3** below). This technique allows examination of a number of genes, and identifies those genes that are affected (“up-regulated” or “down-regulated”) by the UDBG treatment.

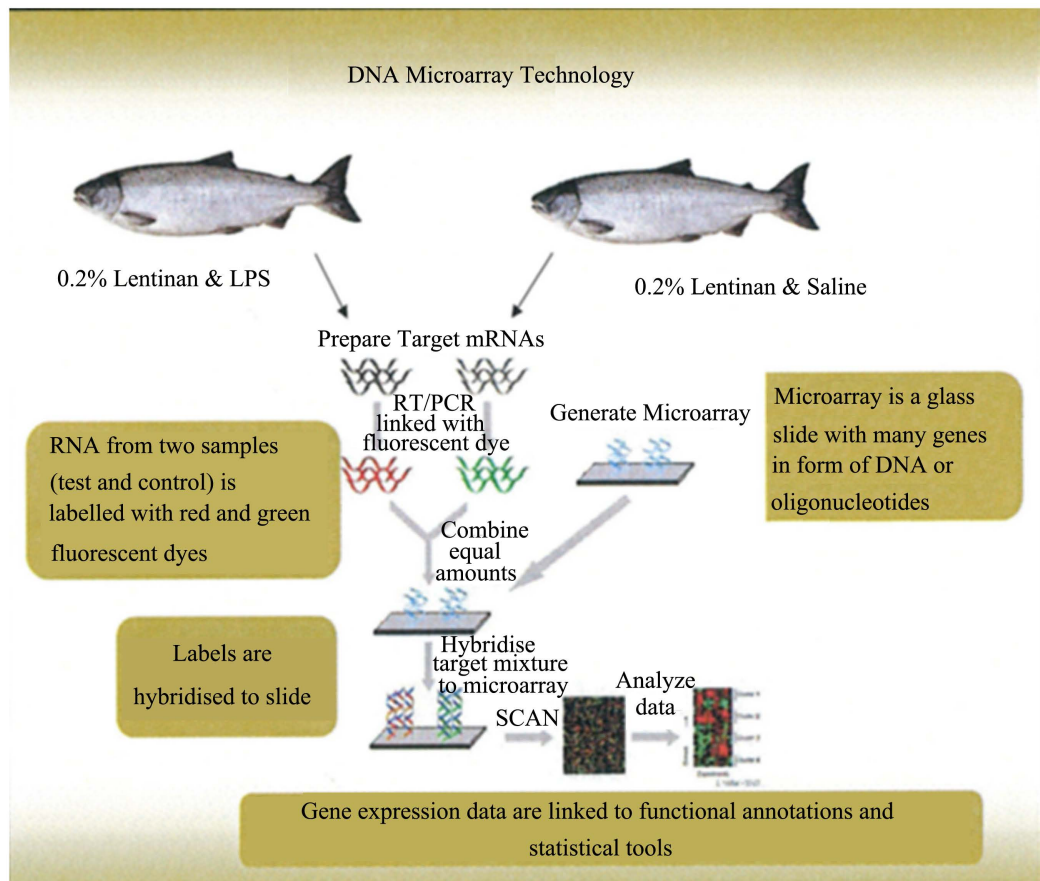


Figure 2. Gene expression influenced by LentiGuard administration using dna microarray technology.

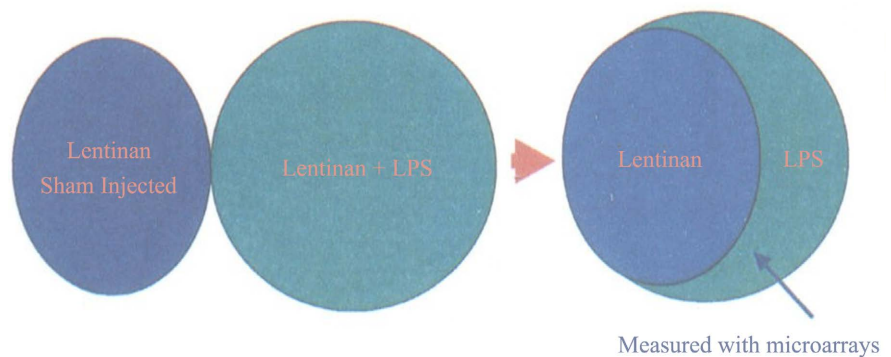


Figure 3. Subtractive hybridization.

Results and Discussion—Trout Studies

The results, as obtained through “Microarray Technology,” demonstrated that UDBG given to LPS-challenged trout positively influenced the level of transcription of several genes relevant to the immune system and overall health. The experimental findings indicated that UDBG: 1) modifies inflammatory responses, 2) makes responses more flexible and versatile, 3) protects from cellular stress, and 4) enhances “effector” mechanisms. Importantly, there was no evidence of immune system over-activity.

The fish ate approximately 5% of their body weight each day, and the body weights of these fish ranged from 500 to 1000 grams. Thus, a 500 gram fish ate 25 grams of feed per day, which contained 50 mg of UDBG in the 2% group, or 100 mg of UDBG in the 4% group.

The insult of the LPS endotoxin to the trout caused several immune system responses similar to those seen in an infective challenge. There were systemic and local inflammatory responses to the challenge including sensing, transport, signal transduction, and induction of inflammatory genes (messengers) and effectors (“disease-fighting” immune system components). There were also immune system cellular and tissue changes, including differentiation of macrophages antigen presentation (effectors), migration of neutrophils to infection site, and destruction and clearance of infection-damaged cells.

After isolation of RNA from the animals, a subtractive hybridization process was performed to obtain the transcripts that are specific for the animals treated with both LPS and UDBG. Target mRNAs were extracted and RT/PCR was used to label cDNA, using a red fluorescent dye for the UDBG + LPS group, and using a green fluorescent dye for the control group. The labelled cDNA from one group was allowed to hybridize with RNA from the other group (*i.e.* subtractive hybridisation) to obtain labelled cDNA unique for each group. This was finally allowed to hybridize to oligonucleotides corresponding to a large number of relevant genes on the slide. The slide is then scanned and the differences between the control group and the test (*i.e.*). The gene expression data are linked to functional annotations and are assessed statistically for levels of significant differences.

The results of the data indicate that with regard to mediators and perception, cytokines and chemokine responses were similar and appropriate in both groups, but Interleukin-1 (IL-1) receptor-like protein was shown to significantly increase, prostaglandin D synthase was significantly reduced as was LPS binding protein. IL-1 and prostaglandins are critical mediators of inflammation and the observed effects on gene transcription levels indicate a net anti-inflammatory effect. These results are consistent with earlier findings of independent researchers.² Additionally the responses to interferon-related genes were similar and appropriate in both groups, but the trout treated with UDBG experienced advantageous increases in gene signal transduction, and decreases in regulation of certain gene expression. Complement and ROS metabolism were both significantly increased in the group fed the UDBG; and cellular stress as measured by protein folding and cytoskeleton motor proteins were significantly reduced (“effectors”).

An important question regarding the immune system of “recovering” animals treated with UDBG is whether the inflammatory genes “switch off”, and whether there is reconstruction of extra-cellular mass. If the genes do not switch off, there is a danger of an auto-immune response occurring. The data from this study clearly indicate that the immune system does switch-off appropriately, and there wasn’t any immune system over-activity.

The net anti-inflammatory effect observed in this experiment strongly de-

monstrates the beneficial effect of UDBG. The injected LPS is initially responsible for a systemic inflammatory response, due to LPS being a “pathogen-associated molecular pattern” that is recognized by the very important innate immunity pattern recognition receptor, TLR4, leading to an inflammatory response. However, as there are no pathogens present in the fish, the inflammatory response is inappropriate, and should be contained quickly. This is exactly what is observed.

With regard to the commercial implications, it is tempting to speculate that we would see the same effect in the analogous, but commercially much more important, situation where a fish feed based on soybeans also leads to an inappropriate inflammation.

Conclusions—Trout Studies

The results demonstrate that undenatured beta-glucans given to LPS-challenged animals influence the level of transcription of several genes relevant to the immune system and overall health. The experimental findings indicate that:

- UDBG modifies inflammatory responses.
- UDBG makes responses more flexible and versatile.
- UDBG protects from cellular stress.
- UDBG enhances “effector” mechanisms.
- UDBG does not cause immune system over-activity.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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