IMMUNE MECHANISMS OF SHRIMP: FORM, FUNCTION AND PRACTICAL APPLICATION

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ABSTRACT

Shrimp possess immune systems that, although quite complex, are substantially different than vertebrate immune systems. There is no true specific immunity (no true antibodies and substantially less lymphocyte heterogeneity), though some aspects of specific immunity (inducibility) appear to be present in some cases. Shrimp possess both humoral and cellular responses, although they are less specialized than vertebrate immune responses. Instead there is an innate immunity characterized by a diverse array of humoral factors that originate and/or reside in the hemocytes and are only released during the immune response. Hemocytes play an important role in the cellular immune response, including clotting, non-self recognition, phagocytosis, melanization, encapsulation, cytotoxicity, and cell-to-cell communication. Clotting of hemolymph is a critical mechanism to protect shrimp from excessive loss of body fluids, as well as to sequester and immobilize invading microorganisms. Certain foreign substances such as endotoxin (LPS) and beta (β) 1-3 glucans cause hemocyte lysis, which releases enzymes that initiate clotting through the prophenoloxidase (proPO) system. Clotting and coagulation first serve to locally immobilize microbial invaders. Antimicrobial factors (antibiotics, lectins, and other defensive enzymes) are then released from granules contained in the hemocytes to effect killing of the pathogens prior to phagocytosis. Once the invader is phagocytized and encapsulated, the processes of melanization render it inert and prepare the foreign material for ejection during subsequent molts. In recent years, there has been a considerable amount of work done to determine if certain compounds can be used to stimulate the immune system in a non-specific fashion to increase innate resistance to diseases. Many substances have been identified that have been shown to increase resistance to a variety of pathogens in laboratory and field trials.

FORM AND FUNCTION

General Characteristics of the Immune Response in Shrimp

Shrimp have a semi-open circulatory system. Hemolymph moves through a series of arteries to various organs, and from there into the interstitial spaces of the hemocoel (body cavity) and subsequently to the gills (for oxygenation) and back to the heart for distribution (Cameron and Magnum 1983). The fluid within this system is called hemolymph because there is no separation between the circulatory and lymphatic system in crustacea (Martin and Hose 1992).

Hemocytes play an important role in the cellular immune response, including clotting, non-self recognition, phagocytosis, melanization, encapsulation, cytotoxicity, and cell-to-cell communication (Söderhäll 1999). Clotting (coagulation) of hemolymph is an important mechanism to protect an animal from excessive loss of hemolymph (e.g. following a puncture wound or removal of an appendage) and to immobilize microbial invaders. Specific foreign substances such as endotoxins (lipopolysaccharides, LPS; and peptidoglycans, PG) and β–glucans, cause hemocytes to lyse. Lysed hemocytes release enzymes that initiate clotting through the proPO system (Söderhäll et al. 1996). Humoral factors (antibiotics, lectins, and other defensive enzymes) are then released from granules contained in the hemocytes to effect killing of the pathogens prior to phagocytosis. Once the invader is phagocytized and/or encapsulated, the process of melanization renders it inert and prepares the foreign material for ejection during subsequent molts. Thus, shrimp use a combination of both humoral and cellular responses in their defense against microbial invaders.

Cellular Components (Hemocytes) Involved in the Immune Response

Hemocytes of shrimp can be divided into three morphologically distinct types, based upon the quantity and sizes of granules contained within: agranulocytes (hyalinocytes), semigranulocytes, and granulocytes (Johansson et al. 2000). Agranulocytes are the most variable type of hemocyte and are responsible for phagocytosis and initiating coagulation. In the

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presence of foreign chemicals such as endotoxin or β-glucans, agranulocytes lyse, releasing cytoplasmic factors that cross-link clotting proteins in the hemolymph (Söderhäll and Thornqvist 1997). Agranulocytes have also been implicated in the release of chemicals needed for tanning of the cuticle following molting. Semigranulocytes are intermediate in appearance between agranulocytes and granulocytes, and are important in cell-to-cell communication, encapsulation of particles too large to be phagocytized, and in the storage and release of the proPO system and other cytotoxic chemicals (Johansson et al. 2000). Granulocytes are the largest of the hemocytes and are primarily involved in the storage and release (degranulation) of proPO and lysosomal enzymes (Table 1).

Table 1. Crustacean hemocytes[a]

Hemocyte type	Function in Immunity
Agranulocyte	Phagocytosis[b]
Semigranulocyte	Phagocytosis
	Encapsulation
	Storage and release of the proPO system
	Cytotoxicity
Granulocyte	Storage and release of the proPO system
	Cytotoxicity

a Adapted from Johansson et al. 2000

b Only granulocytes are involved in phagocytosis in penaeid shrimp according to Hose and Martin (1989).

The hemocyte count can vary greatly in response to environmental stress, infection and endocrine activity during the molting cycle (Smith and Ratcliffe 1980; Smith and Söderhäll 1983; Hauton et al. 1997). Hemocyte counts may also decrease near molting, with starvation, and with blood loss (Noga 2000). Experimental injection of fungal cell wall preparation or β1-3 glucan causes a rapid decrease in the number of hemocytes, followed by a slow recovery in crayfish (Persson et al. 1987). Differential hemocyte counts may also vary. In *Marsupenaeus japonicus*, agranulocytes were the dominant cell immediately before and after molting, whereas they decreased during the intermolt period (Sequeira et al. 1995). Evidence of the importance of hemocytes in the immune response is demonstrated by the increased susceptibility of crustaceans to infection after experimental depletion of circulating hemocytes (Söderhäll and Smith 1986) and their depressed numbers in many disease states (Noga 2000).

Humoral Factors Involved in the Immune Response

Many humoral immune factors have been described in shrimp and other crustaceans (Smith and Chisholm 1992). Several of these described factors that originate and/or reside in the hemocytes and are released during the immune response. These factors are primarily non-self recognition factors that include a variety of defensive enzymes, lectins, lipoproteins, antimicrobial peptides, and reactive oxygen intermediates.

Lectins

Lectins are non-enzyme proteins that bind to specific carbohydrates expressed on cell surfaces. The type of carbohydrate to which they bind determines their specificity. Lectin activity (as defined by the agglutination of foreign particles such as bacteria) has been identified in the hemolymph of several penaeid shrimp species (Ratanapo and Chulavatnatol 1990; Vargas-Albores et al. 1993; Marques and Barracco 2000). Some lectins act as opsonins and bind to foreign agents, facilitating their removal by phagocytosis (Marques and Barracco 2000). Many require calcium as a cofactor. There is some evidence that lectins are inducible in shrimp after exposure to infectious agents or trauma (Middlebrooks et al. 1994), although their specific structure and function has not been unequivocally demonstrated. For example, while Ratanapo and Chulavatnatol (1990) found elevated levels of lectins in *Penaeus monodon* infected with *Vibrio vulnificus*, this was not observed in all infected shrimp and Sritunyalucksana et al. (1999) could not induce an increase in concentration either *in vivo* or *in vitro* by using β-glucan, LPS, peptidoglycan, or other commercial stimulants. While lectins have a number of advantages as a health indicator, such as stability and ease of collection and measurement, their uncertain function makes interpretation of changes in circulating levels difficult (Noga 2000).

Defensive Enzymes

The best-studied enzymatic system of crustaceans is the phenoloxidase cascade. Phenoloxidase (PO) catalyses the oxidation of phenols to quinones followed by several intermediate steps that lead to the production of melanin, a brown pigment (Sugumaran 1996). Melanization is involved in the process of tanning of the soft cuticle during the post-molt period, in wound healing, and in defense reactions (encapsulation) against invading microorganisms (Sritunyalucksana and Söderhäll 2000). This pigment can be recognized as dark brown spots in the cuticle of shrimp that have been injured. In defensive reactions, the proPO cascade is activated by extremely low concentrations (picogram/l) of cell wall components (LPS of gram negative bacteria, peptidoglycan (PG) of gram positive bacteria, and β-glucan of fungi). Some of the intermediates that are produced during the formation of melanin are toxic or inhibitory to fungal growth and possibly other microbes (Söderhäll and Ajaxon 1982). In penaeid shrimp, the

enzymes of the proPO system are localized in the semi-granular and granular hemocytes (Perazzolo and Barracco 1997).

Lipoproteins

Hemolymph lipoproteins (LPs) are responsible for the transport of lipids that provide a major source of energy in growing shrimp. As shrimp eat, the lipids present in the feed are digested, absorbed, and transported to the hepatopancreas for storage (Dall et al. 1990). Due to the hydrophobic nature of lipids, they must first associate with proteins to form LPs for transport through the aqueous hemolymph (Kanost et al. 1990). Two types of LPs have been isolated from penaeid shrimp: sex specific LPs found in mature females undergoing ovarian maturation and non-sex specific LPs found in both sexes (Yepiz-Plascencia et al. 2000a). Shrimp LPs can be separated into different classes according to their hydrated density: high density (HDL) and very high density (VHDL) (Teshima and Kanazawa 1980). Shrimp LPs are also involved in defense reactions. It has been shown that the β-glucan binding protein (BGBP) of *Litopenaeus vannamei* and *Farfantepenaeus californiensis*, which is involved in the recognition of β-glucan and the amplification of the phenoloxidase reaction, is the same protein as HDL. This enzyme has recently been immunolocalized to the hepatopancreatic tubule cells (Yepiz-Plascencia et al. 2000b), thus providing evidence for its dual role in both lipid adsorption and storage and as a modulator (via β-glucan) of the immune response via the diet.

Antimicrobial Peptides

Broad-spectrum antimicrobial activity is present in the hemolymph and appears after stimulation of hemocyte clotting and lysis (Khoo et al. 1999; Noga 2000). Of the activities that have been identified all are small polypeptides of various molecular weights. These peptides are often broad-spectrum (Noga et al. 1996; Destoumieux et al. 1997) and probably protect against many infectious agents. A family of related peptides have been characterized in *L. vannamei* and termed penaeidins (Destoumieux et al. 1997). These peptides have chitin-binding properties (Destoumieux et al. 1999) as well. As has been observed with other immune response factors, it seems clear that they function in several roles, in antimicrobial defense, wound healing and synthesis of chitin which would be particularly important to shrimp exposed to microbial infections during the molting process (Bachère et al. 2000).

Reactive Oxygen Intermediates

Another important defense mechanism of hemocytes is the production of superoxide and hydrogen peroxide (reactive oxygen intermediates or ROI). This response, termed the respiratory burst, is an aerobic process, which generates free radicals having microbiocidal activity (Noga 2000). In *L. vannamei*, ROI can be induced by zymosan (yeast cell walls), LPS (bacterial cell walls), and laminarin, a component of macroalgae and suppressed by exposure, *in vitro*, to propiconozole, a fungicide (Munoz et al. 2000). In *P. monodon*, production of ROI has been induced by β-glucan (fungal cell wall) and zymosan (Song and Hsieh 1994) as well as methylparathion, an insecticide (Bodhikhaksha and Weeks-Perkins 1994). Differences observed in the level of activity between *L. vannamei* and *P. monodon* to these stimulants may be related to age and to the physiological state or immune status of individuals (Munoz et al. 2000). *In vivo* administered immune stimulants (Sung et al. 1994) have been shown to confer enhanced protection against bacterial infections by stimulating ROI production. Conversely, a decrease in ROI production may impair immunity by suppression of the same mechanism.

Environmental Factors Affecting the Immune Response

Environmental stress induces changes in the immune status of shrimp. Immune competence can be estimated by measuring hemocyte counts, proPO activation, agglutination responses, phagocytic indices and the release of ROI as discussed above. See Noga (2000) and Le Moullac and Haffner (2000) for excellent recent reviews on the subject. Circulating hemocyte counts can vary non-specifically according to the natural rhythms of both the animal and its environment. Environmental stress can modulate the turnover of hemocytes in hematopoietic tissues (Johnson 1980) and low circulating numbers is correlated with a decreased resistance to pathogens (Persson et al. 1987; Le Moullac et al. 1998). In cases of hypoxia, a decrease in hemocyte number is explained by the differential decrease in agranulocytes and semigranulocytes while little change is seen in granulocyte numbers (Le Moullac et al. 1998). The phagocytic activity of hemocytes is also affected by hypoxia (Direkbusarakom and Danayadol 1998). ProPO gene expression is apparently seriously affected by ammonia concentration (Le Moullac and Haffner 2000). Future research is needed to elucidate the obvious direct and indirect effects of both acute and chronic patterns of environmental stress on the immune system of shrimp, especially the effects of temperature and developmental stage of the animal.

Studies of the various activities described above have been aimed primarily at elucidating the basic immune mechanisms of shrimp and other crustaceans or for optimizing immunostimulation protocols for the enhancement of immunity in shrimp aquaculture.

APPLICATIONS

As discussed, most of the work strongly suggests that the immune system of shrimp is largely non-specific in nature, and that cell wall carbohydrates of bacteria and fungi are strong elicitors. Work in mammals and marine vertebrates suggests the likelihood that there will be other elicitors that are not necessarily carbohydrates. However, it is very important to make a distinction between compounds that act as nutrients - such as vitamins and vitamin precursors -and compounds that act directly to stimulate the immune system of the animal.

Many compounds have been shown to impact the immune system of shrimp. These include astaxanthin and Vitamin C (Merchie et al. 1998), nucleotides and nucleotide precursors (Devresse 2000), as well as a myriad of others. These likely act by ensuring that the shrimp have the required levels of nutrients to mount a strong immune response. They are not acting as antigenic materials and probably do not act by direct stimulation of the immune system.

Bacterial cell wall carbohydrates include LPS and PG. These are both structural components of bacteria, with gram-positive bacteria usually having more PG than LPS and gram-negative bacteria, and vice-versa. Fungal carbohydrates belong to a diverse class of polymeric glucose molecules. Specific β1-6 branched with a β1-3 glucose backbone molecules are required for optimal activity (Raa 1996).

Lipopolysaccharides (LPS)

Vibrio whole cell suspensions and their extracts have 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 (Lightner 1983). Composed of lipids and carbohydrates, these cell wall components are usually the first structures that invading bacteria present to the hosts' immune system. Classically referred to as endotoxins, LPSs have been the subject of thousands of papers and are known to exert both specific and non-specific effects on the immune system of all animals, and potent non-specific effects in crustaceans.

The earliest experiments examining the potential benefits of exposure of shrimp to dead bacterial preparations were reported in the early 1980's. For many years this interest was largely academic. The rapid global expansion of shrimp farming with its consequent severe profit limiting disease issues in many areas has stimulated much more interest in recent years.

The first published observations on the impact of LPS on shrimp date back to the early 1980's. Crowder (1982) reported on work done in the labs of Don Lewis and Addison Lawrence at Texas A&M in April of 1981. Postlarval *Litopenaeus stylirostris* were exposed to a dead suspension of a *Vibrio* bacterium 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 8-10% greater production. One hundred treated and 100 non-treated animals were brought back to the lab and were temperature stressed. 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 5,000 for non-treated shrimp. Though critical experimental details were not provided, this article marks the beginning of a body of work conducted with dead suspensions of vibrio that have shown that LPS based material can exert a potent productivity increasing impact on the culture of shrimp.

Many subsequent studies (Table 2) have confirmed that every species of shrimp tested responds in some manner to exposure to these materials. These are studies where animals were challenged by exposure to virulent pathogens to determine the effectiveness of the treatment. There are many other studies that have looked at how these materials impact components of the immune response, such as phagocytosis, clotting, prophenoloxidase activation, and others.

Most of the materials tested are Vibrio-based using single strains. Several report the use of polyvalent preparations (Kou et al. 1989, Bechteller et al. 1996, Teunissen et al. 1996). Though protective benefits were noted with polyvalent preparations, when the non-specific nature of the immune response is considered along with the wide diversity of potential bacterial pathogens, the need for this type of approach may be questionable.

Among the many benefits reported are:

a. Increased resistance to challenge with virulent bacterial and viral pathogens
b. Increased growth rates
c. Increased resistance to stress
d. Protective benefit seen in lab to at least 50 days from a single exposure
e. Increased survival of larval stages (not necessarily related to bacterial exposure)

There is little doubt from these studies that LPS based materials can provide a substantial degree of non-specific protection under controlled conditions in the laboratory. Results of the use of these materials in the field are a little more difficult to assess. There is strong evidence that their use can ben-

Table 2. Observations on impact of LPS based non-specific immune stimulants on disease susceptibility of various shrimp species.

Citation	Date	Species	Bacterial Strain	Comments
Crowder	1982	*L. stylirostris*	*Vibrio* sp.	100 fold increase in lethal dose when challenged with virulent bacteria
Lewis and Lawrence	1983	*L. setiferus*	*V. alginolyticus*	1000 fold increase in 48 hr LD50
Itami et al.	1989	*M. japonicus*	*Vibrio* sp. NU-1	Injection 69% survival, Immersion 71% survival, Spray 63% survival Control 21% survival
Kou et al.	1989	*P. monodon*	Mixed *Vibrio* sp.	Substantial protection in PLs and broodstock
Song and Sung.	1990	*P. monodon*	*V. vulnificus*	Enhanced growth-no challenge
Sung et al.	1991	*P. monodon*	*V. vulnificus*	Enhanced growth-no challenge
Itami et al.	1991	*P. monodon*	*Vibrio* sp. NU-1	Oral fed to zooea: Approx doubling of survival to mysis.
Itami et al.	1992a	*M. japonicus*	*Vibrio* sp. NU-1	Bath at 1:100 dilution 50% survival Control 0% survival
Itami et al.	1992b	*M. japonicus*	*Vibrio* sp. NU-1	Demonstrated heat stability of active component
Laramore	1992	*L. vannamei*	*Vibrio* sp.	<u>Nursery (36 days post stocking)</u> Immersion 77.4 % survival 699 lbs/ac Control 64.8 % survival 567 lbs/acre <u>Growout (116 days at harvest)</u> Immersion 67.6% survival 519 lbs/ac Control 50.8% survival 372 lbs/acre
Horne et al.	1995	*P. monodon*	*Vibrio* sp.	Immersion 38% survival Control 21% survival Oral 30-70% survival Control 20% survival
Bechteller and Holler		1995	*P. monodon*	Mixed Vibrio sp. Field evaluation w/ possible efficacy
Teunissen et al.	1996	*P. monodon*	Mixed *Vibrio* sp.	Significant protection in PL's, 10, 20 and 30 days post exposure
Newman	1997	*L. vannamei*	*Vibrio* sp.	<u>Nursery (28 days post stocking)</u> Immersion 25, 69, 80 % survival Controls 20, 52, 64 % survival <u>Nursery (50 days post stocking)</u> Immersion 87, 59, 87 % survival Controls 83, 39, 63 % survival <u>Anti-viral activity</u> against TSV
Zafran et al.	1998	*P. monodon*	*V. harveyi*	<u>Treated larval shrimp</u> Immersion 61.5,64.7,66.8,70.2% survival Control 53.2%
Devarja et al.	1998	*P. monodon*	*V. harveyi and* β	Oral administration-no challenge
Alabi et al.	1999	*P. indicus*	*V. harveyi*	<u>Immersion at zooea 3</u> 78.8, 44.2 % survival Control 63.8, 6.5 % survival
George et al.	1999	*P. monodon* *F. indicus*	*V. alginolyticus*	Immersion one log increase in 96hr LD50
Vici et al.	2000	*M. rosenbergii*	*Vibrio*, Photobacterium sp.	Immersion 90.8, 90.7% survival Control 66.7, 59.7% survival
Takahashi et al.	2000	*M. japonicus*	LPS from *P. agglomerans*	Anti-viral activity Immersion 75, 64.7, 52.9 % survival (three different dosage levels) Control 0 % survival

efit shrimp in terms of increased growth rates, increased survival (presumably due to disease resistance), and, for the shrimp farmer, the bottom line (Newman 1999). However, they do not always perform consistently, and few field-based studies show high levels of statistical significance (S. Newman, personal observations). However, there is wide spread agreement, based on trials with large numbers of animals, that the use of bacterial- based materials provides a cost effective benefit and many farms have incorporated their use as a component of their Standard Operating Procedures.

Lab studies have shown that LPS can be effective in the feed, by immersion, spray or injection. Injection is impractical except for the possible exception of broodstock, and has been shown to have no impact on fecundity. The mechanics of semi-intensive culture make immersion and oral applications the plausible approaches. It has been shown that bathing shrimp in high dilutions of concentrated cell suspensions can impact shrimp to harvest. However, in areas where the shrimp are being stressed frequently or where there are high pathogen loads, immersion needs to be followed by oral application.

Glucans

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 β1-3 with β1-6 side chains.

Some of the data suggest that feeding a combination of LPS and glucans may provide a greater benefit than either alone. During 1996, a set of experiments was performed at AgroMarina de Panama in which *L. vannamei* were fed a combination of a marine bacterial extract and a β1-3 glucan (Aqua In Tech Inc, Lynnwood, WA). Approximately 30 days post stocking, the animals were fed a diet containing both of these materials for six days on and seven off. This was done for the entire life cycle until harvest. A total of six, ten-acre ponds 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 (Figure 1). The fed animals showed on average an 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. However, the survivals in these instances were low and the environment was deteriorated. The differences noted between these groups were statistically significant ($P<0.05$). Devarja et al. (1998) reported similar findings, suggesting that combinations of bacterial cell wall material and glucans might be more effective than either alone.

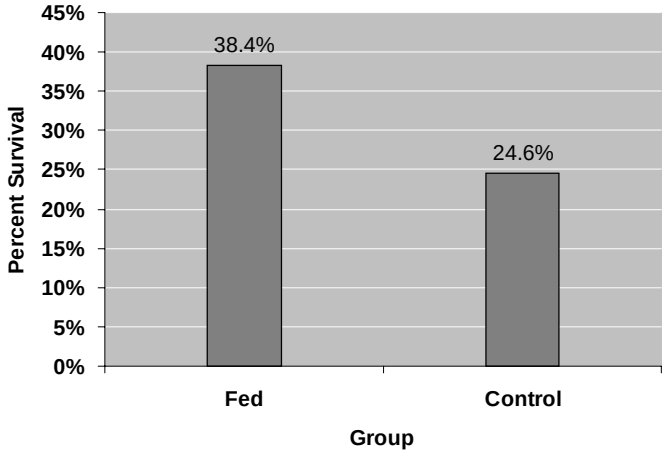

Figure 1. Results of oral field trials conducted in Panama with *L. vannamei*

The ability of glucans to impact the mammalian immune system has been studied for some time and, as with LPS, they have been found to have anti-cancer, anti-viral and antibacterial activity (Raa 1996). Much remains to be learned about their mechanisms of action, and there have been occasional adverse reports associated with their use, despite the fact that one of the most common sources is from the yeast *Saccharomyces cerevisiae*, consumed widely by humans.

There are several features about the glucans that warrant caution when they are used. Many glucans are ineffective (Raa 1996). Furthermore they are apparently readily digested by crustaceans (Glass and Stark 1995) and need to be effectively protected from digestive enzymes. Though the primary source for most of the commercially available preparations is bakers or brewers yeast, almost all of the benefits ascribed to those products being sold are based on data from a non-yeast source (Itami et. al. 1992), or from more highly purified materials that are not sold in the market place because of their cost (Sung et al. 1994). Glucans are also heat labile, and are readily broken down at milling temperatures above 130°C.

The first published reports regarding the use of glucans in shrimp is that of Itami et al. (1992), who noted that Kuruma prawns fed 50 or 100 mg/day of dried *Schizophyllum commune* (VitaStim™-Taito, Tokyo, Japan) cells demonstrated an increase in cellular immune function. No challenges were reported. Song et al. (1997) noted that the tolerance of glucan-treated shrimps was slightly enhanced to stresses like handling, transport and ammonia, but the growth and survival rates of treated and untreated shrimps were not significantly different in their experiments.

Table 3. Observations on impact of β-glucan based non-specific immune stimulants on disease susceptibility of various shrimp species.

Citation	Date	Species	Source	Comments
Itami et al.	1992	*M. japonicus*	*Schizophyllum commune*	
Sung et al.	1994	*P. monodon*	*Saccharomyces*	Resistance to *Vibrio vulnificus*. Immersion at three levels. Ten days post exposure 2 mg/ml 44.4% survival 1 mg/ml 100% survival 0.5 mg/ml 100% survival Control 45.5% mortality. Effect disappeared by day 18
Unknown	1995	*P. monodon*	*Schizophyllum commune*	Fed Z1 to PL1 at 2 g/kg of feed. Challenged PL2 with V. harveyi. 96 h later 4% of controls survived and 16% of the fed groups.
Liao et al.	1996	*P. monodon*	*Schizophyllum commune*	Fed three levels in diet for ten and twenty days prior to challenge with *V. damsela*. Ten days: Control at 8 days post challenge 0% survival. 0.2 g/kg 90% survival, 2 g/kg 90% survival, 10 g/kg 90% survival Twenty days Control: 0% survival, 0.2 g/kg 70%, 2 g/kg 60%, 10 g/kg 70%
Song et al.	1997	*P. monodon*	*Saccharomyces cerevisiae* Glucan precipitated with Ethanol, centrifuged and sonicated.	Fed at 0.1%. PL 66-no protection against *V. vulnificus* (possible over challenge), against WSSV 59% survival in fed and 40% in controls. PL 113 Noted increased tolerance to handling and ammonia stress. *V. vulnificus* challenge 95%,58% survival control 33%, 13% survival. WSSV 54%, 24% survival vs control 0% survival.
Takahashi and Itami	1997	*M. japonicus*	*Schizophyllum commune*	Oral. Fed at 10/mg/kg BW/day for 30 days. Challenged by i.m. injection of virulent *Vibrio sp.* Fed-72% survival Control 48% survival
Chang et al.	1999	*P. monodon*	*Schizophyllum x* commune	Resistance to WSSV. Fed at 2 g/kg diet for 15 day (starting at PL15). 12.2% survival in fed group and 0% survival in controls. Juveniles fed 20 days. 20% survival in fed group and 0% survival in controls.

Most of the peer reviewed work showing that β-glucans are effective tools for impacting the immune response of shrimp has been done with dried *Schizophyllum commune* (Table 3). The results show that the effect is non-specific in nature and a very strong anti-bacterial effect has been noted (Liao et al. 1996). The observed anti-viral effect does not appear to be as strong as that noted with bacterial materials. Also the duration of the effect, by immersion, is not as long as that observed repeatedly with animals exposed to bacterial based materials, 18 days (Sung et al. 1994) or so contrasted with more than 50 days. Overall the data suggests that the use of β-glucans is simply not as effective as the use of LPS or, as shown below, PG.

Peptidoglycan (PG)

PG is a cell wall component of many bacteria, though it is found in greater amounts in gram-positive bacteria. Boonyaratpalin et al. (1995) reported the effects of PG from *Brevibacterium lactofermentum* on the growth, survival, the immune response and tolerance to stress in *P. 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 2). In the challenge, ten shrimp were injected with the Yellowhead Baculovirus (YBV) and survivals compared with non-fed animals. It appeared that there 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 five days of a daily

salinity stress. 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).

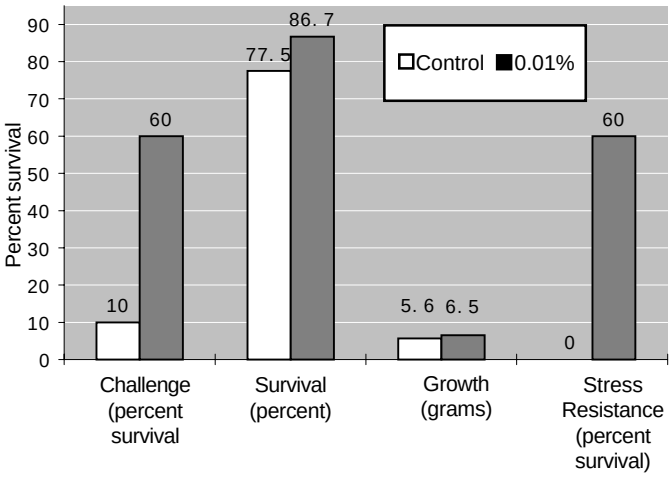

Figure 2. Effects of PG from *Brevibacterium lactofermentum* on the growth, survival, the immune response and tolerance to stress in *P. monodon* (from Boonyaratpalin et al. 1995).

Itami et al. (1998) noted that PG from another source (whole cell material) protected shrimp against the White Spot Syndrome Virus (WSSV) (Figure 3). Three different feeding regimes were used. Animals were fed 0.2 mg of PG per kg/ body weight/day. The feeding regime used in number one (one in the table below) was seven days with PG followed by seven without. This was repeated for 95 days until the experiments were terminated. The second group was fed the diet daily, and the third group two days on and five days off. The groups were small, twenty shrimp each, and they were challenged by a continuous waterborne exposure to the virus initiated at the onset of the experiment. A substantial protective effect was noted. This material is routinely used in conjunction with an algal extract in Japan to offset the impact of the Japanese variant of WSSV (PRDV) in commercially cultured *M. japonicus* (T. Itami, personal communication).

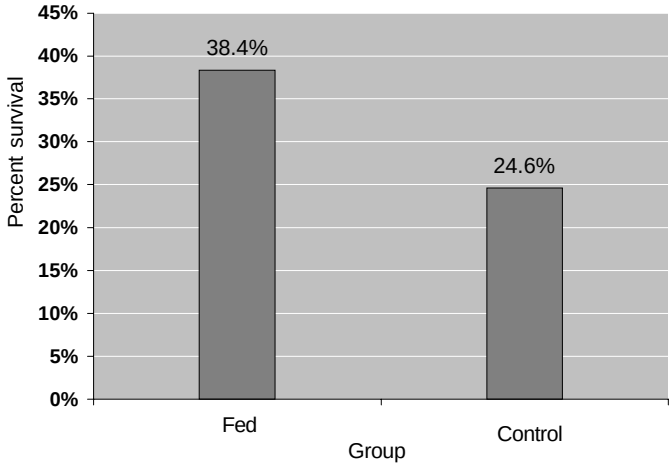

Figure 3. Results of three PG feeding regimes on *M. japonicus* survival to WSSV (from Itami et al. 1998).

CONCLUSION

The cell walls of microorganisms are the first components of pathogens that the shrimp's immune system comes in contact with. Their use as tools for increasing the ability of shrimp to respond to a subsequent encounter with a pathogen is slowly finding a consistent role; though unfortunately many of the materials sold today have little or no scientifically valid data to substantiate their effectiveness. The ability to stimulate a broad range of largely non-specific responses should allow the refinement of those tools that work and the development of new tools that can help the shrimp protect itself against the onslaught of pathogens. However, it is important to recognize that any benefit that they may confer will be minimal in degraded environments or without the implementation of appropriate disease management strategies.

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