EVALUATION OF PARAMYLON AS AN ALTERNATIVE β-1,3-GLUCAN

FOR AQUACULTURED FISH SPECIES

A Dissertation

by

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ABSTRACT

Four separate studies were conducted with different finfish species, to explore the immunomodulatory properties of paramylon, a microalgae-derived linear form of β-1,3-glucan, as an alternative to the widely available and branched yeast-based β-1,3-1,6-glucan products.

In trials I and II, innate immune parameters were investigated in juvenile red drum (*Sciaenops ocellatus*) and Nile tilapia (*Oreochromis niloticus*) exposed *ex vivo* and *in vivo* to a crude microalgae-based commercial product, which provided 50% (wt/wt) of paramylon, compared to β-1,3-1,6-glucan products derived from yeast. Results verified the significant ($P < 0.05$) immunostimulatory properties of these compounds with isolated phagocytes from both species. Red drum and Nile tilapia fed the commercial paramylon product demonstrated a significantly ($P < 0.05$) increased total hemolytic activity and extracellular superoxide anion production, respectively, when fish were fed 100 mg $kg^{-1}$ compared to fish fed the basal diet. In trial III, the immunostimulatory efficacy was compared between purified paramylon and zymosan, the yeast-derived β-1,3-1,6-glucan, when delivered as a feed additive or injected intraperitoneally to hybrid striped bass(*Morone chrysops* × *M. saxatilis*). Juvenile fish fed both beta-glucans at 50 and 100 mg $kg^{-1}$ diet had significantly ($P < 0.05$) increased respiratory burst by isolated phagocytes compared to fish fed the basal diet. Fish fed diets with paramylon at 50 mg $kg^{-1}$ had increased plasma levels of immunoglobulin. The intraperitoneal injection trial exposed sub-adult hybrid striped bass to β-glucan solutions (10 mg β-glucan $kg^{-1}$ body weight). All immunological profiles from whole blood and plasma were modulated

differently depending on the source of β-glucan. In trial IV the synergistic potential between vitamin C and paramylon was evaluated regarding immunological responses of hybrid striped bass *ex vivo* and *in vivo*. The *ex vivo* model was more responsive than *in vivo*, and showed most of the immunological parameters were significantly ($P< 0.05$) enhanced either by vitamin C, the β-glucan or their combination. The feeding trial demonstrated increased production of reactive oxygen species and health-related intestinal enzyme activities of juvenile hybrid striped bass fed these two supplements. The compiled results from these studies support the supplementation of paramylon as a novel β-glucan additive to enhance immunological responses of farmed fish.

# DEDICATION

To my parents,

Melcia and Emilio,

for their unconditional love,

and dedication to their children

and to my sister Daniela,

for her support and care

Thank you!

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To my advisor Dr. Delbert M. Gatlin III for providing me the opportunity to pursue my Ph.D. at Texas A&M University, for his guidance, and patience. To my committee members, Dr. Michael F. Criscitiello, Dr. Michael E. Hume, and Dr. Thomas H. Welsh Jr., for their time, guidance, and for sharing their knowledge.

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# CONTRIBUTORS AND FUNDING SOURCES

## Contributors

This work was supervised by a dissertation committee consisting of Dr. Delbert Gatlin (Department of Wildlife and Fisheries Sciences) as chair and advisor, and Dr. Michael Criscitiello (Department of Veterinary Medicine and Biomedical Sciences), Dr. Michael Hume (USDA-ARS), and Dr. Thomas H. Welsh (Department of Animal Science), as committee members.

Dr. Hume assisted during the experimental infection for Chapter II, and also analyzed the gel images of the denaturing gradient gel electrophoresis analyses for Chapters III and V.

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NOMENCLATURE

| | |
|---|---|
| ACDP | Acid phosphatase |
| ACP | Alternative complement pathway |
| ALKP | Alkaline phosphatase |
| ANOVA | Analysis of variance |
| APCs | Antigen presenting cells |
| BCS | Bovine calf serum |
| BG | Beta-glucan |
| BHI | Brain heart infusion broth |
| CFU | Colony forming units |
| CMC | Carboxymethyl cellulose |
| DE | Digestible energy |
| DGGE | Denaturing gradient gel electrophoresis |
| DMF | Dimethyl formamide |
| DMSO | Dimethyl Sulfoxide |
| ECSA | Extracellular superoxide anion |
| FBS | Fetal bovine serum |
| FE | Feed efficiency |
| GIT | Gastrointestinal tract |
| HBSS | Hank's Balanced Salt Solution |
| HSB | Hybrid striped bass |
| HSD | Honestly significant difference |
| HSI | Hepatosomatic index |
| ICSA | Intrecellular superoxide anion |
| Ig | Immunoglobulin |

| | |
|---|---|
| IPF | Intraperitoneal fat |
| L-15 | Leibovitz cell culture medium |
| MS-222 | Tricaine methanesulfonate |
| MTT | Thiazolyl blue tetrazolium bromide |
| NADPH | Adenine dinucleotide phosphate |
| NBT | Nitroblue tetrazolium |
| NFE | Nitrogen free extract |
| OD | Optical density |
| PAMP | Pathogen-associated molecular pattern |
| PBS | Phosphate buffer saline solution |
| PCE | Protein conversion efficiency |
| PMA | Phorbol myristate acetate |
| PRE | Protein retention efficiency |
| PRR | Pattern recognition receptor |
| P/S | Penicillin/Streptomycin |
| PSE | Pooled standard error |
| PWG | Percentage of weight gain |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| SE | Standard error |
| SOD | Superoxide dismutase |
| TAN | Total ammonia nitrogen |
| Vit. C | Vitamin C |
| WG | Weight gain |

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# CHAPTER I

# INTRODUCTION

Disease outbreaks are recognized as a major bottleneck for aquaculture production and trade. Outbreaks impact directly on the economic revenue of the aquaculture sector in several countries [1]. It is estimated that losses due to pathogenic diseases can reach deficits of billions of dollars in global aquaculture each year [2]. To prevent these losses, fish farms rely on chemotherapeutics, but their usage is becoming more restrictive in animal production, especially for aquaculture. The restrictions happen due to the risk of having drugs and antibiotics leached into the environment, potentially resulting in the emergence of resistant bacterial strains. The worldwide concern over the development of antibiotic-resistant bacteria led to the banishment in 2006 of their use in the European Union for livestock animals [3, 4]. Moreover, some of the substances commonly used to medicate animals may have carcinogenic properties and long degrading periods (*e.g.*, malachite green, pesticides, and antibiotics); therefore, potentially impacting the adjacent fauna and flora, or even bioaccumulating in the trophic chain [5, 6].

Depending on farming conditions, the culture water can be a dense microbial environment that continually exposes farmed fish to a wide range of waterborne pathogenic organisms (*e.g.*, bacteria, viruses, fungi, protozoa, and helminths) and the primary routes for infection are mucosal surfaces of skin, gills, eyes, and the gastrointestinal tract (GIT) [7]. It is believed that the GIT is the main route of infection for bacterial diseases such as streptococcosis, vibriosis, furunculosis, and enteric septicemia in fish [8]. In addition, the increased demand for alternative protein ingredients to replace fishmeal in aquafeeds has led to higher inclusion of plant feedstuffs [9], and these ingredients may contain anti-nutritional factors and secondary

metabolites that can impair the functional competency of the GIT against pathogen translocation [10, 11].

The use of selected non-antibiotic feed additives seems to be an environmentally sound strategy to reduce the incidence of disease by augmenting the immunological responses prior to an infection, thereby overcoming the indiscriminate use of antibiotics and chemotherapeutics in aquaculture [12, 13]. One example of such an immunomodulating additive is β-glucans (BGs), which are bioactive compounds that have long been used in animal husbandry [14-16]. BG can be naturally found as cell wall components in bacteria, microalgae, macroalgae, yeast, fungi, and plants [17-19]. When supplemented in fish diets, theoretically, these molecules cannot be digested and absorbed due to the β-glycosidic linkages between the glucose molecules. The repeating patterns of their structures, also shared by microbial pathogens, have been termed as pathogen-associated molecular patterns (PAMPs), which are recognized by the host cell pattern recognition receptors (PRR), eliciting an inflammatory cascade that, ultimately, leads to the enhancement of innate immune responses [14].

Many aspects of the immunological response can be improved by dietary supplementation of BGs, such as the phagocytic activity of leukocytes, the release of cytokines (IL-1, IL-6, TNF-α, and interferons), production of reactive oxygen species, and antigen processing [15, 18]. The BG's molecular weight, solubility, polymer charge, chemical structure, solution conformation and degrees of branching play a role when activating different cell membrane receptors (*e.g.*, dectin-1, complement receptor 3, and toll-like receptors) [18-20]. For instance, high molecular weight BGs can activate leukocytes stimulating their phagocytic, cytotoxic and anti-microbial activities, while low molecular weight BGs may affect leukocytes only when they are previously stimulated by cytokines [21, 22]. Most studies with aquatic

organisms have focused on testing BGs either derived from yeast or using the yeast itself as a functional ingredient [23]. However, the integrity and purity of yeast-derived BGs may be affected during the extraction and preparation of these molecules, and consequently influence their immunostimulatory properties [17, 24].

In this context, *Euglena gracilis* appears to be a suitable alternative BG source compared to more traditional yeast-derived BGs. *Euglena* is a microalgae that can be applied as a bioremediation tool and treat wastewater from agriculture and the sugar industry [25]. Under heterotrophic conditions, this microalgae can store large quantities of Paramylon, a linear polymer of β-1,3-glucan, in their cytoplasm [26, 27]. The high purity and crystallinity of *Euglena* BG facilitate the isolation and purification processes, thereby decreasing extraction costs [25]. In addition, crude *Euglena* can also be considered as a feed additive, not only for supplying Paramylon BGs but also for being a highly nutritious food source. Crude Euglena products can provide relatively high levels of protein, minerals, polyunsaturated fatty acids, and vitamins, and thus may be considered a promising nutraceutical candidate for the aquafeed industry [26, 28-30]. Based on the immunostimulation recently observed in different fish species supplementation with BG, I hypothesize that even greater efficacy may be achieved by supplementing these products with nutrients related to health, such as vitamin C.

Unlike with several farmed terrestrial animal species, vitamin C or ascorbic acid is an essential nutrient for fish. These aquatic animals cannot synthesize this compound endogenously; therefore, vitamin C is required to be provided in the diet [31]. Vitamin C is involved in several physiological functions such as growth, development, reproduction, and tissue repair, as well as immunological and stress responses [32]. Fish leukocytes and lymphoid organs have been observed to accumulate vitamin C concentrations 50 to 100 times higher than that of circulating

plasma [32, 33]. It is hypothesized that the antioxidant properties of vitamin C may enhance immune cells by protecting them against free radicals produced by normal metabolism or stress exposure [34, 35].

As previously mentioned, considerable attention has been drawn to dietary supplementation of immunostimulants as prophylactic measures to prevent diseases. Several studies have addressed the benefits of BGs on animal health, and the enhanced effects on innate and adaptive immune systems have been demonstrated for several fish species. However, only a few studies have addressed alternative sources of BGs, and compared different BGs in aquaculture species. Limited studies compared the interaction between immunostimulants and nutrients that play roles in the immune response, such as vitamin C. Therefore, the goals of the compiled research were three-fold: First, to evaluate the immunomodulatory properties of purified Paramylon and crude *Euglena gracilis* supplementation on isolated phagocytes, and evaluate the short-term effects of dietary crude *Euglena gracilis* product as a source of Paramylon, using red drum and Nile tilapia as animal models; secondly, to compare the exposure of BG from *Euglena gracilis* and *Saccharomyces cerevisiae* on hybrid striped bass; and fourthly, to compare possible interactions of BGs with vitamin C *ex vivo* and *in vivo* using hybrid striped bass as an animal model.

# CHAPTER II

# THE EFFECT OF Β-1,3-GLUCAN DERIVED FROM *Euglena gracilis* (ALGAMUNE™) ON THE INNATE IMMUNOLOGICAL RESPONSES OF NILE TILAPIA (*Oreochromis niloticus* L.). [1]

## 2.1 Introduction

The use of feed additives to enhance the innate immune system is a strategy that aims to reduce the incidence of diseases, thereby overcoming the indiscriminate use of antibiotics and chemotherapeutics in aquaculture [12, 13]. The worldwide concern of developing bacterial strains resistant to antibiotics should be further stressed for aquacultural production because medicated feeds, and antibiotics directly administered in the water can readily leach into the environment [36]. Therefore, the effective application of non-antibiotic feed additives to enhance immunity and reduce disease incidence continues to be explored.

One such feed additive is β-glucan, which are all naturally occurring polysaccharides having glucose as their building blocks linked by beta-linkages [15, 17]. β-glucans are found naturally in plants, yeasts, fungi, algae, protozoa and bacteria [14, 20, 37]. Pathogenic microorganisms also share these polymers in their cell walls. Therefore, β-glucans can be termed pathogen-associated molecular patterns (PAMPs) [3, 16]. Moreover, when there are interactions of these polysaccharides with membrane receptors of fish leukocytes, an intracellular signaling

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[1] *Reprinted: F.Y. Yamamoto, F.J. Sutili, M. Hume, D.M. Gatlin III, The effect of β-1,3-glucan derived from *Euglena gracilis* (AlgamuneTM) on the innate immunological responses of Nile tilapia (*Oreochromis niloticus* L.). Journal of Fish Diseases 41 (2018) 1579-1588. Copyright 2019, with permission from John Wiley and Sons, Inc. Wiley Company.

cascade occurs, ultimately leading to the production of cytokines and anti- or pro-inflammatory responses [18, 20, 38].

Differences in β-glucan molecules such as degrees of branching, molecular mass, and solubility can influence how the immune system will be affected [14, 20]. Most studies with aquatic organisms have focused on testing β-glucans either derived from yeast or using the yeast itself as a functional ingredient [23]. However, the integrity and purity of yeast-derived glucans may be affected during the extraction and preparation of these molecules, and consequently influence their immunostimulatory properties [17, 24].

In this context, *Euglena gracilis* appears to be a suitable alternative β-glucan source. *Euglena* is a microalgae that can be used as a bioremediation tool to treat waste water from agriculture and the sugar industry [25]. Under heterotrophic conditions, this algae can store large quantities of Paramylon, a linear polymer β-1,3 glucan, in their cytoplasm [26, 27, 39]. The high purity and crystallinity of *Euglena* β-glucan facilitates the isolation and purification processes at low cost [25]. In addition, crude *Euglena* can also be considered as a feed additive, not only for supplying Paramylon, but also for being a highly nutritious food source. It can provide high levels of protein, minerals, polyunsaturated fatty acids, and vitamins, thereby making it a promising nutraceutical candidate for the aquafeed industry [26-30].

Beneficial responses have been reported for different animal models when exposed to Paramylon. Rats fed supplemental doses of Paramylon, presented hepatoprotective properties when challenged with carbon tetrachloride, and suppressed atopic dermatitis lesions [40, 41]. A preventive effect against colon cancer and increased levels of organic acids in the cecal contents were observed in mice fed Paramylon for 11 weeks [29]. Rainbow trout (*Oncorhynchus mykiss*) fed Paramylon had up-regulated expression of antimicrobial peptides genes (cathelicidins) and

IL-1β [39]. Brine shrimp *Artemia* sp. had enhanced stress resistance when exposed to Paramylon granules in the culture water [42].

The present study aimed to evaluate the immunomodulatory ability of Algamune™ (a crude extract of dried *Euglena gracilis*) and purified Paramylon when incubated with isolated phagocytes of Nile tilapia *ex vivo*. After the immunomodulation of the isolated tilapia phagocytes was appraised, a feeding trial evaluating graded doses of Algamune™ in Nile tilapia diets was conducted, followed by a *Streptococcus iniae* disease-challenge.

## 2.2 Material and methods

### *2.2.1 Ex vivo trial*

#### 2.2.1.1. Fish and phagocyte isolation

Twelve healthy adult Nile tilapia (*Oreochromis niloticus*) (402 ± 12 g, average ± standard error (SE)) were obtained from Louisiana Specialty Aquafarms (Robert, LA) and were held in 1,000-L circular fiberglass tanks as a part of a freshwater recirculating aquaculture system. The recirculating system included a settling chamber, biological and mechanical filtration, and aeration was provided through air diffusers. Water quality parameters were steady and suitable for tilapia culture. Fish were maintained by feeding a commercial diet containing 32% crude protein and 6% lipid (Rangen, Angleton, TX), twice daily to apparent satiation. During sampling, fish were euthanized with an overdose of tricaine methanesulfonate (>250 mg $L^{-1}$) (MS-222, Western Chemical Inc, Ferndale, WA, USA) [43]. Kidney tissues were aseptically excised, pooled into a composite sample, and stored in 50 ml of cold Leibovitz cell culture medium (L-15, cat# 4500-372, Corning®, Corning, NY, USA) supplemented with 2% fetal bovine serum (FBS), 10 units $mL^{-1}$ of heparin, and 100 units $ml^{-1}$ of penicillin/streptomycin (#cat L5520,

F2242, H6279, and P4333, respectively; Sigma Aldrich, St. Louis, MO, USA). Phagocyte isolation followed the procedures established by Secombes [44] with modifications by Sealey and Gatlin III [34]. Briefly, the tissues were homogenized using a Potter-Elvehjem glass homogenizer, and the homogenate suspension was filtered using a sterile 100-µm mesh nylon filter (cat# 21905-026, VWR, Radnor, PA, USA). The filtered suspension was centrifuged at 1,000 × *g* for 10 min at 4˚C and the cell pellet re-suspended in cold, sterile phosphate-buffered saline (PBS, cat# P1644, Sigma Aldrich). This procedure was repeated three times. The resulting cell suspension was layered on a Percoll 34/51% gradient (cat# P1644, Sigma Aldrich) and centrifuged at 400 × *g* for 25 min at 4˚C. The layer of cells resting at the 34/51% interface were carefully collected and washed three times as previously mentioned. Cells were re-suspended in 0.1% L-15, and cell viability was assessed by staining with 0.4% trypan blue and recovered samples were used only when survivability was higher than 95%. Cells were enumerated using a hemocytometer under a light microscope, and the concentration was adjusted to $2 \times 10^7$ cells $mL^{-1}$ by adding 0.1% L-15. A 96-well flat-bottom microplate (351172, Falcon, Le Pont de Claix, France) was seeded with 100 µL of the phagocyte suspension in each well.

2.2.1.2. β-glucan and phagocytes culture conditions

Commercial preparations of dried crude *Euglena gracilis* (Algamune™, Algal Scientific Corporation, Plymouth, MI, USA) and purified β-glucan Paramylon (cat# 89862, Sigma Aldrich) were solubilized using 0.1 N NaOH and one-half serial dilutions in 0.1% L-15 to yield final concentrations of 0, 8, 16, 32, 64 and 128 µg $mL^{-1}$ for each glucan source. The potential effects of pH change on the cell culture media were diminished by maximizing the amount of each β-glucan source. Thus, a high volume of cell culture media was necessary to achieve final

concentrations. Sixteen wells of the microplate were used as replicates for each source and inclusion level combination. Microplates were incubated with gentle agitation for 24 h at room temperature (~24˚C). After incubation, the cell culture media was removed, and wells were washed twice with room temperature sterile PBS and samples were prepared for the following analyses.

2.2.1.3. Intracellular superoxide anion (ICSA)

In order to measure the intracellular $O_2^-$ production by phagocytes, 100 µL of nitroblue tetrazolium solution (1 mg of NBT $mL^{-1}$, cat# N6876, Sigma Aldrich) with 1 µg $mL^{-1}$ of phorbol myristate acetate (PMA, cat# P8139, Sigma Aldrich) in phenol- red-free Hank's Balanced Salt Solution (HBSS) [45] was added to each well. By reducing the NBT, with intracellular anions, formazan granules are formed within the cell. After 45 min of incubation, the media were removed, and cells were fixed in 100% methanol for 10 min. After the fixation, wells were washed three times with 70% methanol and allowed to air-dry in the dark for 1 h. The reduced formazan in each well was solubilized by adding 120 µL of 2M KOH (2M) and 140 µL of dimethyl sulfoxide (DMSO, D8418, Sigma Aldrich). Plates were read using a multi-scan spectrophotometer at 620 nm, and the absorbance was expressed as intracellular superoxide anion production (ICSA).

2.2.1.4. Extracellular superoxide anion (ECSA)

The ability to secrete extracellular $O_2^-$ by phagocytes was measured by reduction of ferricytochrome C (cat# C2506, Sigma Aldrich). Ten wells with layered cells were covered with 2 mg $mL^{-1}$ of ferricytochrome C solution containing 1 µg $mL^{-1}$ of PMA for each β-glucan

concentration. Four wells were used as a negative control by adding 300 units $mL^{-1}$ of superoxide dismutase (SOD, cat# S2515, Sigma Aldrich) to the ferricytochrome solution to prevent oxidation. Plates were read immediately and after every 15 min until 60 min at 550 nm using a multi-scan spectrophotometer. The concentration of extracellular superoxide anion (ECSA) was calculated as following [46]: ECSA nmol $O_2^- = [(\Delta \text{ Absorbance after 45 min} \times 100) \div 6.3]$.

*2.2.2. Experimental diets and feeding trial*

A basal diet (Table 1) was formulated to meet the known nutrient requirements of tilapia according to Zychowski et al. [47], and a premix containing cellulose and dried crude *Euglena gracilis* (Algamune™, Algae Scientific Inc.) at 100 g $kg^{-1}$ was thouroghly mixed into the basal diet at the expense of cellulose to give a final concentration of 100, 200, 400 and 800 mg $kg^{-1}$ diet. The feed mixture was further mixed in a V-mixer (Blend master, Buflovak, NY, USA) machine for 30 min and blended with oil and water using an industrial mixer (A-200 Hobart meat grinder, OH, USA). The resultant moistened mash was cold-pelleted through a 3-mm die plate and dried at room temperature with forced ventilation for 24 h. The dried pellets were ground and sieved to the appropriate size (~10 mm length) to feed the fish. All diets were analyzed for proximate composition following AOAC [48] procedures.

Three hundred advanced juvenile tilapia (68.0 ± 1.46 g), obtained from the same supplier as mentioned for the *in vitro* trial, were stocked in 20, 110-L glass aquaria operating as a freshwater recirculating system equipped with a settling chamber, UV-filtration, biological and mechanical filtration. Aeration was supplied to each tank by diffusion through air stones connected to a central regenerative blower system. Water quality parameters were measured

twice weekly and maintained within optimal levels for tilapia culture. Water temperature and dissolved oxygen were measured using a YSI-ProODO (YSI Inc., Yellow Springs, OH, USA) and these parameters remained steady throughout the trial. Total ammonia-nitrogen (TAN) and nitrite were measured spectrophotometrically (Hach Inc, Loveland, CO, USA). Water quality measurements throughout the trial are summarized as follows: temperature: 27.2 ± 0.29 ˚C; dissolved oxygen, 4.89 ± 0.15 mg $L^{-1}$; TAN, 0.34 ± 0.14; nitrite, 0.048 ± 0.010 mg $L^{-1}$; and pH, 7.86 ± 0.16 (values reported as mean ± SE). A 12:12 hour photoperiod was maintained using fluorescent lighting controlled by timers. Fish were conditioned with the basal diet for 1 week prior to initiating the trial. Treatments were assigned to each aquarium in quadruplicate using a completely randomized design. Fish in each aquarium were fed twice daily at a fixed rate ranging from three to two percent of biomass over a 3-week period.

Table 1: Formulation and analyzed proximate composition of the basal diet fed to Nile tilapia

| Ingredient | g $kg^{-1}$ |
|---|---|
| Menhaden Meal[1] | 110 |
| Soybean Meal | 492 |
| Soybean Oil | 48 |
| Vitamin Premix[2] | 30 |
| Mineral Premix[3] | 40 |
| Dicalcium phosphate | 10 |
| Dextrinized starch[4] | 203 |
| Glycine | 10 |
| DL-Methionine | 2 |
| CMC[5] | 20 |
| Celufil[6] | 35 |
| **Proximate composition** | **(%)** |
| Moisture | 7.8 |
| Crude Protein | 35.0 |
| Lipid | 8.4 |
| Ash | 8.6 |

[1] Omega Protein Abbeville, LO, USA

[2,3] Same as in Moon and Gatlin III [49]

[4,5,6] MP Biomedicals, Solon, OH, USA

2.2.2.1. Production performance, sample collection, and immune analysis

Production parameters were measured as:

Percentage weight gain of the initial weight (%WG = 100 × ((final weight – initial weight) ÷ initial weight)

Feed efficiency (FE) = weight gain ÷ dry feed intake

Survival (%) = 100 × (number of surviving fish ÷ initial number of fish)

At the end of the third week, four fish were randomly chosen from each aquarium, and had blood collected from a caudal puncture with heparinized tuberculin syringes and euthanized immediately with an overdose of MS-222 [43]. Head and trunk kidneys from the four fish were dissected and pooled for each aquarium in a 15-mL tube containing L-15 enriched medium (containing 2% of bovine calf serum (BCS) and 100 units of penicillin/streptomycin $mL^{-1}$). Phagocyte isolation followed the same protocol as previously mentioned for the *ex vivo* trial.

Extracellular and intracellular reactive oxygen species produced by the cells were measured as described in the *ex vivo* section. Reactive oxygen species from blood phagocytes were measured by incubating 50 µL of whole heparinized blood with 2 mg $mL^{-1}$ of NBT for 30 min, formazan granules were suspended using N-N dimethylformamide (DMF; cat # 227056, Sigma Aldrich), and read in the spectrophotometer at 545 nm [50]. The remaining blood was centrifuged for 10 min at 10,000 × *g*, and plasma samples were aliquoted in different vials and frozen at -80˚C prior to analysis. Plasma lysozyme activity was measured by a turbidimetric reduction assay using a *Micrococcus lysodeikticus* (cat # M3770, Sigma Aldrich) suspension in PBS, and pH adjusted to 6.1 [51] which was previously determined to be the optimum activity for Nile tilapia. The hemolytic assay was performed according to Sutili et al. [52] with minor modifications. Briefly, 100 µL of plasma were incubated with 25 µL of goat erythrocytes in a

96-well U-shaped microplate at room temperature (~24˚C) for 2 hours. After incubation, 150 μL of ice cold PBS was added to stop the hemolytic activity of the complement proteins and plates were centrifuged at 3,000 × *g* for 5 min. Each sample analysis was performed in duplicate, and as a positive and negative control, erythrocytes were incubated with distilled water or PBS for total hemolysis or no lysis, respectively. Plasma total protein and total immunoglobulins were measured following the method of Siwicki et al. [50].

2.2.2.2. Bacterial challenge

After sampling on the third week of feeding the experimental diets, 10 fish from each aquarium were randomly selected and transferred to a flow-through culture system and confined in a manner that each experimental unit would be represented as the same setup as in the indoor trial. Fish were injected intraperitoneally 0.1 mL with a previously estimated LD 50 dose of virulent hemolytic *Streptococcus iniae* (*S. iniae* was kindly donated by Dr. John P. Hawke of Louisiana State University). The bacteria were cultured in Brain-Heart Infusion broth (BHI, cat#53286, Sigma Aldrich) and incubated overnight at 27°C. The bacterial broth was centrifuged at 2,000 × *g* for 10 min, then washed with sterile PBS and resuspended to adjust the absorbance of 0.700 at 620 nm, to give a final concentration of 2 x $10^8$ colony forming units (CFU) $mL^{-1}$ (validated by the previous LD 50) [53]. Colony forming units were confirmed by a ten-fold serial dilutions in sterile PBS and plating on BHI agar (cat# 70138, Sigma Aldrich). Mortality was recorded daily for 7 days, and deceased animals were removed. Brain tissue and head kidneys from these animals were aseptically dissected and spread on BHI agar to confirm mortality by *S. iniae* infection.

### *2.2.3. Statistical analysis*

*Ex vivo* and *in vivo* data were validated for homogeneity of variances by the Brown-Forsythe test [54] and subjected to one-way ANOVA ($\alpha$=0.05), PROC GLM of SAS (version 9.4, SAS Institute, Cary, North Carolina, USA). If significant differences were identified ($P<0.05$), data were subjected to polynomial contrasts to verify the patterns that most adequately fit the results. When significance differences were found, data were subjected to linear or quadratic regression, chosen by lesser P value. Intracellular superoxide anion *in vitro* was subjected to a linear broken-line regression (PROC NLIN). If no regression pattern could be detected, a Duncan test was performed for pairwise comparisons of means. Survivability after the bacterial challenge was compared by Kaplan-Meier survival analysis with Log-rank test of Sigma Plot (version 10.0, Systat Software, San Jose, CA, USA).

## 2.3 Results

### *2.3.1. Ex vivo* assays

Significant differences were observed for both β-glucan sources for ECSA and ICSA production by head-kidney phagocytes in a dose-dependent manner. Responses to graded Paramylon increments in the cell culture medium was best explained by a non-linear broken line regression, reaching a plateau after 16.21 µg $mL^{-1}$ for ICSA (Figure 1). In constrast, cells incubated with Algamune™ presented a linear upward response ($R^2$=0.53) for ICSA. Both Algamune™ and Paramylon showed a linear upward response for ECSA ($R^2$=0.63 and $R^2$=0.41, respectively) (Figure 2).

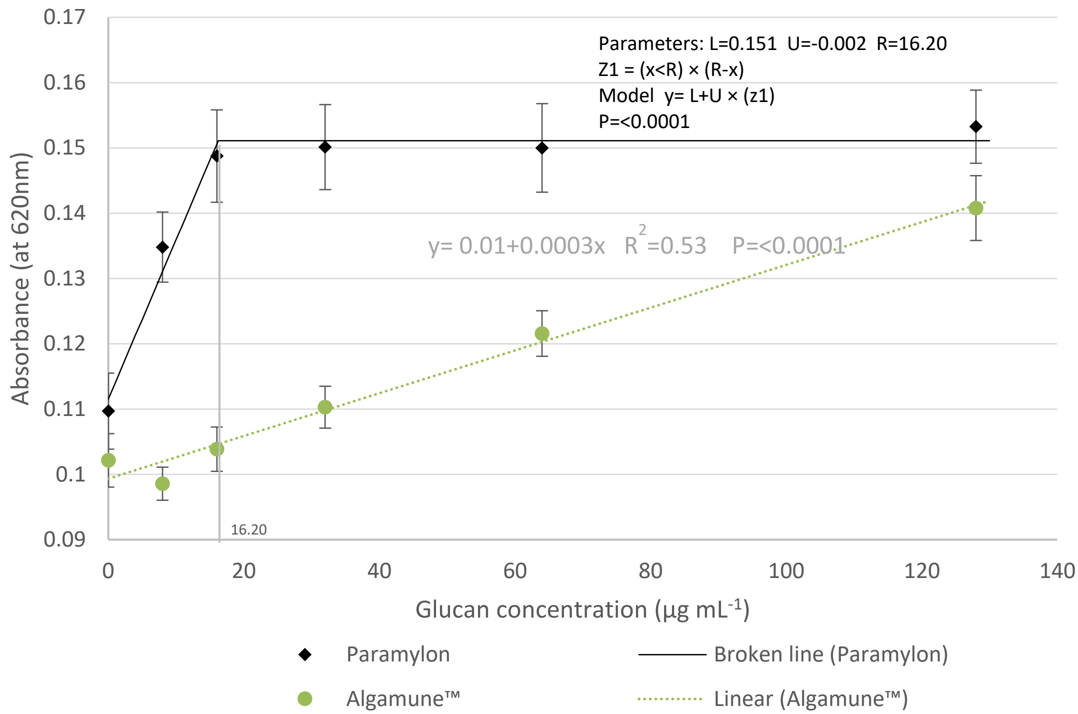


Figure 1. Intracellular anion superoxide production by isolated leukocytes incubated with Algamune™ and Paramylon and as measured by reduction of nitroblue tetrazolium

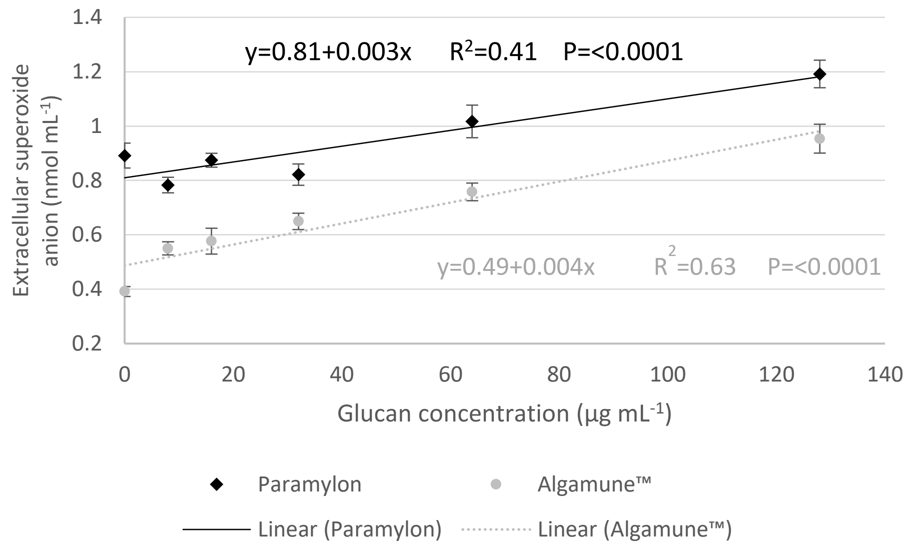


Figure 2: Extracellular anion superoxide production of isolated Nile tilapia leukocytes incubated with Algamune™ and Paramylon and as measured by reduction of Ferricytochrome C

*2.3.2. Production and immune parameters, and bacterial challenge for the in vivo trial*

No significant differences were observed for the production parameters (WG, FE, and survival) after the 3-week feeding period (Table 2). Respiratory burst of whole blood phagocytes, plasma lysozyme and antiprotease activity, total plasma protein and total immunoglobulin, as well as ICSA of isolated phagocytes were also similar among the various treatments (Table 3). Significance was found for ECSA of isolated phagocytes ($P<0.01$) (Figure 3) but no dose-response model could be established. Fish fed diets supplemented with 200 mg $kg^{-1}$ had significantly higher ECSA production when compared to those fed the basal diet and 100 mg $kg^{-1}$. Experimentally infected fish presented progressively clinical signs of *Streptococcus* infection. Fish developed skin melanisation, lethargic behavior by not consuming feed and resting close to the water surface, erratic and disoriented swimming, opacity of the cornea, and uni- or bi-lateral exophthalmia. Nevertheless, no differences were observed for survival after challenge with the virulent *Streptococcus iniae* strain ($P=0.78$) (Figure 4).

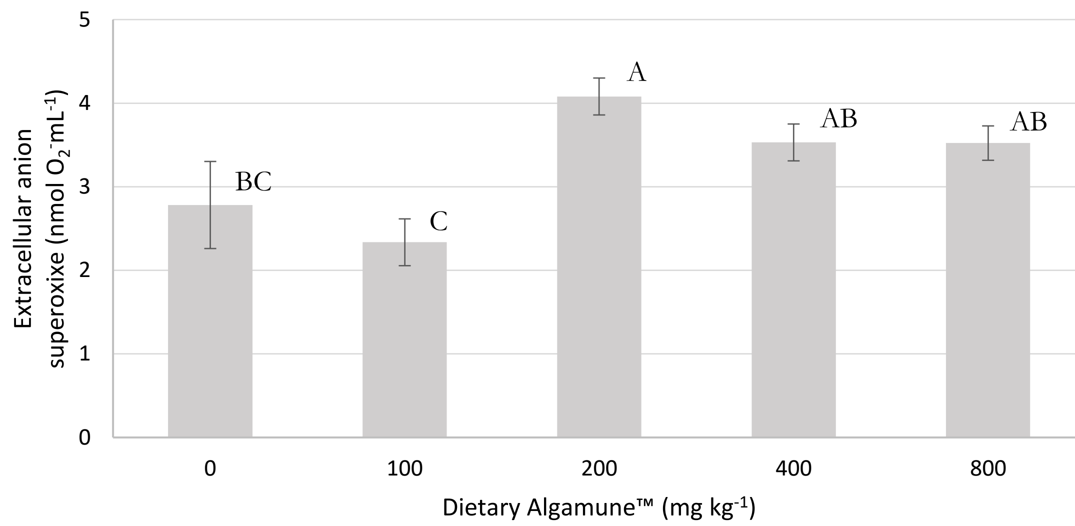


Figure 3: Extracellular superoxide anion production of Nile tilapia fed diets containing Algamune™

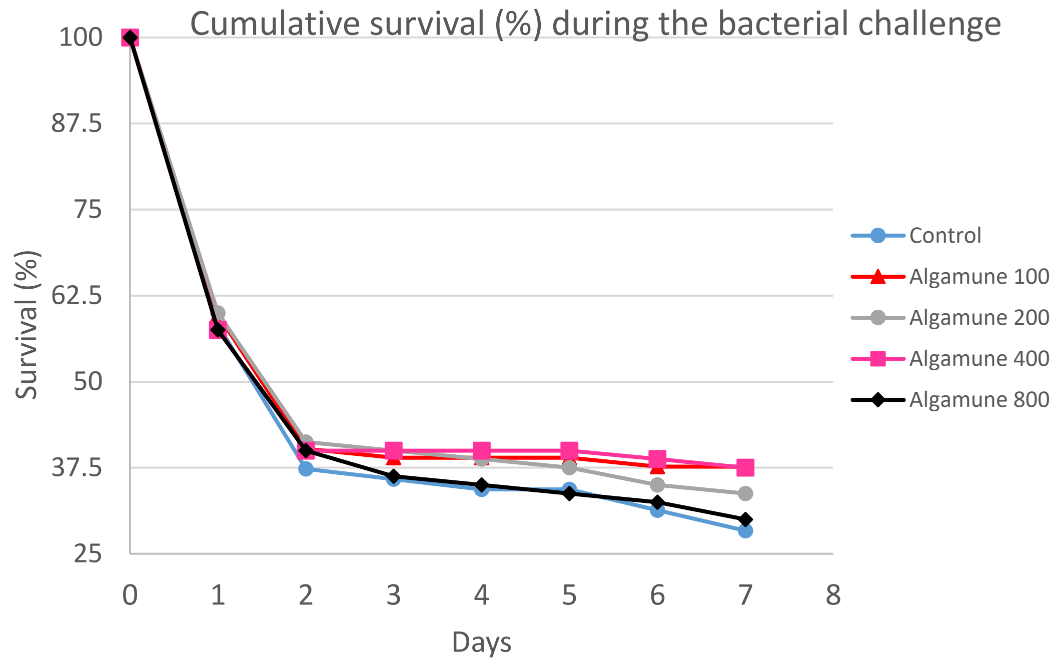


Figure 4: Cumulative survival rate of Nile tilapia fed Algamune™ for 3 weeks graded doses of Algamune™ and challenged by intraperitoneal inoculation of a virulent strain of *Streptococcus iniae* (P=0.78)

Table 2: Performance of Nile tilapia fed graded inclusion levels of Algamune™ for 21 days

| Algamune™ concentration (mg $kg^{-1}$) | 0 | 100 | 200 | 400 | 800 | P value | PSE |
|---|---|---|---|---|---|---|---|
| Survival (%) | 98.35 | 98.35 | 100 | 100 | 98.35 | 0.73 | 0.54 |
| Weight gain (%) | 35.08 | 31.97 | 33.55 | 38.07 | 34.83 | 0.23 | 12.65 |
| Feed efficiency | 0.47 | 0.45 | 0.46 | 0.51 | 0.46 | 0.78 | 0.02 |

PSE: Pooled standard error

Table 3: Immunological responses of Nile tilapia fed graded inclusion levels of Algamune™ for 21 days

| Algamune™ concentration (mg $kg^{-1}$) | 0 | 100 | 200 | 400 | 800 | P value | PSE |
|---|---|---|---|---|---|---|---|
| NBT (mg mL $blood^{-1}$) | 2.61 | 2.99 | 2.86 | 2.65 | 2.54 | 0.24 | 0.07 |
| Intracellular superoxide anion (abs 620 nm) | 0.229 | 0.161 | 0.140 | 0.214 | 0.229 | 0.53 | 0.20 |
| Hemolytic activity (Hemolysis %) | 62.63 | 70.30 | 73.33 | 67.76 | 72.90 | 0.83 | 2.74 |
| Lysozyme activity (Units $mL^{-1}$) | 475 | 536 | 454 | 395 | 447 | 0.47 | 23.98 |
| Total plasma protein (mg $mL^{-1}$) | 41.35 | 42.14 | 52.84 | 43.69 | 49.7 | 0.14 | 1.74 |
| Total plasma immunoglobulin (mg $mL^{-1}$) | 19.06 | 22.53 | 25.19 | 21.17 | 25.19 | 0.36 | 1.12 |

PSE: Pooled standard error

2.4 Discussion

In aquacultural production, the functional feed concept is based on developing a balanced nutritive diet with supplementation of additives that can improve health and disease resistance of cultured fish [15, 55]. The commercial product of *Euglena gracilis,* Algamune™, is a potential aquafeed supplement that can fit into this scope. Not only can it supply high-quality nutrients, it also provides an alternative source of β-glucan, the linear β-1,3 Paramylon [25, 26, 28]. Whereas most research to date has focused on evaluating the immunomodulatory effects of β-glucan derived from *Saccharomyces cerevisiae*, the present study verified the immunomodulatory effects of an alternative source of β-glucan derived from *Euglena* directly to isolated Nile tilapia phagocytes and through supplementation of graded doses in the diet.

Respiratory burst is an antimicrobial response from the immunological repertoire of phagocytes. During phagocytosis, hightened consumption of molecular oxygen takes place,$O_2$ is reduced by membrane-associated NADPH oxidase, hence generating superoxide anions ($O_2^-$) that are further converted to the bactericidal hydrogen peroxide ($H_2O_2$) by superoxide dismutase [56, 57]. The ability of β-glucan to interact with phagocyte receptors (such as dectin-1, toll-like receptors, complement receptor 3) can result in the assembly of NADPH oxidase within the cytosol, and also a cascade of intracellular signaling, that ultimately leads to NF-κB pathway activation [18, 20, 56, 58]. In the present study, the incubation of the two Paramylon sources resulted in the production of ECSA by the phagocytes in a linear, dose-dependent response, with the purified Paramylon having higher production than Algamune™. Interestingly, ICSA reached a plateau after 16 $\mu g\ ml^{-1}$ for Paramylon; whereas, Algamune™ still followed a linear increase. When incubating the same Algamune™ and Paramylon with red drum phagocytes, an optimum point was observed for ICSA at 0.16 and 2.24 $mg\ L^{-1}$, respectively [59]. An apparent plateau for

reactive oxygen species response also was observed when porcine neutrophils were incubated with 400 and 800 μg $ml^{-1}$ of Paramylon [60]. In contrast, an overdose of Paramylon was not observed even when 1,000 μg $ml^{-1}$ of Paramylon were supplemented to Barramundi (*Lates calcarifer*) macrophage culture media, with this concentration yielding the highest value of reactive oxygen species measured by relative chemiluminescence [61].

Orally administrated β-glucans can interact with the gut mucosal immune system, eliciting intestinal inflammation, hence increasing the mucosal immune responses [62], and activity of leukocytes such as macrophage antigen presenting cells (APCs) and intraepithelial lymphocytes [20, 22, 63]. The mechanisms of how dietary β-glucan is uptaken by APCs has not been fully characterized for fish. Nevertheless, it has been reported that when labeled laminaran (branched β (1,3-1,6) glucan) was administered perianally to Atlantic salmon (*Salmo salar*), fluorescent vesicles were found in the epithelial cells of the intestine and macrophages from the anterior head kidney [64, 65]. It is suggested that APCs, presumably macrophage-like cells [63], transport the glucans as antigens to the head kidney and upregulate the nonspecific and specific immune responses in this lymphoid organ. For example, when feeding commercial β-glucans to rainbow trout, upregulation of many genes associated with the immune system in the head kidney were observed (*e.g.* lysozyme, TNF-α, TGF-β) [39, 66, 67]. In the present study an elicited immunological response after the dietary exposure to Algamune™ was observed in the form of increased extracellular and intracellular production of ROS by isolated phagocytes from the head kidney. These same findings were also reported for snapper (*Pagrus auratus*), salmon and hybrid striped bass (*Morone saxatilis* × *M. chrysops*) when fed β-glucan derived from brewer’s yeast [55, 68].

Other innate immune parameters were not enhanced while feeding Algamune™ to Nile tilapia. Similar results were observed for red drum fed Algamune™ where most immunological parameters were not affected, but plasma total hemolytic activity, which was higher for fish fed 100 and 200 mg $kg^{-1}$ [59]. However, reduced P value were observed for some parameters, such extracellular anion superoxide and plasma lysozyme for red drum. The same trend was observed in the present study, plasma total protein (P=0.14) and whole blood NBT (P=0.24), presenting higher values for the intermediate concentrations of Algamune™. Perhaps if the samples were taken at different time points, significant results might have been observed. Bricknell & Dalmo [69] stated that the most effective strategy to enhance the immunological responses via the diet is by pulse feeding the supplemented dies for a short period of time, usually 4-6 weeks, to not desensitize the animals or induce tolerance to the immunostimulants. On the other hand, rainbow trout fed a commercial β-glucan product, Immunogen®, had a higher lysozyme activity, hemolytic activity from serum at 15 and 45 days when compared to the control group, but agglutination antibody titer only at day 45 [66]. Rainbow trout fed Macrograd® had some immune genes upregulated at 15 days but not at 30 days [67]. Hence, this raises the question if the variance found for NBT and plasma total protein would be reduced if samples were taken at different time points, earlier, or later than 3 weeks.

In the present study, the cumulative survivability of Nile tilapia fed Algamune™ and challenged with a virulent strain of *Streptococcus iniae* was not statistically different from the basal group. Nevertheless, fish fed intermediate concentrations of Algamune™ had numerically higher survivability than the basal group, which may diminish economical losses if translated to industrial farm settings. No enhanced survivability was also reported for Nile tilapia fed yeast-derived beta glucans and further challenged intraperitoneally with the same pathogen as

performed by the present study [70-73]. However, it is noteworthy to mention it was also observed by those authors that cumulative mortality was not significantly influenced by the addition of β-glucan in the diet, but survivability was also substantially increased when compared to the basal group.

In contrast, supplementing graded levels of Macrogard®, a commercial yeast-based β-glucan, to hybrid striped bass improved resistance to *Streptococcus iniae* when the animals were immersed in a bacterial suspension [74]. Dietary supplementation of two different sources of β-glucan also appeared to confer better survivability for Nile tilapia after challenged by a virulent strain of *Streptococcus agalactiae* [75]. Intraperitoneally injected β-glucan from fungi (schizophyllan and scleroglucan) also enhanced the survivability of yellowtail (*Seriola quinqueradiata*) against *Streptococcus* sp. [76].

The pathogenesis of streptococcosis for teleost fish is still not fully understood [77]. It is suggested that the infection starts by colonization of the external or gastrointestinal tissues followed by invasion of the bloodstream [78]. Corroborating this hypothesis, a study conducted with *Oreochromis* sp. and *Streptococcus agalactiae* demonstrated that, presumably, streptococcosis is initiated in the gut at the primary site of infection [79]. Moreover, *Streptococcus iniae* seems to have the ability to evade the bactericidal mechanisms of macrophages after being phagocytosed, surviving and multiplying within the intracellular contents and finally leading to apoptosis of the macrophages [78]. Thus, exposing these animals to the pathogen directly by intraperitoneal injection may have hampered the accurate assessment of the immunoprotection conferred by dietary Algamune™, once other factors of the mucosal innate immunity associated with the gastrointestinal tract were bypassed. However, this speculation should be better addressed by further studies.

The use of biological modifiers, such as β-glucans, during stressful conditions or prior to an insult has been shown to be a beneficial strategy to enhance the immune responses of cultured fish, and thereby reduce the susceptibility to diseases. The ability of crude *Euglena gracilis* as Algamune™ and purified Paramylon to enhance ROS production was corroborated by the *ex vivo* model and the immunomodulatory properties of dietary Algamune™ were ascertained by increasing the ability of isolated phagocytes of Nile tilapia to produce ROS. However, it is recommended that additional trials be conducted to evaluate the immunological effects of dietary Algamune™ at different time points and, if the survivability is in fact improved when feeding Nile tilapia this dietary supplement. Perhaps, if the route of exposure to the pathogen is altered, a statistically significant immunoprotection, conferred by Algamune™, might be observed.

# CHAPTER III

# B-1,3 GLUCAN DERIVED FROM *Euglena gracilis* AND ALGAMUNE™ ENHANCES INNATE IMMUNE RESPONSES OF RED DRUM (*Sciaenops ocellatus* L.).[2]

## 3.1.Introduction

Beta glucans (BGs) are bioactive compounds that have long been used in aquaculture: either as feed additives, vaccine adjuvants or in prophylactic baths [14-16]. They can be naturally found as cell wall components in bacteria, microalgae, macro algae, yeast, fungi, and plants [17-19]. When supplemented in fish diets, theoretically, these molecules are not digestible due to β-glycosidic linkages between their glucose molecules. The repeating patterns of their structures, a feature also shared by microbial pathogen and termed pathogen-associated molecular patterns (PAMPs), are recognized by the host's cell pattern recognition receptors (PRR), eliciting an inflammatory cascade that, ultimately, leads to the enhancement of innate immune responses [14].

Different immunological parameters can be triggered by BG. The glucan's molecular weight, solubility, polymer charge, chemical structure, solution conformation and degrees of branching play a role when activating different cell membrane receptors [18, 20]. For instance, high molecular weight BG can activate leukocytes stimulating their phagocytic, cytotoxic and antimicrobial activity, while low molecular weight BG may affect leukocytes only when they were previously stimulated by cytokines [21, 22]. Several studies have been conducted assessing

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[2] Reprinted: F.Y. Yamamoto, F. Yin, W. Rossi Jr, M. Hume, D.M. Gatlin III, β-1,3 glucan derived from *Euglena gracilis* and Algamune™ enhances innate immune responses of red drum (*Sciaenops ocellatus*), Fish & Shellfish Immunology 77 (2018) 273-279. Copyright 2019, with permission from Elsevier Science.

benefits of dietary BG on fish health. The enhanced effect on the innate and adaptive immune systems has been demonstrated for several species. However, according to the most recent comprehensive reviews of Meena et al. [15] and Petit and Wiegertjes [22], most studies focused on the use of the autolyzed yeast *Saccharomyces cerevisiae*, the plentiful by-product from the brewery industry; while only a few studies evaluated immune responses to alternative sources of BG.

*Euglena gracilis* is a freshwater microalgae species that has increasingly received attention from researchers because it can be used as a biotechnological tool to remediate industrial pollution and to synthesize high-value nutrients [80]. Depending on the culture conditions, it retains a large concentration of vitamins, wax esters, protein and polyunsaturated fatty acids [27, 80, 81]. When *Euglena* is cultured under heterotrophic conditions, it can store up to 90% of the cell mass as linear β-1,3 glucan (Paramylon), therefore making it a promising nutraceutical candidate [42, 80]. Feeding diets supplemented with Paramylon to rainbow trout (*Oncorhynchus mykiss*) improved their innate and adaptive immune responses after challenged with *Yersinia ruckeri* [39]. It also has been documented that mice fed Paramylon had anti-inflammatory properties and alleviated chronic dermatitis and enhanced hepatic protection [40, 41].

The red drum (*Scieanops ocellatus*) is a promising aquaculture species due to its rapid growth and ready acceptance of artificial diets [82, 83]. Its commercial production has continued to increase in recent years in United States for stock enhancement and food production as well as for seafood production in other countries such as China [83]. The objective of this study was twofold: firstly, to assess the immunological responses of isolated red drum phagocytes to graded doses of Algamune™ and the purified Paramylon as a standard reference; and secondly, to

evaluate the *in vivo* effects of supplemental Algamune™ in the diet of red drum with respect to production performance, immunological responses, and gut microbiota composition.

## 3.2 Materials and methods

### *3.2.1. Ex vivo* trial

#### 3.2.1.1. Fish and β-glucan

Advanced juvenile red drum were used for the *ex vivo* experiment. Prior to the experiment, 12 healthy red drum with a mean weight of ~100 g were held in a 1200-L circular fiberglass tank as part of a recirculating system (flow of 1.1 L $min^{-1}$) containing brackish (7 ppt) water. Fish were fed a commercial diet containing 40% crude protein and 12% lipid twice daily to apparent satiation. The recirculating system included a common settling chamber, biological and mechanical filtration, and aeration was provided through air diffusers connected to a central regenerative blower system. The β-1,3 glucan evaluated in the ex vivo and in vivo trials was derived from crude *Euglena gracilis* (Algamune™, Algal Scientific Corporation) and purified Paramylon (Sigma Aldrich Co.).

#### 3.2.1.2. Phagocytes isolation

Fish were euthanized by an overdose (~250 mg $L^{-1}$) of tricaine methanesulfonate (MS-222, Tricaine-S, Western Chemical Inc) [43]. Head and trunk kidneys were aseptically excised and stored in cold Leibowitz cell culture media 2% (L-15 media enriched with 2% bovine calf serum, 100 units $ml^{-1}$ Penicillin/Streptomycin). All tissues were pooled into a composite sample for the isolation of phagocytes following the procedure of Secombes [44], with minor modifications of Sealey and Gatlin [34]. Briefly, the kidneys were homogenized using a sterile

Potter-Elvejhem glass homogenizer, and the homogenate suspension was filtered using sterile 100-µm mesh filter. The filtered suspension was centrifuged at 1000 x *g* for 10 min and washed thrice with cold phosphate buffer saline (PBS, Sigma Aldrich Co.) at pH 7.4. The homogenate was layered onto a 34/51% Percoll gradient (Sigma Aldrich Co.), then centrifuged at 400 x *g* for 25 min at 4°C. The layer of cells resting at the 34/51% interface was collected using a Pasteur pipette and washed twice (1000 x *g*; 10 min; 4°C) with cold PBS, then resuspended in Leibowitz cell culture media 0.1% (L-15 media 0.1% FCS with 100 units mL-1 penicillin/streptomycin). Cell viability was assessed using trypan blue (0.4%) and only used when survivability was greater than 95%. Cells were enumerated under a light microscope and concentration was adjusted to approximately $2 \times 10^7$ cells $mL^{-1}$ by resuspending the cells in L-15, 0.1% FCS media. Phagocytes were layered in a 96-well flat-bottom microplate by adding 100 µL of cell suspension yielding a final concentration of $2 \times 10^6$ cells per well.

3.2.1.3. β-Glucan solutions and culture conditions

Each source of β-(1,3) glucan (Algamune™ and Paramylon) were pre-dissolved in a 0.1 N sodium hydroxide solution and then serially diluted with L-15, 0.1% FCS media to yield final concentrations of Algamune™ and Paramylon of 0.2, 0.4, 0.8, 1.6, 3.2 mg $L^{-1}$. A β-(1,3) glucan-free solution of L-15, 0.1% FCS media was used as the basal level or control (0.0 mg $L^{-1}$). The amount of each β-glucan source to be pre-dissolved in 0.1 N sodium hydroxide was maximized so that a high volume of L-15, 0.1% FCS media would be necessary for the dilutions; thereby diminishing any potential effects of the pre-dissolving base. Sixteen wells were used as replicates for each source and concentration. Plates were incubated with gentle agitation for 24 h at room

temperature. After incubation, the media was removed from the plate and wells were washed twice with PBS at room temperature and prepared for the following analyses.

3.2.1.4. Detection of extracellular $O_2^-$

Twelve wells with layered cells, incubated as described above, were covered with 100 µL of 2 mg $mL^{-1}$ ferricytochrome C (Sigma Aldrich Co.) with 1 µg $mL^{-1}$ phorbol myristate acetate (PMA, Sigma Aldrich) in phenol red-free Hank's Balanced Salt Solution (HBSS) for detection of extracellular superoxide anion (ECSA) as described by Secombes [44]. For each concentration, four replicate wells were used as a negative control by adding to the ferricytochrome solution 300 units $mL^{-1}$ of superoxide dismutase (SOD) (Sigma Aldrich Co.) to prevent oxidation. Plates were read immediately (time 0) and after every 15 min until 1 h using a multi-scan spectrophotometer at 550 nm. Final concentration of extracellular superoxide anion production (ECSA) was calculated according to Pick and Mizel [46] using the following formula: nmol of anion superoxide $O_2^-$= [(Δ Absorbance after 45 min × 100) ÷ 6.3]

3.2.1.5. Detection of intracellular $O_2^-$

Phagocytes were incubated with 100 µL of nitroblue tetrazolium (NBT, cat # N6876, Sigma Aldrich) solution (1 mg $mL^{-1}$ with 1 µg PMA $mL^{-1}$) in phenol red-free HBSS for 45 minutes at room temperature (Secombes [44] modified by Caipang et al. [45]). After the incubation period, the media was removed, and cells were fixed in methanol 100% for 10 min. After the fixation interval, the wells were washed thrice with 70% methanol and allowed to air-dry in the dark for 1 h. The reduced formazan in each well was solubilized in 120 µL of 2 M potassium hydroxide and 140 µL of DMSO (Sigma Aldrich Co.) and mixed vigorously. Plates

were read at 620 nm as described above and the intracellular superoxide anion production (ICSA) was expressed as the absorbance average.

3.2.1.6. Lysozyme activity

The lysozyme activity of kidney phagocytes was evaluated by using a turbidimetric assay [51]. Briefly, the culture medium supernatant used to incubate the phagocytes with the BG sources was collected and incubated with a *Micrococcus lysodeikticus* suspension in PBS. *Micrococcus* suspension pH was adjusted to 5.2 as previously established in this laboratory for best activity of this enzyme in red drum. The reduced absorbance was measured at 450 nm after 4 min of incubation. Quantification of lysozyme activity was done as per standard definition of one unit of lysozyme activity from chicken egg hen lysozyme (L6876, Sigma Aldrich) corresponding to the linear decrease in optical density (OD) at 450 nm of 0.001 per minute [84].

3.2.1.7. Bactericidal assay

The ability of red drum phagocytes to kill *Streptococcus iniae* was evaluated at different BG source concentrations using the method described by Secombes [44] and modified by Shoemaker et al. [85]. The procedure to prepare the phagocyte layers on the cell culture plates were the same as described above, but with no addition of Penicillin and Streptomycin to the L-15 culture media. An overnight culture of *Streptococcus iniae* in Brain Heart Infusion broth (BHI, cat # 53286, Sigma Aldrich) was washed thrice with PBS and bacteria concentration was adjusted under a light microscope to give a final concentration of $1 \times 10^8$ cells $mL^{-1}$. In each well, 20 µL of the bacterial suspension was added, and plates were centrifuged to bring bacteria and cell into contact. As a control, two wells from each treatment were treated with cell- culture-

grade water to promote the lysis of the bottom attached phagocytes. After 5 h of incubation, the supernatant was removed, and phagocytes were lysed by adding 50 µL of 0.2% Tween 20 (H285, Mallinckrodt) in distilled water. Bacterial growth was estimated by adding 100 µL of fresh BHI broth and incubating at 27˚C overnight under sterile conditions. After the incubation period, each well received 10 µl of thiazolyl blue tetrazolium bromide (MTT, cat # M2128, Sigma Aldrich, 5 mg $mL^{-1}$) and the plate was incubated for another 15 minutes. Bactericidal activity was measured as follows: (Control 100% survival – overnight cultured Bacteria ÷ Bacteria 100% survival) and data were presented as mean % killing per treatment.

### *3.2.2. In vivo trial*

#### 3.2.2.1.Experimental diets and feeding trial

An Algamune$^{TM}$-free basal diet was formulated to contain 400 g crude protein $kg^{-1}$, 120 g lipid $kg^{-1}$, and an estimated 14.6 MJ digestible energy (DE) $kg^{-1}$ (Table 4) meeting all known nutrient requirements of red drum [83]. The DE was estimated based on the standard physiological fuel values of 16.7, 16.7, and 37.7 MJ $kg^{-1}$ for soluble carbohydrates (NFE), protein and lipid, respectively. A premix containing celullose and dried *Euglena gracilis* (Algamune™) at 100 g $kg^{-1}$ was supplemented into the basal diet at the expense of cellulose to produce four additional diets containing the β-1,3 glucan source at 100, 200, 400, and 800 mg $kg^{-1}$. Diet ingredients were mixed for 30 minutes in a V-mixer and blended with oil and water using an industrial mixer with a meat grinder attachment. The moist mash of each diet was cold pelleted through a 3-mm die plate and dried for 48 h using forced air at room temperature. Finally, dry diet strands were ground to the appropriate size using a food chopper, sieved to

Table 4: Formulation and analyzed proximate composition of the basal diet fed to red drum in the *in vivo* experiment.

| Ingredients | $g\ kg^{-1}$ |
|---|---|
| Menhaden meal[1] | 290 |
| Soybean meal | 407.5 |
| Menhaden oil | 50 |
| Vitamin premix[2] | 30 |
| Mineral premix[3] | 40 |
| Dextrinized starch[4] | 120 |
| Carboxymethyl cellulose[5] | 20 |
| Celufil[6] | 42.5 |
| Proximate composition | |
| Moisture | 11.5 |
| Protein | 409.2 |
| Lipid | 117.7 |
| Ash | 133.0 |

[1]Omega Protein, Abbeville, Louisiana, USA
[2,3]Same as in Moon and Gatlin [86]
[4,5,6]MP Biomedicals, Solon, Ohio, USA

remove fines and stored at -20 °C until fed. The proximate composition of the diets was analyzed following AOAC [48] procedures.

Juvenile red drum were obtained from Texas Parks and Wildlife Department and were maintained at the Aquacultural Research and Teaching Facility. Fifteen fish with a mean initial weight of 26.6 g were stocked in each of 20, 110-L glass aquaria operating as a recirculating system. Aeration was supplied to each aquarium through air stones connected to a central regenerative blower system. Water temperature was maintained steady (25-27 ˚C) throughout the feeding trial by conditioning ambient air. Quadruplicate aquaria were randomly assigned to each of the experimental diets. Red drum were fed twice daily and feed ration was adjusted weekly according to the percentage of aquarium biomass for 3 weeks. Water quality parameters were monitored every 2 days throughout the feeding trial and were as follows (mean ± standard error): temperature 26.5 ± 0.08 ˚C, dissolved oxygen = 6.15 ± 0.33 mg $L^{-1}$, salinity 5.33 ± 0.33 g $L^{-1}$, total ammonia nitrogen = 0.11 ± 0.04 mg $L^{-1}$, nitrite nitrogen 0.037 ± 0.015 mg $L^{-1}$, and pH = 7.6 ± 0.07. A 12 h photoperiod was maintained using fluorescent lighting controlled by timers.

3.2.2.2. Production performance parameters and sample collection

At the end of the feeding trial, fish from each aquarium were group weighed and counted after an overnight fast. Production parameters were computed as follows:

Weight gain (WG) (% of initial) = 100 × [(final weight – initial weight) ÷ initial weight]

Feed efficiency (FE) = weight gain ÷ dry feed intake

Survival (%) = 100 × (number of surviving fish ÷ initial number of fish)

Blood from four fish from each tank was collected with 1.0-mL heparinized syringes with 28-gauge needles. The same fish were euthanized as described for the ex vivo assays and head

and trunk kidney tissues were aseptically excised and stored in Leibowitz cell culture media, 2% (L-15 media enriched with 2% FCS and 50 units $mL^{-1}$ of penicillin - 0.05 mg $mL^{-1}$ of streptomycin).

3.2.2.3. Immune parameters for whole blood, plasma, and isolated leucocytes

Respiratory burst of blood leucocytes was determined by the NBT reduction to diformazan followed by re-suspension with N-N dimethyl formamide according to Siwicki et al.[50]. Lysozyme activity of the plasma was measured by a turbidimetric assay according to Ellis [51]. The activity of lysozyme was defined by the decrease in absorbance of the mixture of *Micrococcus lysodeikticus* (M3770 ATCC 4698, Sigma Aldrich) in PBS and plasma. *Micrococcus* suspension pH was adjusted as described for the *ex vivo* trial. The total hemolytic activity of the complement system was measured according to Castro et al. [87] with minor modifications as described by Sutili et al. [52]. ECSA and ICSA were performed as described for the *ex vivo* trial.

3.2.2.4. Intestinal digesta DNA extraction and Denaturing Gradient Gel Electrophoresis (DGGE) Analysis

To evaluate potential effects of supplemental Algamune™ on red drum intestinal microbiota profile, four fish per aquarium were euthanized (as previously described) at 4 h postprandial and digesta were aseptically collected by stripping out the intestinal contents into sterile microfuge tubes using sterilized tweezers. The microfuge tubes were immediately flash frozen in liquid nitrogen and stored at -80˚C freezer pending analyses. Genomic DNA was extracted from each sample using a commercial kit Bio-Rad Aqua Pure DNA isolation (Bio-Rad,

Hercules, CA, USA) following the instructions provided by the manufacturer with minor modifications by Burr et al. [88]. Isolated DNA was quantified from an aliquot using a Nanodrop® spectrophotometer ND-1000 at 280 nm, and DNA was concentrated or diluted as needed to obtain a final concentration of 50 ng of DNA $\mu L^{-1}$. Samples were amplified using bacteria-specific PCR primers of conserved regions flanking the variable V3 region of 16S rDNA according to the methodology proposed by Hume et al. [89]. Five microliters of pooled PCR products from each dietary treatment was combined with an equal volume of 2X loading buffer (bromophenol blue 0.05%, xylene cyanol 0.05%, glycerol 70%). A total of 7 µL of the mixture was then loaded into each well of a polyacrylamide gel [8% v/v acrylamide-bisacrylamide (Bio-Rad Laboratories, Richmond, CA, USA) ratio of 37.5:1]. Electrophoresis was performed for 17 h at 60 V using a DCode Universal Mutation Detection System (BioRad, Hercules, CA, USA). Then, the gel was stained with SYBR Green I (1: 10,000 dilution) for 30 minutes and digitalized.

### *3.2.3. Statistical analysis*

All resulting data from the *ex vivo* and *in vivo* experiments were validated for homogeneity of variances (Levene's test), and subjected to orthogonal polynomial contrasts to verify the patterns that most adequately described the results. When significances were found, parameters were subjected to linear or quadratic regression, chosen by the lesser P value ($P<0.05$). These statistical analyses were performed using SAS® software (SAS Institute Inc. Cary, NC, USA). When dose-dependent responses could not be identified, data were submitted to one-way ANOVA, and multiple comparisons of means were performed by Duncan test to determine differences among treatments ($P<0.05$). Data from the *ex vivo* study also were subjected to a

factorial ANOVA using JMP® (SAS Institute Inc. Cary, NC, USA) excluding the control to assess the effects of the different β-Glucan sources against the incremental doses.

Fragment analysis pattern relatedness of DGGE results was determined using Molecular Analysis Fingerprinting software (v 1.6; BioRad, Hercules, CA, USA), using Dice's similarity coefficient (SC, %) for indicating relative similarity of dendrogram band patterns as follow: SC < 80% = different populations; 80 ≤ SC ≥ 95% = similar populations; SC > 95% = identical populations.

## 3.3 Results

### *3.3.1. Ex vivo assays*

Results for the ex vivo assay showed that both Paramylon and the Algamune™ had significant effects (P<0.001) on ECSA and ICSA production, and bactericidal activity against *Streptococcus iniae* in a dose- dependent manner. Bactericidal activity for Algamune™ supplementation followed a second order polynomial relationship ($R^2$= 0.48), and the maximum level was estimated to be 1.1 mg $L^{-1}$ (Fig. 5). On the other hand, cells incubated with Paramylon had an upward linear response ($R^2$=0.21). The ECSA production did not show differences for the Paramylon treatment, but a downward quadratic relationship ($R^2$=0.22) was observed for the Algamune™ treatment (Fig. 6). Significant effects for the glucan source (P<0.0001) were detected, with phagocytes treated with Paramylon having the higher ability to produce ECSA (Table 5). For ICSA production, both Algamune™ and Paramylon results were best explained by second order polynomial models, having highest ICSA production at 0.16 and 2.24 mg $L^{-1}$ ($R^2$=0.19 and 0.82), respectively (Fig. 7). Differences were detected for the different concentrations (P<0.02) (Table 5).

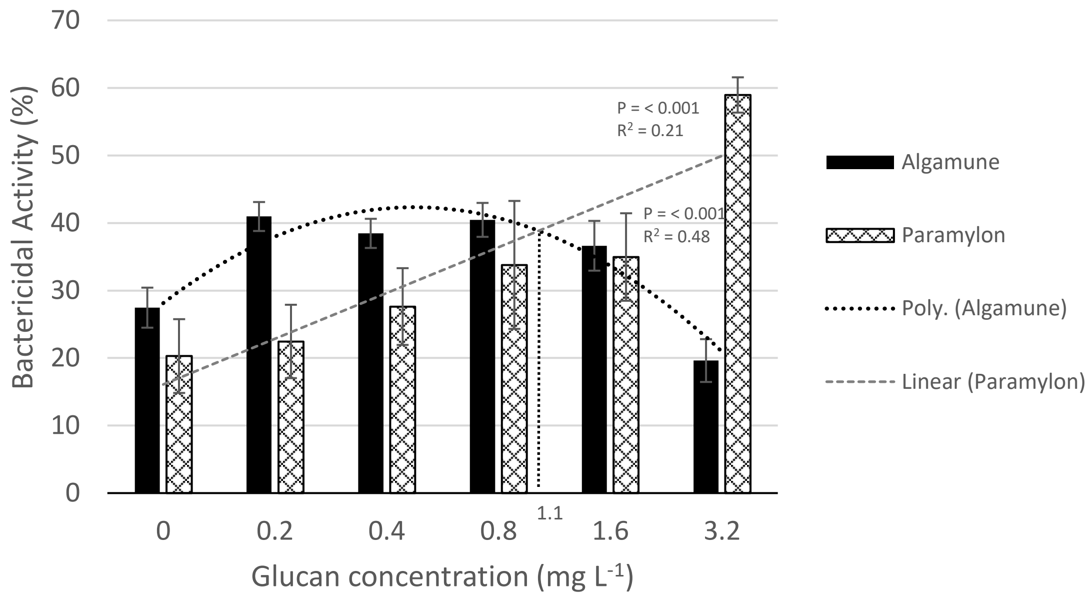


Figure 5: Bactericidal activity of Red Drum isolated phagocytes incubated with graded doses of Paramylon and Algamune$^{TM}$ against a virulent strain of *Streptococcus iniae*

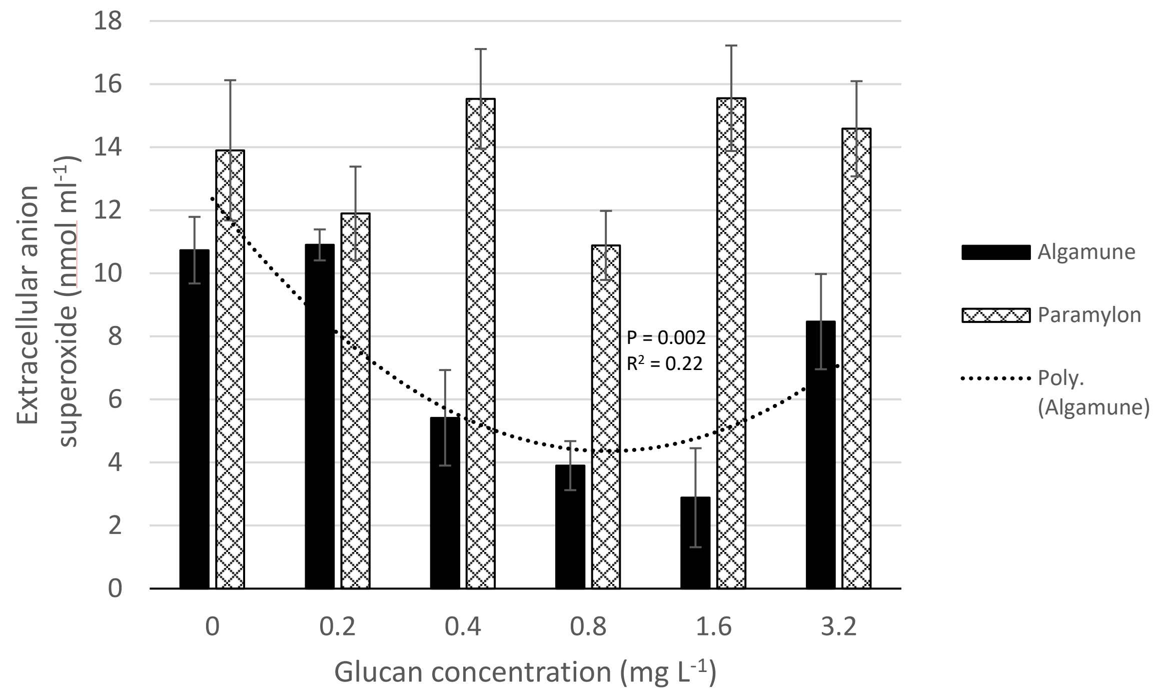


Figure 6: Extracellular anion superoxide production by isolated phagocytes incubated with graded doses of Algamune$^{TM}$ and Paramylon measured by the reduction of ferricytochrome C.

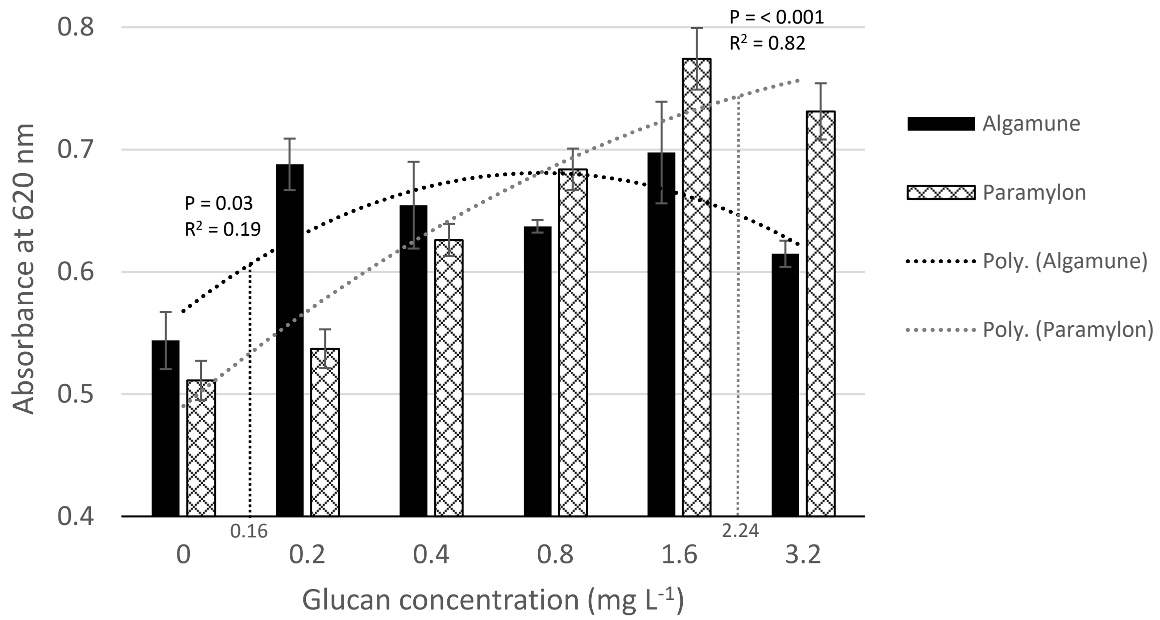


Figure 7: Intracellular anion superoxide production by isolated red drum phagocytes incubated with graded doses of Algamune™ and Paramylon measured by reduction of nitroblue tetrazolium

Table 5: *Ex vivo* effects of various β-glucan levels and sources on the immunological responses of red drum isolated phagocytes

| Type of β-glucan supplemented in the culture media | β-glucan concentration (mg $L^{-1}$) | Bactericidal activity (%) | ICSA (Abs. 620 nm) | ECSA (nmol $O_2^-$ $mL^{-1}$) |
|---|---|---|---|---|
| Control | 0 | 23.9 | 0.528 | 12.3 |
| Algamune™ | 0.2 | 41.0 | 0.688 | 10.9 |
| | 0.4 | 38.5 | 0.655 | 5.4 |
| | 0.8 | 40.5 | 0.637 | 3.9 |
| | 1.6 | 36.6 | 0.698 | 2.9 |
| | 3.2 | 19.6 | 0.615 | 8.5 |
| Paramylon | 0.2 | 22.5 | 0.537 | 11.9 |
| | 0.4 | 27.6 | 0.626 | 15.5 |
| | 0.8 | 33.8 | 0.684 | 10.9 |
| | 1.6 | 35.0 | 0.774 | 15.6 |
| | 3.2 | 59.0 | 0.731 | 14.6 |
| P value | | | | |
| β-Glucan source | | 0.916 | 0.512 | <0.0001 |
| Concentration in the media | | 0.146 | 0.021 | 0.446 |
| Glucan source × concentration | | <0.0001 | 0.0001 | 0.453 |
| Pooled Standard Error (PSE) | | 1.95 | 0.01 | 0.83 |

*3.3.2. Productive, immune parameters and microbiota of digestive tract contents from the in vivo trial*

There were no significant differences in productive parameters (WG, FE, and survival) of red drum fed diets containing graded levels of Algamune$^{TM}$ β-glucan for 3 weeks (Table 6). Immune parameters such as NBT, lysozyme activity, ECSA, and ICSA were similar among all treatments (Table 7). Significance ($P<0.002$) was found for the total hemolytic assay, but a dose response model could not be established for this parameter. By mean comparison, fish fed the diets supplemented with Algamune$^{TM}$ β-glucan at both 100 and 200 mg kg$^{-1}$ had significantly higher total hemolytic activity when compared to the other treatments. Although no significant differences were detected for ECSA and lysozyme activity, the former was marginally significant ($P = 0.08$), while the highest values for both parameters were observed in red drum fed diets supplemented with Algamune$^{TM}$. The DGGE dendrogram showed that the intestinal microbial community of red drum was unaffected (SC > 95%) by the dietary treatments (Fig. 8).

Table 6: Production performance of red drum fed diets with graded inclusion levels of Algamune™ for 21 days

| Algamune™ concentration (mg $kg^{-1}$) | 0 | 100 | 200 | 400 | 800 | P value | PSE |
|---|---|---|---|---|---|---|---|
| Weight gain (%) | 49.6 | 51.1 | 59.2 | 51 | 52.8 | 0.18 | 1.37 |
| Feed efficiency | 0.64 | 0.65 | 0.74 | 0.65 | 0.67 | 0.3 | 1.6 |
| Survival (%) | 88.3 | 78.3 | 85 | 91.7 | 90 | 0.65 | 0.03 |

Table 7: Immunological responses of red drum fed diets with graded inclusion of Algamune™ for 21 days

| Algamune™ concentration (mg $kg^{-1}$) | 0 | 100 | 200 | 400 | 800 | P value | PSE |
|---|---|---|---|---|---|---|---|
| NBT mg $ml^{-1}$ of blood | 6.4 | 6.44 | 6.38 | 6.41 | 6.58 | 0.46 | 0.05 |
| ECSA (nmol $O_2^-$ $ml^{-1}$) | 0.665 | 0.36 | 0.465 | 0.819 | 0.413 | 0.08 | 0.06 |
| ICSA (Abs. at 620 nm) | 0.082 | 0.079 | 0.084 | 0.078 | 0.081 | 0.55 | 0.001 |
| Lysozyme activity (units $ml^{-1}$) | 166.67 | 230 | 358.88 | 334.84 | 354.16 | 0.12 | 29.01 |
| Total hemolytic activity (%) | $53.96^{B}$ | $72.97^{A}$ | $70.01^{A}$ | $54.65^{B}$ | $66.44^{AB}$ | 0.006 | 2.16 |

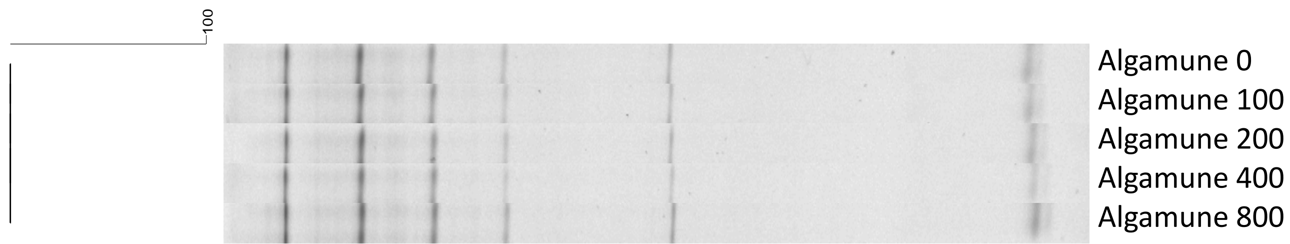


Figure 8: Dendrogram of red drum gut microbiota after 3 weeks of feeding the experimental diets, showing identical populations (Dice's similarity coefficient > 95%).

## 3.4 Discussion

β-Glucans are considered biological response modifiers, and they have been used as feed additives for prophylactic management to enhance fish immunological responses prior to a pathogenic insult. Brewers' yeast, the abundant brewery by-product is an established feed additive and the source of BG that holds the longest record research in aquafeeds [14]. Whereas many studies focused on testing brewery residues from *Saccharomyces cerevisiae*, the present study showed that the alternative source Algamune™, a commercial additive produced from *Euglena gracilis*, and the purified β-glucan from *Euglena* (Paramylon) can also enhance immune responses of red drum both *ex vivo* and *in vivo*.

Exposing naïve phagocytes directly to BG facilitates the activation of the cells upon the intracellular signaling initiated by the PAMP with the PRR receptors (*e.g.*, dectin 1, BG receptors, type 3 complement, and toll-like receptors) or indirectly by cytokines signaling produced by activated phagocytes. Hence, the increased bactericidal activity observed in the present study can be associated with elicited responses of free radical production and/or increased phagocytic activity following BG exposure [20]. On the other hand, decreased bactericidal activity was observed as the concentration of Algamune™ increased in the culture media. We presume the intracellular content of dried *Euglena*, such as antioxidant vitamins (*e.g.*, tocopherol), [80] may have been re-suspended after the basic digestion and scavenged the ECSA produced by the phagocytes, thus hampering the ability to eliminate the bacteria. An increased phagocytic activity was observed when different BG sources were incubated with circulating phagocytes from hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) and Japanese eel (*Anguilla japonica*) [90]. Isolated barramundi (*Lates calcarifer*) macrophages were activated by Paramylon *in vitro* [61].

The reduction of molecular oxygen into superoxide anion $O_2^-$ by the enzyme NADPH oxidase on the cell membrane is an asset of phagocytes to combat foreign elements, an event known as respiratory burst [57]. In the present study, the reactive oxygen species (ROS) produced intracellularly followed an upward trend as the concentration of both Algamune™ and Paramylon increased in the cell culture media. Similar results were observed by Kudrenko et al. [61] when incubating graded concentrations of Paramylon and other BG sources with barramundi macrophages. Bridle et al. [91], using three commercial BG with isolated macrophages of Atlantic salmon (*Salmo salar*) also observed an increasing response of intracellular ROS, but found no differences in lysozyme activity in the cell culture supernatant.

No differences were observed for the ECSA when the phagocytes were incubated with Paramylon and a descending second order polynomial regression was observed for the Algamune™ treatment. As mentioned before, for the bactericidal assay, the antioxidants nutrients may have played a role by scavenging the ROS and hindering the reduction of cytochrome C. Whereas ECSA measures the ability to release ROS to the medium, ICSA gauges the ability of phagocytes to reduce NBT to formazan granules by ROS intracellularly. Along with NBT, a limited amount of culture medium also could have been engulfed. Thus, the concentration of these antioxidant agents in the phagosome would be restricted and not hamper ROS reduction properties.

Previous studies reported improved weight gain of experimental fish fed diets supplemented with β-glucans. Red sea bream (*Pagrus aurata*) [92], yellow croaker [93], snapper [68], rohu [94], rainbow trout [95], koi [96], and mirror carp [97] displayed higher growth performance when supplementation ranged from 0.09 to 2% v/v and the trials lasted from 3 to 12

weeks. The results from the current study did not show significant effects on weight gain and feed efficiency among the treatments.

Interestingly, for the feeding trial, the reduction of NBT by the oxidative radical production of whole blood phagocytes was not affected by dietary Algamune$^{TM}$. The production of ECSA and ICSA by isolated phagocytes was also unaffected by the treatments, but a marginally significant response ($P = 0.08$) was noticed for ECSA. Perhaps, a significant difference would be observed for this parameter had sampling been performed during a different time. The responses of respiratory burst when collected at different time points are not very consistent in the literature. While some authors reported a peak of respiratory burst in response to dietary BG between 21 and 28 days [97, 98], others reported a higher response at 42 days [94]. Even when an immune fatigue was noted beyond the 6$^{th}$ week, a higher production of ROS was observed at day 56 [93]. Therefore, not only the length of administration but also the distinct physiological characteristics of different fish species used as biological models may impact the outcomes of such assays.

Dalmo and Bogwald hypothesized that supplemental BG could induce an inflammatory process *in situ* and consequently enhance resistance against enteric pathogens. Another comprehensive review by Swennen et al. [99] presupposed that the bacterial community in the gut would assist in the digestion and breakage of the BG molecule into short chain fatty acids, thereafter sparing glutamine for immune responses. These hypotheses are yet to be proven because these represent only a handful of studies when compared to many others reporting no beneficial effects. In fact, the unobserved changes in gut microbiota composition of red drum fed diets supplemented with Algamune$^{TM}$ in our study corroborate the latter. No differences were

also observed for the plasma lysozyme activity of rainbow trout when fed diets supplemented with Paramylon prior to a bacterial challenge with *Yersinia ruckerii* [39].

The complement system is a set of about 30 distinct proteins in the plasma, and it is a major humoral response of innate immunity [100, 101]. It plays an important and versatile role for the immune system because it can opsonize, recruit phagocytes, and directly kill pathogens by assembling the membrane attack complex [102]. The latter was evaluated in the present study as the total hemolytic assay, and dietary Algamune™ at the concentration of 100 and 200 mg $kg^{-1}$ enhanced the ability to disrupt the cell membrane of a foreign organism. Similar results were observed by Bagni et al. [103] while feeding diets supplemented with Macrogard® at 0.1% to sea bass (*Dicentrarchus labrax*); although in that study the enhancement of the alternative complement pathway (ACP) activity of seabass was only observed for 15 days but not for 45 days. Also noteworthy is the inconsistency of results on ACP activity reported for different species. For instance, while the ACP activity of rainbow trout fed Macrogard® supplemented diets for 2 weeks was unaffected [104], a reduced ACP activity was reported for seabream after feeding *Saccharomyces cerevisiae* for 6 weeks [98]. Therefore, the discrepancy in the results obtained for this parameter may be explained by the different factors in question such as: fish species, BG source, and length of administration.

In conclusion, the supplementation of *Euglena gracilis* as Algamune™ and purified Paramylon enhanced the innate immune responses of cultured red drum phagocytes mediating production of ICSA and increased bactericidal activity ex vivo. Despite undetected effects on production performance and intestinal microbiota composition, the dietary supplementation of Algamune™ was found to enhance in a moderate fashion the total hemolytic activity of red drum plasma. A long-term feeding trial evaluating the effects of dietary *Euglena gracilis* on the

immune system of red drum and a comparison between this additive and other BG sources are suggested.

CHAPTER IV

# IMMUNOMODULATORY EFFECTS OF B-GLUCANS DERIVED FROM *Euglena gracilis* OR *Saccharomyces cerevisiae* FOR HYBRID STRIPED BASS (*Morone chrysops* × *M. saxatilis*)

## 4.1 Introduction

Disease outbreaks are recognized as a significant bottleneck for aquaculture production and trade. They impact directly on the economic revenue of the aquaculture sector in several countries [1]. It is estimated that losses due to pathogenic diseases can reach deficits of billions of dollars in global aquaculture each year [2]. Moreover, the use of chemotherapeutics, such as antibiotics, is becoming more restrictive for animal production, especially for aquaculture [16], due to the risks of having these medications leached into the environment, potentially raising resistant pathogenic bacterial strains and undermining the drug's effectiveness [4, 36, 105]. The use of immunostimulants as feed additives appears to be an environmentally sound strategy to reduce the use of chemotherapeutics and the incidence of disease by augmenting the host's immunological responses prior to infection [12, 13]. One example of such an immunomodulating additive is beta-glucans (BGs), which are bioactive compounds that have long been used in animal husbandry [14, 15, 18].

Various BGs are natural immunostimulant molecules present in several life forms, from single-cell organisms, such as bacteria, yeast, and microalgae, to more complex multicellular organisms such as algae, plants, fungi and protozoans [15, 17, 18]. The repeating pattern of their structure can be recognized as a pathogen-associated molecular pattern (PAMP), which triggers the immunological system upon interaction with the pathogen recognition receptors (PRR) of

fish leukocytes [20, 106, 107]. The intracellular signaling upon recognition of a PAMP elicits the inflammatory cascade by activating transcription factors, leading to the production of pro-inflammatory cytokines, which ultimately augments the immune responses [14, 15, 38]. As such, the use of BGs as immunostimulants may serve as a prophylactic tool to strategically prevent diseases by enhancing the immunological responses of farmed fish prior stressful events or seasonal pathogen outbreaks.

The most conventional BG source in animal feeds is the by-product from the brewing industry, the yeast (*Saccharomyces cerevisiae*) [16, 108, 109]. When supplemented in fish diets, the brewer's yeast not only can provide branched β-1,3/1,6 glucan molecules, but also some nutrients and other compounds that can enhance the immunological responses of the host [16, 110]. Zymosan is a reference product extracted from the yeast cell wall, which consists of a mannose-rich proteoglycan attached to the branched β-1,3/1,6 structure [17, 108, 111, 112].

An alternative source of BGs for animal feeds may be provided from crude *Euglena gracilis*, a freshwater microalgae that can serve as a bioremediator for agriculture waste, and synthesize high-value nutrients [80, 113]. Paramylon is a unique linear β-1,3 glucan molecule produced by Euglenoids, which it is stored within its cytoplasm and used as a metabolic substrate under sub-optimal conditions [27, 113]. When cultured under a heterotrophic environment, *Euglena* can store large quantities of Paramylon, and due to the water-insoluble properties of this molecule and its unusual high degree of crystallinity, it is possible to recover Paramylon BG at a lower cost when compared to other glucan sources [25, 26, 80, 114].

Hybrid striped bass (*Morone chrysops* × *M. saxatilis*) is an important carnivorous fish cultured in North America for both recreational fisheries and food production [115]. The hybrid vigor exhibited by this cross results in superior growth performance in captivity, as well as

enhanced disease and stress resistance which are desirable characteristics for cultured fish [116]. The objective of this study was two-fold: firstly, to evaluate the dietary supplementation of two different BGs, Paramylon and Zymosan, at 50 mg and 100 mg $kg^{-1}$ of diet on growth performance and immunological responses of hybrid striped bass; and secondly, to evaluate the immunological responses of the hybrid striped bass when exposed to Paramylon and Zymosan intraperitoneally.

## 4.2 Material and methods

### *4.2.1. Experimental diets and feeding trial*

Hybrid striped bass (HSB) fingerlings were kindly donated by Keo Fish Farm (Keo, Arkansas, USA) and transported to the Aquacultural Research and Teaching Facility of the Texas A&M University System (College Station, TX, USA). Fish were acclimated to local conditions and fed a 400 g $kg^{-1}$ crude protein, 140 g $kg^{-1}$ crude fat commercial diet (EXTR 400, Rangen Inc., Angleton, TX, USA) prior to commencement of the feeding trial. Three hundred fish with average weighing 15.4 ± 0.1 g (average ± standard error, SE) were equally distributed in 20, 110-L glass aquaria operating as a recirculating system, which included a common settling chamber, biological and mechanical filtration. Aeration was provided continuously by air stones connected to a regenerative blower system. Water temperature was conditioned by the ambient air and kept steady between 25 and 26°C throughout the feeding trial, and salinity was maintained at 2 mg $L^{-1}$ using synthetic marine sea salt (Red Sea Salt, Red Sea USA, Houston, TX, USA) to balance the hardness and alkalinity of the well water source. A 12:12 h photoperiod was maintained using fluorescent lighting controlled by timers.

A basal diet was formulated to meet all known requirements of HSB [115, 117], containing 420 g crude protein $kg^{-1}$, 100 g lipid $kg^{-1}$, and an estimated digestible energy of 12.7 MJ $kg^{-1}$ (Table 8). Purified supplies of Paramylon (Sigma-Aldrich Co.) and Zymosan (cat# Z4250, Sigma-Aldrich Co.) were added to the basal diet at the expense of cellulose to yield final concentrations of 50 and 100 mg $kg^{-1}$ for each source of BGs, resulting in a total of five dietary treatments. The dry ingredients of each treatment were mixed in a V-Mixer machine (Blend master, Buflovak) for 30 min, and then blended with fish oil and water using an industrial mixer (A-200 Hobart meat grinder). The resultant moistened mash was cold-pelleted through a 3-mm die plate and dried at room temperature with forced ventilation for 24 h. The pellets were collected on the following day, ground and sieved to appropriate size for the fish, and the resultant fines were sub-sampled for proximate analysis [48]. Quadruplicate aquaria were randomly assigned to each experimental diet, and fish were fed twice daily according to a percentage of total biomass per aquarium. Fish were group weighed weekly, and daily rations were adjusted on a dry-matter basis. This approach aimed to provide the same daily feed ration for all treatments, which was close to apparent satiation without overfeeding. Water quality was measured thrice a week, and parameters were kept within ranges suitable for HSB culture (Hodson, 1990). Water dissolved oxygen and temperature were measured using an optical dissolved oxygen meter (ProOdo, YSI, OH, USA), water pH was measured using a portable pH meter (Pocket Pro pH tester, Hach Company, Loveland, CO, USA), water salinity was measured using a portable salinity meter (EC170, Extech, Boston, MA, USA), and total ammonia- and total nitrite-nitrogen dissolved in culture water was measured photometrically (Hach DR 2000 spectrophotometer and test reagents, Hach Company). All parameter results are summarized as follows (mean ± SE): temperature = 25.8 ± 0.1˚C, dissolved oxygen = 6.84 ± 0.1 mg $L^{-1}$,

Table 8: Formulation and analyzed proximate composition of the basal diet used for the comparative feeding trial for hybrid striped bass

| Ingredients | g $kg^{-1}$ |
|---|---|
| Menhaden Meal[1] | 302.7 |
| Soybean Meal[2] | 390.5 |
| Menhaden Oil[3] | 48.3 |
| Vitamin Premix[4] | 30.0 |
| Mineral Premix[5] | 40.0 |
| Dextrinized starch[6] | 110.0 |
| CMC[7] | 20.0 |
| Celufil[8] | 58.5 |
| **Proximate composition** | **g $kg^{-1}$** |
| Moisture | 89.9 |
| Crude protein | 428.6 |
| Lipid | 96.5 |
| Ash | 131.5 |

[1,3] Omega Protein Corporation

[2] Producers Cooperative Association, Bryan TX

[4,5] Same as in Moon & Gatlin III [86]

[6,7,8] MP Biomedicals

pH = 8.04 ± 0.05, salinity = 2.35 ± 0.26 mg $L^{-1}$, total ammonia nitrogen = 0.08 ± 0.01 mg $L^{-1}$, nitrite nitrogen = 0.035 ± 0.01 mg $L^{-1}$.

*4.2.2. Sample collections and production performance parameters*

All procedures performed were approved by the Institutional Animal Care and Use Committee at Texas A&M University. At the end of the $4^{th}$ week of feeding, three fish were randomly selected from each aquarium and blood was collected from a caudal puncture with heparinized tuberculin syringes and fish were euthanized immediately with an overdose (~300 mg $L^{-1}$) of MS-222 (Western Chemical Inc) [43]. Respiratory burst from the whole blood was evaluated by measuring the ability of circulating phagocytes to reduce NBT (Sigma-Aldrich Co.) to formazan granules [50]. Briefly, 50 µL of whole blood from each fish were incubated with 50 µL of 2 mg $mL^{-1}$ of NBT dissolved in PBS (Sigma Aldrich Co.) in a U-shaped-bottom, 96-well microplate for 30 min sheltered from light. After the incubation period, 50 µL of the mixture was aliquoted, and the formazan granules within the phagocytes were resuspended in 1 mL of *N-N* DMF (Sigma Aldrich Co.). The cell debris was separated by centrifugation at 3,000 × *g* for 5 min and supernatant aliquoted and read in a spectrophotometer at 545 nm. The remaining blood was centrifuged at 10,000 × *g* for 10 min, and plasma was aliquoted into plastic microtubes and stored frozen at -80˚C for other analyses described below.

The plasma lysozyme activity was measured by the reduction of absorbance when 10 µL of plasma from each fish (in duplicate) was incubated with 200 µL of a *Micrococcus lysodeikticus* (Sigma Aldrich) suspension in PBS solution (200 mg $L^{-1}$) [51]. Each sample was incubated in a 96-well, flat-bottom microplate (cat# 351172, Falcon, Le Pont de Claix, France) and absorbance at 450 nm was read at time 0 and after 4 minutes. The lysozyme activity was

expressed as the decrease of absorbance using the following formula: [(Absorbance at 4 min – at 0 min/4)/0.001] ×100. Plasma antiprotease activity was measured by Ellis [118] with modifications described by Magnadóttir et al. [119]. Briefly, this assay measured the ability of HSB plasma to inhibit trypsin digestion (20 µL of 5 mg $mL^{-1}$ trypsin cat# T4799 , Sigma Aldrich) for 10 min after which samples were further incubated with 250 µL of 2% azocasein solution (cat# A2765, Sigma Aldrich), and 200 uL of PBS (pH adjusted to 7) for 1 h. The reaction was terminated with 10% trichloroacetic acid (TCA, cat# T6399, Sigma Aldrich), centrifuged at 4000 × *g* for 5 min and the azo dye was solubilized with 1N NaOH and absorbance read at 440 nm. The percentage of trypsin inhibited was expressed by comparing each plasma sample to a 100% digested control sample: Antiprotease activity (%) = 100 – (Absorbance sample × 100) / (Absorbance 100% control). Plasma protein and plasma immunoglobulin were measured using the procedure established by Siwicki et al. [50].

Head kidney tissue from the three fish per aquarium were aseptically dissected and pooled in a 15-mL tube containing Leibovitz cell culture medium (L-15, cat# 4500-372, Corning, NY, USA) enriched with 2% of Bovine Calf Serum (BCS, cat# 12133C, Sigma Aldrich Co.) and 50 units $mL^{-1}$ of Penicillin and 0.05 mg $mL^{-1}$ of Streptomycin (Sigma Aldrich Co.). Phagocyte isolation followed the method established by Secombes [44] with minor modifications proposed by Sealey and Gatlin [34]. Pooled head kidney samples from each aquarium were mechanically disaggregated with glass Potter Elvehjem homogenizers, and the homogenate suspension was filtered with sterilized 100-µm nylon mesh (cat# 21905-026, VWR, Radnor, PA, USA). The filtered suspension was then centrifuged at 1,000 × *g* for 10 min at 4˚C, and the resultant cell pellets were washed with ice-cold autoclaved PBS (cat# 97062-732, VWR). This procedure was repeated thrice, and the resultant phagocyte suspension was layered in a 34/51% Percoll gradient

(Sigma Aldrich Co.) and centrifuged at $400 \times g$ for 25 min at 4˚C. The layer of cells resting at the 34/51% interface was collected with a sterile pipette tip and centrifuged/washed three times as previously described. The cells were then resuspended with L-15 0.1% BCS, and cell viability was assessed by 0.4% trypan blue staining with recovered samples having survivability higher than 95%. Phagocytes were counted under a light microscope using a hemocytometer and concentration of the samples was adjusted to $2 \times 10^7$ cells $mL^{-1}$ by diluting the samples with L-15 0.1% BCS. The diluted samples were aliquoted, and 100 µL of the cell suspension was layered in a 96-well, flat-bottom microplate, in a manner that each aquarium had their pooled cells layered in eight wells of the microplate. The ability of the pooled isolated phagocytes to produce superoxide anion radicals was measured by reduction of ferricytochrome C (cat# C2506, Sigma Aldrich Co.) [44]. Each aquarium was represented by six wells of layered cells which were incubated with 2 mg $mL^{-1}$ of cytochrome C solution in PBS supplemented with 1 µg $mL^{-1}$ of PMA (Sigma Aldrich Co.). Additional two wells were used as a negative control by incubating the same cytochrome C/PMA solution supplemented with 300 units $mL^{-1}$ of SOD (Sigma Aldrich Co.) to prevent oxidation. Plates were read immediately at 550 nm and every 15 min until an absorbance plateau was reached. The concentration of extracellular superoxide anion (ECSA) was measured as follows:

ECSA nmol $O_2^-$ = [(Δ Absorbance after 30 min × 100) ÷ 6.3].

The intracellular superoxide anion (ICSA) production was measured by the ability of the isolated phagocytes attached to the flat bottom of the microplate to engulf and reduce 100 µL of NBT (1 mg $mL^{-1}$) to formazan granules as established by Secombes [44] and modified by Caipang et al. [45]. After incubating for 45 min, the media was removed, and the phagocytes

were fixed with methanol and lysed with 140 μL of DMSO (Sigma Aldrich Co.) and 120 μL of potassium hydroxide 2M. Plates were read using a multiscan spectrophotometer at 620 nm.

At the end of the $8^{th}$ week, the same sampling procedures for immunological parameters were performed as mentioned previously, but with four fish randomly sampled per aquarium. Growth performance also was computed as follows:

% Weight gain of the initial weight (%WG) = 100 × [(final weight – initial weight) ÷ initial weight]

Feed efficiency (FE) = weight gain ÷ dry feed intake

Survival (%) = 100 × (number of surviving fish ÷ initial number of fish)

In addition, three fish per aquarium were euthanized with an overdose of MS-222 (~300 mg $L^{-1}$) and immediately stored frozen at -20˚C for measurement of whole-body proximate composition and computation of protein conversion efficiency according the following formula: [(Final body weight (g) × final body protein (%)) – (initial weight (g) × initial body protein (%)] ÷ protein intake (g) × 100. Whole-body were analyzed using the same procedures for analyzing the experimental diets.

*4.2.3. Evaluation of the two different sources of BG administered intraperitoneally*

A second experiment evaluated the immunological responses of advanced juvenile HSB (average weight ~330 g) after intraperitoneal injection with a solution of 10 mg of Paramylon or Zymosan per kg of fish body weight, or with only sterile PBS serving as the control. Each BG source was homogenized in PBS with a handheld homogenizer (PT1200 E, Kinematica AG, Luzern, Switzerland) for 1 min to yield a final concentration of 1 mg per mL of autoclaved PBS solution. A total of 30 fish were distributed evenly and conditioned for a week in six circular

fiberglass round tanks (~1200 L), arranged in two rows of three tanks, which operated as a recirculating system with similar components and layout as mentioned above for the feeding trial. Treatments were randomly distributed to each row of tanks, which was considered as a statistical block. Fish were netted out and placed in plastic tubs to be anesthetized with 150 mg of MS-222 per L of culture water. After presenting the clinical signs of sedation, fish were individually weighed and injected the β-glucans solutions or PBS according to their body weight. Following 7 days after injection, five fish were sampled again, sedated with the same procedure, and blood was collected with heparinized syringes. This procedure was performed twice and each collection consisted of a statistical block. Whole blood respiratory burst and plasma lysozyme, antiprotease, total protein, and total immunoglobulin were measured with the same procedures as mentioned for the feeding trial. Total hemolytic activity of the plasma also was performed for this trial as an additional immunological response. This procedure was performed according to Sutili et al. [52] with minor modifications. Briefly, after being sedated with MS-222 (100 mg $L^{-1}$), 1 mL of Nile tilapia (*Oreochromis niloticus*) tilapia blood was collected with heparinized tuberculin syringes and erythrocytes were washed and centrifuged thrice with PBS and the final pellet of red blood cells was diluted 10 times with PBS. The tilapia erythrocyte suspension (30 µL) was incubated with 15 µL of hybrid striped bass plasma in U-shaped-bottom microplates at room temperature (~24°C) for 2 h. After incubation, 240 µL of ice-cold PBS was pipetted to stop the hemolytic activity of the complement proteins and the plate was centrifuged at 3,000 × *g* for 5 min. As positive and negative control, erythrocytes were incubated in deionized water or PBS for total hemolysis or no lysis, respectively.

*4.2.4. Statistical analysis*

Resulting data from the feeding trial were treated as a completely randomized design, subjected to one-way ANOVA using JMP software (SAS Institute Inc., Cary, North Carolina, USA) and validated for homogeneity of variance using the Brown-Forsythe test [54]. When statistical significance ($P<0.05$) was found, a Tukey HSD test was performed for pairwise comparison of means. For the evaluation of BGs administered intraperitoneally, data were treated according to a completely randomized block design, having the disposition of the tanks. Individual fish were treated as the experimental units, and resulting data were also validated for homogeneity of variance by the Brown-Forsythe test. When significance ($P<0.05$) was detected, the Tukey HSD test was also used for comparison of means.

## 4.3 Results

No differences were observed for growth performance, condition indexes, feed efficiency and survival of HSB fed the various diets (Table 9). However, it is noteworthy to mention that reduced P values ($P=0.16$) were observed for weight gain and feed efficiency, indicating a trend for better performance for fish treated with 50 mg of Paramylon $kg^{-1}$ diet. Although differences were also not detected for whole-body proximate composition and protein conversion efficiency (PCE) (Table 10), significant differences were observed for some of the immunological parameters when the fish were sampled after the $4^{th}$ week of feeding. The ability to produce reactive oxygen species extracellularly was higher for the fish treated with 100 mg $kg^{-1}$ diet of either Paramylon or Zymosan when compared to those fed the basal diet. For the ICSA, fish fed the diet with Paramylon at 50 mg $kg^{-1}$ had the highest production of reactive oxygen species

Table 9: Growth performance, condition indexes, feed efficiency and survival of hybrid striped bass after 8 weeks of feeding the basal diet or experimental diets containing Paramylon (P) or Zymosan (Z) at 50 or 100 mg $kg^{-1}$

| | Basal | P 50 | P 100 | Z 50 | Z 100 | P value | PSE |
|---|---|---|---|---|---|---|---|
| Growth performance (%) | 376.2 | 395.7 | 380.8 | 381.8 | 379.7 | 0.16 | 2.65 |
| Hepatosomatic Index (%) | 2.45 | 2.55 | 2.53 | 2.53 | 2.54 | 0.89 | 0.03 |
| Intraperitoneal Fat Ratio (%) | 5.82 | 5.61 | 6.30 | 6.00 | 5.52 | 0.27 | 0.12 |
| Feed Efficiency | 0.68 | 0.70 | 0.68 | 0.68 | 0.68 | 0.16 | 0.00 |
| Survival (%) | 100 | 100 | 100 | 100 | 100 | - | 0 |

Table 10: Proximate composition of the whole body of hybrid striped bass expressed as g kg-1 and protein conversion efficiency (PCE) after 8 weeks of feeding the basal diet or experimental diets containing Paramylon (P) or Zymosan (Z) at 50 or 100 mg $kg^{-1}$ diet

| | Basal | P 50 | P 100 | Z 50 | Z 100 | P value | PSE |
|---|---|---|---|---|---|---|---|
| Moisture (g $kg^{-1}$) | 683.6 | 689.5 | 681.8 | 679.6 | 688.3 | 0.21 | 0.33 |
| Protein (g $kg^{-1}$) | 166.2 | 166.0 | 161.1 | 164.6 | 165.6 | 0.36 | 0.19 |
| Lipid (g $kg^{-1}$) | 104.8 | 97.6 | 104.4 | 105.0 | 98.5 | 0.36 | 0.38 |
| Ash (g $kg^{-1}$) | 39.2 | 40.1 | 40.3 | 38.4 | 39.0 | 0.78 | 0.12 |
| PCE (%) | 21.8 | 22.0 | 21.9 | 21.9 | 21.1 | 0.63 | 0.45 |

intracellularly, followed by those fed Paramylon at 100 mg $kg^{-1}$. In contrast, fish fed the two levels of Zymosan were not statistically different from those fed the basal diet. The other immunological parameters, such as whole blood NBT, antiprotease, lysozyme, protein, and total immunoglobulin, were not statistically different (Table 11). After the $8^{th}$ week of feeding, differences were observed for ECSA, ICSA, and total immunoglobulin (Table 12). Fish fed Zymosan at 50 mg $kg^{-1}$ diet had the highest value of ECSA; whereas, all other treatments were not significantly different from those fed the basal diet. The isolated phagocytes from fish fed both levels of Paramylon, and Zymosan at 50 mg $kg^{-1}$ produced significantly more ICSA when compared to those fed the basal diet. A higher concentration of circulating immunoglobulins in the plasma also was observed for fish fed the two diets supplemented with Paramylon compared to fish fed the basal diet. No differences were observed for whole-blood NBT, plasma antiprotease, or lysozyme activities, or total protein concentration. However, a marginal significance was observed for plasma lysozyme ($P=0.15$), with all fish fed diets supplemented with BGs having numerically higher values than those fed the basal diet.

Table 11: Immunological responses of hybrid striped bass fed the basal diet or experimental diets containing Paramylon (P) or Zymosan (Z) at 50 or 100 mg kg$^{-1}$ diet for 4 weeks.

| | Basal | P 50 | P 100 | Z 50 | Z 100 | P value | PSE |
|---|---|---|---|---|---|---|---|
| ECSA ($O_2^-$ nmol mL$^{-1}$) | 1.28$^{B}$ | 1.32$^{AB}$ | 1.37$^{A}$ | 1.31$^{AB}$ | 1.37$^{A}$ | 0.02 | 0.01 |
| ICSA (Absorbance at 620 nm) | 0.110$^{C}$ | 0.138$^{A}$ | 0.137$^{AB}$ | 0.111$^{C}$ | 0.120$^{BC}$ | <0.001 | 0.00 |
| NBT (Absorbance at 545 nm) | 0.748 | 0.747 | 0.733 | 0.754 | 0.750 | 0.90 | 0.01 |
| Antiprotease activity (%) | 67.7 | 65.4 | 66.2 | 67.2 | 66.8 | 0.90 | 0.75 |
| Lysozyme (U mL$^{-1}$) | 686 | 694 | 727 | 672 | 675 | 0.96 | 24.0 |
| Protein (mg mL$^{-1}$) | 80.2 | 90.4 | 80.2 | 88.2 | 79.3 | 0.23 | 1.96 |
| Total immunoglobulin (mg mL$^{-1}$) | 36.6 | 35.4 | 37.4 | 38.1 | 32.7 | 0.34 | 1.92 |

Table 12: Immunological responses of hybrid striped bass fed the basal diet or experimental diets containing Paramylon (P) or Zymosan (Z) at 50 or 100 mg $kg^{-1}$ for 8 weeks.

| | Basal | P 50 | P 100 | Z 50 | Z 100 | P value | PSE |
|---|---|---|---|---|---|---|---|
| ECSA ($O_2^-$ nmol $mL^{-1}$) | $1.53^{B}$ | $1.67^{AB}$ | $1.58^{AB}$ | $1.83^{A}$ | $1.56^{B}$ | 0.02 | 0.03 |
| ICSA (Absorbance at 620 nm) | $0.181^{B}$ | $0.245^{A}$ | $0.235^{A}$ | $0.235^{A}$ | $0.211^{AB}$ | 0.01 | 0.01 |
| NBT (Absorbance at 540 nm) | 0.607 | 0.571 | 0.601 | 0.596 | 0.618 | 0.23 | 0.01 |
| Antiprotease activity (%) | 44.0 | 41.6 | 39.4 | 47.7 | 45.4 | 0.57 | 3.65 |
| Lysozyme (U $mL^{-1}$) | 631 | 687 | 723 | 750 | 746 | 0.15 | 37.65 |
| Protein (mg $mL^{-1}$) | 123.8 | 127.2 | 132.8 | 133.3 | 125.8 | 0.19 | 3.43 |
| Total immunoglobulin (mg $mL^{-1}$) | $55.8^{B}$ | $68.45^{A}$ | $70.3^{A}$ | $66.0^{AB}$ | $61.4^{AB}$ | <0.01 | 3.02 |

All immunological responses of whole-blood and plasma of fish were affected by intraperitoneal exposure to the different BGs (Table 13). Whole blood respiratory burst and plasma antiprotease activity were higher for fish injected with Zymosan compared to those injected with Paramylon or PBS. Total hemolytic activity of the plasma of fish injected with Paramylon was higher compared to those treated with Zymosan or the control treatment. In contrast, plasma lysozyme activity and plasma protein concentrations were lower for fish injected with Zymosan compared to the other treatments. Plasma total immunoglobulin concentration was higher for the fish injected with Paramylon compared to the control group and those injected with Zymosan.

Table 13: Immunological responses of hybrid striped bass whole blood and plasma after 7 days when injected intraperitoneally with different β-glucans and only PBS serving as the Control.

| | Control | Paramylon | Zymosan | P value | Block P value | PSE |
|---|---|---|---|---|---|---|
| NBT (Absorbance at 545 nm) | 0.683$^{B}$ | 0.701$^{B}$ | 0.734$^{A}$ | <0.01 | 0.02 | 0.01 |
| Hemolytic activity (%) | 79.6$^{B}$ | 95.0$^{A}$ | 87.2$^{AB}$ | 0.03 | 0.69 | 2.53 |
| Antiprotease activity (%) | 70.5$^{B}$ | 73.9$^{B}$ | 77.2$^{A}$ | <0.01 | 0.27 | 1.3 |
| Lysozyme activity (U $mL^{-1}$) | 1249$^{A}$ | 1368$^{A}$ | 1163$^{B}$ | <0.001 | 0.15 | 30.1 |
| Total protein (mg $mL^{-1}$) | 92.4$^{A}$ | 96.5$^{A}$ | 80.4$^{B}$ | <0.001 | 0.99 | 2.9 |
| Total immunoglobulin (mg $mL^{-1}$) | 49.1$^{AB}$ | 51.9$^{A}$ | 45.7$^{B}$ | 0.04 | 0.92 | 1.6 |

## 4.4 Discussion

The nature of BGs, including their molecular mass, tertiary structure, polymer charge, solution conformation, degree of branching and the solubility, can promote different interactions with leukocyte PRRs and ultimately affect the immune responses of the host [15, 20, 22, 120]. Paramylon is composed solely of glucose subunits linearly linked between 1,3 glucan ligands, and its molecule structure is a unique triple helix conformation [80, 112-114]. In contrast, Zymosan is composed of a mannose proteoglycan attached to a β-1,3 backbone with a branches of β-1,6 glycosidic bonds [17]. The aim of the present study was to compare the immunomodulatory properties of the novel BG Paramylon, extracted from the blue-green microalgae *Euglena gracilis*, with an established BG reference extracted from the cell wall of baker's yeast. Paramylon and Zymosan were delivered orally, as a dietary supplement, and injected intraperitoneally to HSB. Indeed, the immunological responses of HSB were modulated by these BGs in different intensities, and the results showed more responsiveness when the BGs were delivered intraperitoneally.

No differences were observed for production performance and nutritional composition of whole-body tissues, regardless of the nature of the BGs and levels. This result is in agreement with previous studies which evaluated *Euglena gracilis* as a dietary source of Paramylon as a short-term feed additive for teleost fish [59, 121], but not with *Saccharomyces*-derived BG, where some prolonged exposure to the dietary supplement promoted better growth performance of other fish species [75, 96, 122-124]. However, it is unclear if these growth-promoting properties found in those studies were being induced by the BG *per se*, or if other components of the *Saccharomyces cerevisiae* commercial products, (*e.g.*, nucleotides, mannans, oligosaccharides) were playing role. It is noteworthy mentioning that, in the present study, HSB

fed Paramylon at 50 mg $kg^{-1}$ diet had numerically improved weight gain and feed efficiency which trended towards significance with a reduced P-value of 0.16.

The production of oxygen radicals can be activated by PAMPs prompting TLR in the surface of the phagocyte cell membranes. Upon this interaction, there is an increased oxygen consumption and reduction to superoxide anion by NADPH-oxidase, an enzyme assembled in the inner surface of the phagocyte [58, 125]. The present study observed an increased production of reactive oxygen species of phagocytes isolated from the head kidney at two collection points. The dietary supplementation of another Euglenoid species (*Euglena viridis*) in the diet for rohu (*Labeo rohita*), also enhanced the production of reactive oxygen species and likely was related to the Paramylon content in this microalgae [126]. When tested on other species, the ability of Paramylon to prompt the respiratory burst was also verified in barramundi (*Lates calcarife*r) macrophages and porcine neutrophils *in vitro* when exposed to Paramylon and Zymosan [60, 61]. Corroborating the present results, Zymosan also triggered an increased gene expression of NADPH oxidase *in vitro* when incubated with rainbow trout (*Oncorhynchus mykiss*) macrophages [127], and respiratory burst and inflammatory gene expression of carp (*Cyprinus carpio*) macrophages was enhanced [128]. Nevertheless, in the present study, no differences were detected in the ability of circulating phagocytes in whole blood to produce reactive oxygen species for fish treated with dietary BGs.

Lysozyme, an antimicrobial enzyme produced and secreted by neutrophils and monocytes, targets a specific linkage in the gram-positive bacterial cell wall to promote its rupture, and it can be detected in the mucus, eggs, organs and circulating blood of fish [16, 129]. Several studies reported an augmented lysozyme activity in the plasma/serum of fish fed dietary BG from *Saccharomyces cerevisiae* [66, 122, 124, 130], and an increased gene expression for

lysozyme in the head kidney also was observed in rainbow trout fed diets supplemented with Paramylon [39]. In contrast, plasma lysozyme activity was not statistically significant at both collection times in the present study. However, at the end of the 8$^{th}$ week of feeding, a reduced P value (0.15) could be observed with higher values for the highest inclusion levels of BGs and 50 mg kg$^{-1}$ of Zymosan. Similar trends also were observed by Li et al. [74] and Yamamoto et al. [59] for HSB and red drum, respectively, where reduced P values were obtained but no statistical differences were detected, with the intermediate doses of dietary BG presenting higher activity values when compared to those fed the basal diet. In addition, two separate studies feeding rainbow trout diets supplemented with BG from *Saccharomyces* also presented numerically higher lysozyme activity but with no statistical differences [67, 131]. Unarguably, the high variance obtained from the replicates limited the detection of statistical differences in this immunological variable, and perhaps if the expression of the lysozyme genes was measured in the leukocytes or the lymphoid organs, a more accurate picture of the dietary BG immunomodulation in this parameter could be observed. Antiprotease in fish serum/plasma can inhibit the activity of bacterial proteolytic toxins that digest the tissue of the host to obtain nourishment from amino acids [132, 133]. But the present study did not detect any differences or trends with regard to dietary BG supplementation on this immunological response of HSB.

The length of administration of dietary BG may potentially cause apprehension to industrial food fish producers because prolonged exposure to BG may cause deleterious effects to the host by, either overstimulating leucocytes and thereby exhausting them and down-regulating immunological responses, or limiting the sensitivity of leucocytes due to continuous exposure to BG [69]. The present study collected samples at weeks 4 and 8, and fish appeared to be immunostimulated by having a higher production of reactive oxygen species of isolated

phagocytes at both collection points. Corroborating with the present results, other studies also presented stimulation of the innate immune system when feeding BG [66, 67, 103, 134]. Interestingly, in the present study, Paramylon induced a higher concentration of total plasma immunoglobulins only at the 8$^{th}$ week. Moreover, comparable findings on total plasma immunoglobulin were reported for rainbow trout fed Macrogard® [50, 135] and barley-derived BG on (infectious hematopoietic necrosis virus (IHNV) neutralizing antibody titers after vaccination [95], and for Caspian trout (*Salmo trutta caspius*) fed BG extracted from brewer's yeast [130]. On the other hand, mice fed *Euglena gracilis* and Paramylon presented significantly lower antibody titers against influenza when compared to the control [136]. It is unclear the underlying causes of elevated levels of circulating immunoglobulins in plasma of HSB fed Paramylon, with no precedent of pathogen exposure or vaccination during the present feeding trial, but in parallel to the current findings, human patients orally treated with BG from baker's yeast presented higher levels of mucosal IgA in their saliva [137, 138]. Perhaps, supplementing Paramylon in the diet also promoted an increased concentration of mucosal immunoglobulins in the plasma, but further research is warranted as no characterization of antibody isotypes was performed in the present study.

Exposing BGs directly to the peritoneal cavity had more pronounced immunostimulatory effects than the feeding trial in the present study. Fish intraperitoneally injected with Zymosan had higher reactive oxygen species production of circulating phagocytes as well as plasma antiprotease activity at day 7 post injection. These results are in accordance with the findings of Selvaraj et al. [139] who also injected Zymosan into common carp (*Cyprinus carpio*) as the species model. In the present study, Paramylon presented a higher percentage of plasma total hemolytic activity than Zymosan and the control group. Contrasting to the present findings,

Japanese eel (*Anguila japonica*) and hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) injected with the same dosage of Zymosan intraperitoneally exhibited enhanced alternative and classical complement pathway when compared to the control, but samples were collected only 2 days after injection [90]. For the remainder parameters evaluated in the present study, fish injected with Zymosan had reduced values compared to fish in the Paramylon and control groups. It is hypothesized that the fish treated with Zymosan may have either down-regulated the immunological responses or their immune system was exhausted by the 7$^{th}$ day. This was observed for the expression of lysozyme genes in Japanese flounder (*Paralychthys olivaceus*) injected with BG with the peak at 12 h followed by a steady decline throughout the week [140].

BGs may be good candidates to replace the traditional oil emulsion and bacterial lipopolysaccharides commonly used as adjuvants, which can leave scars or dark spots in the fish fillet, depreciating its market value [15]. The ability of Paramylon and Zymosan to trigger innate immune responses was demonstrated in the present study, such that they may be prominent adjuvants to augment the efficacy during vaccination to specific antigens.

In conclusion, dietary Paramylon and Zymosan did not enhance growth performance but stimulated the production of reactive oxygen species in HSB at two different time points. Moreover, fish fed Paramylon only showed a higher concentration of immunoglobulins in the plasma after the 8$^{th}$ week. When changing the BG route of exposure from dietary to intraperitoneally, the immunological responses had more attenuated responses. Some immunological parameters in plasma were enhanced depending on the BG, while others were downregulated either by immunosuppression or exhaustion of the immune system. Further research evaluating the efficacy of Paramylon and Zymosan as vaccine adjuvants is suggested.

# CHAPTER V

# B-1,3 GLUCAN AND VITAMIN C SYNERGISM ENHANCES SOME OF THE IMMUNOLOGICAL RESPONSES OF HYBRID STRIPED BASS (*Morone chrysops* × *M. saxatilis*)

## 5.1 Introduction

For the past six decades, aquaculture has become a crucial player in the food industry, supplying more than half of the fish available for human consumption worldwide, and compiling annual growth rates higher than any other type of animal husbandry [141, 142]. However, unlike farmed terrestrial animals, fish and shellfish have a simpler immunological repertoire and are constantly being exposed to opportunistic pathogens that occur naturally in the water [2, 106], to a degree to which largely depends on culture conditions. In addition, the increased global demand for forage fishmeal and resulting price escalation has pressured various sectors of aquaculture to seek alternative protein ingredients [9], many of which contain anti-nutritional factors and secondary metabolites that can impair the functional competency of the gastrointestinal tract (GIT) against pathogen translocation [7, 10]. Thus, developing strategies to optimize the supplementation of feed additives in aquafeeds, such as allying functional nutrients and immunostimulants, appears to be an environmentally sound maneuver to overcome these issues.

β-glucans (BGs) are biological modifiers, consisting of D-glucose monomers as their building blocks linked by β-glycosidic bonds, and are known for their ability to activate the immune system [16, 20]. They can be naturally found in the cell walls of bacteria, yeast, and fungi, and when exposed to fish leucocytes, their pattern recognition receptors (PRRs) recognize

the structures of these molecules as foreign antigens, which elicit immunostimulation by inflammatory cascade, producing and secreting cytokines which ultimately regulate the production of transcription factor nuclear factor κB (NF-κB) [15, 20, 69]. The application of BGs in aquafeeds has been widely demonstrated in benefiting the humoral and cellular immunological responses, growth performance, and modulation of the gut microbiota.

Ascorbic acid, or vitamin C, is an essential micronutrient to be provided in the diets of finfish because they lack the ability to synthesize L-gulono-γ-lactone oxidase, an enzyme necessary for vitamin C biosynthesis [31, 143]. Vitamin C has been demonstrated to play a role in various aspects of the immune system, and their key components, such as leucocytes and lymphoid organs, store high concentrations of this nutrient, ranging from 50 to 100 times higher than that in circulating plasma [32, 144]. It is hypothesized that the antioxidant properties of vitamin C can enhance immunological cells by scavenging free radicals produced by normal metabolism, stress exposure, and respiratory burst during immunostimulation, ultimately protecting these cells from oxidative damage [33, 34]. Moreover, vitamin C can also regenerate the oxidized form of vitamin E within the cell cytoplasm, increasing the antioxidant capability within the intracellular content [145]. For several years it has been recognized that vitamin C fortification can enhance the immunological responses of many animal models against pathogens or cancer cells [146], and it is hypothesized that the addition of vitamin C can further enhance the immunological responses of fish when immunostimulated by BG.

Hybrid striped bass (HSB) is a cross between the anadromous striped bass (*Morone saxatilis*) and the freshwater white bass (*Morone chrysops*). It is an important farm-raised fish in North America, not only for recreational fishing but also for seafood production. The hybrid vigor confers improved growth performance, survival, hardiness to handling, wide tolerance to

water quality, and disease resistance [115, 147]. The objective of this study was two-fold: firstly, to evaluate the immunostimulatory properties of BG and vitamin C *in vitro*, and possibly identify any synergistic effect between these supplements for HSB isolated tissue and cells, and secondly, to analyze possible synergisms *in vivo* during a feeding trial with HSB.

## 5.2 Material and methods

### *5.2.1. Ex vivo trials*

#### 5.2.1.1. Experimental animals, blood collection, culture conditions, and incubation

Twenty-four advanced hybrid striped bass juveniles weighing ~330g on average were held in a circular fiberglass tank with 1,200-L volume operating as a recirculating system, which included a common settling chamber, and biological and mechanical filtration along with ultraviolet (UV) sterilization. Aeration was provided continuously by air stones connected to a regenerative blower. The temperature of the water was maintained by the ambient air at ~26˚C. Fish were fed a commercial diet containing 40% crude protein and 14% crude fat (EXTR 400, Rangen Inc). Prior to tissue collection, fish were netted out and anesthetized with 100 mg of MS-222 (Western Chemical Inc) per liter of cultured water [43]. To ensure reproducibility of the experiment, the *ex vivo* trial sampling was performed twice at two different time points, each with 12 fish.

Approximately 1.2 mL of blood from each fish were collected with a heparinized syringe (sodium heparin, cat#AK3004, Akron Biotech, Boca Raton, FL, USA) through the caudal peduncle and the blood was aliquoted in equal portions of 300 µL to four separate microtubes which contained 300 µL of sterile PBS (VWR), 300 µL of PBS with 200 µg of Paramylon $mL^{-1}$ (Sigma Aldrich Co) as the source of BG, 300 µL of PBS with 200 µM of dehydroascorbic acid

$L^{-1}$ (cat# D-0300-5G, Biosynth International Inc., Itasca, IL, USA) as source of vitamin C, or 300 µL of PBS with 200 µg of dehydroascorbic acid $mL^{-1}$. Dehydroascorbic acid was chosen as a source of vitamin C because leucocytes can favor the uptake of this oxidized form of ascorbic acid across their plasma membrane [32, 148]. When pipetting the whole blood over the various PBS solutions, the final concentration yielded 100 µg $mL^{-1}$ of BG, 100 µM $L^{-1}$ of vitamin C, and both 100 µg $mL^{-1}$ of BG and 100 µM $L^{-1}$ of Vitamin C; PBS only served as a control. The vitamin C concentration was chosen based on the previous study by Sealey & Gatlin III [149] reporting optimal levels of this nutrient in hybrid striped bass plasma for immunological responses. The optimal concentration of BG chosen for the whole blood for reactive oxygen species (ROS) production *in vitro* for HSB was previously reported by Yamamoto et al [150].

An aliquot of the whole blood from each sample was separated in a 96-well round-bottom microplate and incubated with 2 mg of NBT (VWR) per $mL^{-1}$ of PBS solution, to measure the respiratory burst according to Siwicki & Anderson [50] with minor modifications. Briefly, 50 µL of heparinized blood was incubated for 30 min with 50 µL of NBT solution in the 96-well round-bottom microplate sheltered from light. After incubation, formazan granules within the phagocytes were re-suspended in 1 mL of N-N DMF (Sigma Aldrich Co) and the supernatant was recovered after centrifuging the samples at 3,000 × *g* for 5 min. The absorbance of the supernatant was read in a spectrophotometer at 545 nm. The remaining blood was centrifuged for 10 min at 10,000 × *g,* and plasma samples were aliquoted in different plastic microtubes and stored frozen at -80°C for further analysis. Antiprotease activity was measured by the procedures established by Ellis [118] with modifications [119]. Plasma (10 µL) samples were incubated for 10 min with 20 µL of 5 mg trypsin $mL^{-1}$ solution (Sigma Aldrich Co), and further incubated with 250 µL of 2% azocasein solution (Sigma Aldrich Co.) in PBS, and 200 µL of PBS (pH adjusted

to 7) for 1 h. After incubation, proteins were precipitated with 10% trichloroacetic acid (TCA, Sigma Aldrich Co.), and centrifuged at 4,000 × *g* for 5 min. The supernatant was collected and 100 µL was transferred to a 96-well flat-bottom microplate, and the sample had the azo dye solubilized with 100 µL of 1N NaOH. Absorbance was read in a multiscan spectrophotometer at 440 nm.

Soluble plasma proteins were measured using Coomassie blue with the protocol established by Bradford [151] with minor modifications. Briefly, HSB plasma was diluted in a 1:200 ratio, then 10 µL of the diluted samples were pipetted into a 96-well flat-bottom microplate, and incubated with 190 µL of Bradford reagent (cat#5000205, Bio-Rad, Hercules, CA, USA) for 5 min at room temperature. Protein concentration was estimated by using a standard curve with bovine serum albumin (BSA, cat# A2153, Sigma Aldrich) in the same microplate as the samples, and absorbance was read at 595 nm. Total plasma immunoglobulin concentration was measured by precipitating the diluted samples with 12% polyethylene glycol solution (Sigma Aldrich Co) and incubating the samples for 2 h at room temperature [135]. The protein concentration of the samples was read with the Bradford reagent and estimated with a standard curve as previously described. Plasma lysozyme activity was determined by the reduction of absorbance of 200 µL of *Micrococcus lysodeikticus* (Sigma Aldrich Co) suspension in PBS (200 µg $mL^{-1}$) when incubated for 4 min with 10 µL of plasma [51]. Incubation took place in 96-well flat-bottom microplates, and absorbance was read at 450 nm, with lysozyme activity expressed as the decrease of absorbance using the formula:

$$\text{Lysozyme Activity (U ml}^{-1}\text{of plasma)} = [(\Delta \text{ Absorbance}/4 \text{ min})/0.001]\times 100$$

5.2.1.2. Phagocytic cells isolation

After the blood collection, fish were euthanized with an overdose of MS-222 (300 mg $L^{-1}$) and head kidney samples were aseptically excised to isolate phagocytes following procedures established by Secombes [44] with modifications described by Yamamoto et al. [59]. Briefly, head kidneys from the sampled fish were pooled in tubes containing Leibowitz cell culture medium (Corning) enriched with 2% of BCS (Sigma Aldrich Co), and 100 units $mL^{-1}$ of Penicillin and 0.05 mg $L^{-1}$ Streptomycin (P/S) (Sigma Aldrich Co) as well as 10 U $mL^{-1}$ of heparin. Tissues were mechanically disaggregated with an autoclaved Potter-Elvehjem tissue homogenizer, and the resulting suspension was filtered with sterilized 100-µm nylon mesh (VWR). The filtered suspension was centrifuged and washed with sterile ice-cold PBS and layered in a 34/51% Percoll gradient (Sigma Aldrich Co) and centrifuged at $400 \times g$ for 25 min at 4°C with the centrifuge's brakes off. The cells resting in the 34/51% interface were carefully collected with a sterile pipette tip and washed and centrifuged again with ice-cold PBS as previously described. Isolated phagocytes were re-suspended in 10 mL of L-15 0.1% BCS and stained with 0.4% Trypan Blue to assess cell viability, which was consistently higher than 95%. Cells were counted under a light microscope using a hemocytometer, and cell concentration was adjusted to approximately $1 \times 10^7$ cells $mL^{-1}$ in L-15 with 0.1% BCS and layered unto four 96-well flat-bottom microplates by adding 100 µL of cell suspension, yielding a final concentration of $1 \times 10^6$ cells per well.

5.2.1.3. Graded levels of BG and vitamin C incubated with HSB phagocytic cells and culture conditions

A preliminary assay was conducted to establish an optimum level of BG and vitamin C when incubating with HSB phagocytes. Paramylon was homogenized using a handheld homogenizer (PT1200 E, Kinematica AG) for 2 min, and diluted in L-15 medium, enriched with 10% BCS and supplemented with 100 U of P/S $mL^{-1}$ as previously described, to yield a final BG concentration of 800, 400, 200, 100 and 50 µg $mL^{-1}$ when 100 µL were pipetted into the microplate, and with L-15 (10% BCS with 100 U P/S $mL^{-1}$) only serving as the control. Each concentration was pipetted into 16 wells, in two different 96-well flat-bottom plates, and this procedure was carried out twice at separate sampling periods. Phagocytes were incubated with BG for 18 h at room temperature, sheltered from light, with gentle agitation provided by an orbital incubator. After incubation, media was flipped from the plates and wells were washed with sterile PBS and prepared for further analysis. The same procedure was carried out to establish an optimal level of vitamin C, where dehydroascorbic acid was dissolved in L-15 and further diluted in L-15 10% BCS with 100 U of P/S $mL^{-1}$, at the following concentrations: 25, 50, 100, 200 and 400 µg $mL^{-1}$, with no supplementation serving as control. The pH of these solutions had to be adjusted by adding 1N sodium hydroxide (NaOH) to ensure that all culture media was neutral. The incubation process was the same as described for the levels of BG, and further analysis was carried out as described above.

5.2.1.4. Detection on extracellular and intracellular superoxide anion ($O_2^-$)

From the 16 wells with layered cells from each treatment as described above, 100 µL of a 2 mg $mL^{-1}$ of ferricytochrome C (Sigma Aldrich Co.) solution with PMA (Sigma Aldrich Co) in

phenol-red-free Hank's Balanced Salt Solution (HBSS, Sigma Aldrich Co) was pipetted over 12 wells to measure the extracellular superoxide anion (ECSA) production as described by Secombes [44]. The remaining four wells were used as a control with the ferricytochrome solution but supplemented with 300 U $mL^{-1}$ of superoxide dismutase (Sigma Aldrich Co) to scavenge oxidation. Plates were immediately read after pipetting (time 0) and after every 15 min until 1 h using a multi-scan spectrophotometer at 550 nm. The final concentration of ECSA was measured according to Pick and Mizel [46] using the following formula:

$$\text{nmol of } O_2^- = [(\Delta \text{ Absorbance after 30 min} \times 100) \div 6.3]$$

The ability of isolated cells to engulf NBT and to reduce it to formazan granules was measured by incubating 100 µL of NBT solution (1 mg $mL^{-1}$ with 1µg of PMA $mL^{-1}$) for 45 min at room temperature as described by Secombes [44] with minor modifications by Caipang [45]. Cells were then fixed in 100% methanol for 10 min, washed thrice with 70% methanol, and air-dried for 1 h sheltered from light. The formazan granules were resuspended in 120 µL of 2M potassium hydroxide and 140 µL of DMSO (Sigma Aldrich Co) and vigorously mixed with the pipette. Plates were read at 620 nm, and the absorbance was expressed as intracellular superoxide anion production (ICSA).

5.2.1.5. BG, vitamin C and their combination incubated with HSB isolated phagocytes

After obtaining the results from the graded doses of BG and vitamin C, an optimum point was established for both parameters, after which an additional four fish with an average weight of ~162.9 g were euthanized, as described above, and head kidney phagocytic cells were harvested. Isolation procedures were the same as previously described, and phagocytes were layered in four microplates to investigate possible synergistic effects of vitamin C and BG.

Groups of 24 wells were assigned to each treatment and incubated with either 100 µg of BG $mL^{-1}$ in L-15 (10% BCS, 0.1% P/S), 100 µg of vitamin C $mL^{-1}$, or the combination of 100 µg BG and vit. C $mL^{-1}$. Leibovitz 10% BCS with 0.1 % P/S alone served as a control. The media enriched with vitamin C had the pH adjusted with 1N NaOH. Plates were incubated for 18 h, and ICSA and ECSA were assessed. This procedure was performed twice to ensure reproducibility of the results.

*5.2.2. In vivo trial*

5.2.2.1. Experimental fish, culture system, diets and feeding trial

Juvenile HSB were kindly donated by Keo Fish Farms (Keo, AR, USA) and acclimated to the local conditions at the Aquacultural Research and Teaching Facility of the Texas A&M University System (College Station, TX, USA). Fish were a fed a commercial diet containing 400 g $kg^{-1}$ of crude protein and 140 g $kg^{-1}$ crude fat (EXTR 400, Rangen Inc) prior to commencement of the feeding trial. Two hundred and twenty-four fish were equally distributed in 16, 110-L aquaria, resulting in 14 fish per tank. A subsample of 10 fish from the same batch was euthanized with an overdose of 300 mg of MS-222 $L^{-1}$ for measurement of initial whole-body proximate composition. The aquaria operated as a recirculating system containing a common settling chamber for suspended solids removal, a biological filter with plastic bio-balls, an ultraviolet (UV) sterilizer to reduce the microorganism load in the water, and a sand filter for mechanical filtration. The temperature of the water was conditioned by the ambient air and kept steady throughout the trial. Salinity was maintained by the addition of synthetic marine sea salt (Red Sea Salt, Red Sea USA, Houston, TX, USA) to balance the low hardness and alkalinity of

the deep-well water source. A photoperiod 12:12 hours dark:light was maintained by using fluorescent light bulbs controlled by timers.

The basal diet was formulated to meet all known requirements of HSB [115], the previously established minimum vitamin C requirement (50 mg $kg^{-1}$) for normal growth performance [152], and to contain 420 g of crude protein $kg^{-1}$ of diet, 100 g of lipid $kg^{-1}$ and an estimated digestible energy of 12.7 MJ $kg^{-1}$ (Table 14). A total of four diets were manufactured, consisting of the basal diet and singular additions of either 100 mg of Paramylon $kg^{-1}$, 500 mg of L-ascorbic acid $kg^{-1}$ (cat# IC10076991, MP Biomedicals, Solon, OH, USA), or the combination of 100 mg of Paramylon and 500 mg of L-Ascorbic acid $kg^{-1}$. The supplements were added to the diet at the expense of cellulose. The basal diet with no supplementation served as the control. To prepare the diets, ingredients of each treatment were mixed in a V-mixer machine (Blend Master) for 30 min and blended with oil and water by an industrial mixer (A-200 Hobart meat grinder). The resultant mash was cold- pelleted through a 3-mm die plate and dried at room temperature with forced ventilation for 48 h. The pellets were ground and sieved to the appropriate size for the fish, and the resultant fines were sampled and analyzed for proximate composition [48]. Each of the four treatments was randomly distributed to the aquaria with four replicates, and fish were weighed weekly and rations of feed were adjusted according to the percentage of total biomass (initially at 6% of biomass and reduced to 3% by trial termination). This feeding method aimed to provide an equal daily feed ration for all treatments, being close to apparent satiation without overfeeding. Water quality was measured three times a week and parameters were kept within optimum ranges for HSB culture [153]. Water dissolved oxygen and temperature were measured by an optical dissolved oxygen meter (ProOdo, YSI Inc), pH with a portable pH meter (Pocket Pro pH tester, Hach Company), salinity with a

Table 14: Formulation and analyzed proximate composition of the basal diet used in the comparative feeding trial

| Ingredients | g $kg^{-1}$ |
|---|---|
| Poultry by-product meal[1] | 91 |
| Fishmeal[2] | 121.5 |
| Soybean meal[3] | 482.5 |
| Dextrinized starch[4] | 100 |
| Carboxymethyl cellulose[4] | 20 |
| Fish oil[2] | 25 |
| Soybean oil | 30 |
| Vitamin premix[5] | 30 |
| Mineral premix[6] | 40 |
| DL-Methionine[7] | 5 |
| Glycine[4] | 10 |
| Celufil[4] | 45 |

| Proximate composition | g $kg^{-1}$ |
|---|---|
| Moisture | 95.5 |
| Protein | 432.7 |
| Lipid | 103.1 |
| Ash | 98.8 |

[1] Tyson Foods, Springdale, AR, USA

[2] Omega Protein Corporation

[3] Producers Cooperative Association

[4] MP Biomedicals

[5] Modified from Sealey & Gatlin III [152] with 50 mg $kg^{-1}$ of L-Ascorbic acid

[6] Same as in Moon & Gatlin III [86]

[7] Ajinomoto North America Inc., Itasca, IL, USA

portable salinity meter (EC170, Extech), and total ammonia-, and total nitrite-nitrogen determined photometrically using the test reagents from Hach and reading in the DR2000 Spectrophotometer (Hach Company). Water quality parameters were as follows [average ± standard deviation (SD)]: Temperature= 26.2 ± 1.0˚C, dissolved oxygen = 6.95 ± 0.66 mg $L^{-1}$, total ammonia-nitrogen = 0.15 ± 0.1 mg $L^{-1}$, total nitrite-nitrogen = 0.07 ± 0.06 mg $L^{-1}$, pH = 7.76 ± 0.25, and salinity = 3.15 ± 0.20 mg $L^{-1}$.

5.2.2.2. Sample collection, production performance, and immunological analysis

At the end of the eighth week of feeding, fish from each aquarium were group-weighed and counted after being fasted overnight. Four fish were anesthetized with MS-222 (100 mg $L^{-1}$) and blood samples were collected through the caudal vasculature with heparinized tuberculin syringes and euthanized immediately with an overdose of MS-222 (300 mg $L^{-1}$). The euthanized fish were dissected, with the liver and fat in the peritoneal cavity excised and weighed to compute the hepatosomatic index (HSI) and intraperitoneal fat (IPF) ratio. The fish intestine was excised, and divided into three segments, stored in 2-mL centrifuge tubes, flash-frozen in liquid nitrogen and stored at -80˚C until being processed. Another set of three fish was also euthanized, stored at -20˚C, and analyzed for whole-body proximate composition [48].

Production performance measures were computed as follows:

Percent weight gain (PWG) (% of initial) = 100 × [(Final weight (g) - Initial weight (g)) ÷ initial weight (g)]

Protein retention efficiency (PRE) (%) = [(Final body weight (g) × Final body protein (%)) - (initial body weight (g) × initial body protein (%))] ÷ protein intake (g) × 100

Feed efficiency (FE) = weight gain (g) ÷ dry feed intake (g)

Viscerosomatic indices (HSI or IPF) (%) = [liver or IPF weight (g) ÷ body weight (g)] × 100

Survival (%) = 100 × (number of surviving fish ÷ initial number of fish stocked)

Blood samples were processed as previously described for the *ex vivo* trial, with plasma samples aliquoted, and subjected to the same immunological analyses. The head kidneys of four fish from each aquarium were pooled in 12 mL of L-15 (2% BCS with 100 U of P/S and 10 U of heparin $mL^{-1}$) and layered unto six wells in two, 96-well flat-bottom microplates. The measurements of ICSA and ECSA were conducted in separated plates as described for the *ex vivo* trial but using only 2 wells with SOD supplemented with ferricytochrome C as the control for ECSA.

5.2.2.3. Intestinal enzymes related to health

The intestinal segments were weighed in a 2-mL microtube and cold Tris-HCl buffer (50 mM, 20 mM $CaCl_2$, pH adjusted to 7) was added to maintain a ratio of 50 mg of wet intestine tissue $mL^{-1}$ of buffer. The microtubes were placed on ice and tissues were homogenized with a handheld homogenizer (PT1200 E) and centrifuged at 15,000 × *g* for 10 min. The supernatant was collected and aliquoted as 500-µL samples into microtubes and frozen at -80˚C. The activities of alkaline phosphatase (ALKP) and acid phosphatase (ACDP) were determined as described by Castillo et al. [154].

5.2.2.4. DNA extraction of intestinal digesta and denaturing gradient gel electrophoresis (DGGE)

To assess potential dietary effects on intestinal microbial profile, digesta contents were aseptically collected from the intestine by stripping, from the end of the pyloric ceca to the distal intestine, with sterilized tweezers. After the sampling at the end of week 8, the remaining fish were fed the experimental diets for an additional week and two fish were randomly selected 5 h postprandial. Feeding was staggered by 5-min intervals between experimental units to ensure that samples would be collected at the same transit time. The collection procedure was performed as described by Burr et al. [48] with modifications [59]. Fresh samples were immediately flash-frozen in liquid nitrogen and stored at -80°C until being further processed.

After thawing in a water bath, digesta were centrifuged at 5,000 × *g* for 10 min, the resulting pellet was separated from the supernatant, and 0.2 g of the pellet was transferred to a new sterile microtube. The pellet was further digested with 180 µL of lysing buffer (20 mg $mL^{-1}$ lysozyme 20 mM Tris-HCl, pH 8.0; 2 mM EDTA, 1.2% Triton) and incubated in a water bath at 37°C for 30 min. After this step, the genomic DNA isolation protocol was followed according to the manufacturer's (QIAamp DNA Mini kit, Qiagen, Hilden, Germany) instructions. The isolated DNA was quantified using a Nanodrop One (ThermoFisher, Madinson, WI, USA) spectrophotometer at 280 nm, and DNA samples were diluted to 50 ng of DNA $\mu L^{-1}$. The samples were amplified using primers specific for conserved regions of bacteria, flanking the variable V3 region of 16S rDNA [50]. The PCR products from each treatment were pooled, and 5 µL were added with an equal volume of loading buffer 2 (bromophenol 0.05%, xylene cyanol 0.05%, glycerol 70%). From this mixture, 7 µL was loaded onto a polyacrylamide gel (8% v/v acrylamide-bisacrylamide ratio of 37.5:1). Electrophoresis was performed for 17 h at 60 V using a DCode Universal Mutation Detection System (BioRad), after which the gel was carefully

removed and stained with SYBR Green I (1:10,000 dilution) for 15 min and images digitalized. Comparison of samples band patterns was assessed using the Dice percentage similarity coefficient (%SC) and dendrograms were constructed using unweighted pair group method using arithmetic averages (UPGMA) options in Gel Compare II 6.6 (Applied Maths, Inc., Austin, TX, USA).

*5.2.3. Statistical analysis*

*Ex vivo* and *in vivo* data were analyzed using JMP software (v 14.0, SAS Institute, Cary, NC, USA) and SAS software (v 9.4, SAS Institute). The data resulting from the preliminary *ex vivo* trial evaluating graded doses of BG and vitamin C when incubated with HSB phagocytes were subjected to unequally spaced orthogonal polynomial contrasts, and regressions were chosen by the lesser P-value given in the SAS output. All data showed significant ($P<0.05$) differences for the orthogonal polynomial contrasts. However, this set of data also was subjected to a two-slope broken line regression (PROC NLIN), and the regressions models that best represented the data were chosen by the higher $R^2$. The blood incubation trial was subjected to a mixed model of a full 2×2 factorial using Two-way ANOVA with JMP software. The dietary treatments were considered as main factors: BG, vitamin C, and the interaction between the BG and vitamin C; and the variance caused by the individual fish was accounted for in the model. If significance ($P<0.05$) differences were observed for main factors, then a Student t-test was performed for comparison of means. If the interaction between BG and vitamin C was significant ($P<0.05$), the main factors were not analyzed even if there were significant differences detected, and the multiple comparisons of means were performed by Tukey-HSD for all the dietary treatments. The *ex vivo* data from investigating the ICSA and ECSA production and data

generated from the feeding trial were analyzed in the same manner but not having the individual fish in the model.

## 5.3. Results

### *5.3.1. Ex vivo assays*

Results from the *ex vivo* assays demonstrated that graded doses of BG significantly ($P<0.001$) enhanced the production of reactive oxygen species (ROS) extracellularly ($R^2=0.75$) and intracellularly ($R^2=0.88$). Maximum levels were determined by two-slope broken-line regression (Figure 9 and 10), and were estimated to be 100 and 126.5 µg of BG $mL^{-1}$ of cell culture media, respectively. The ECSA production for the graded doses of vitamin C presented a downward quadratic relationship ($R^2=0.40$); thus, an optimum point could not be established (Figure 11). However, for the intracellular parameter, an optimum level was established by a two-slope broken-line regression ($R^2=0.32$) when vitamin C was supplemented at 79.7 µg $mL^{-1}$ (Figure 12).

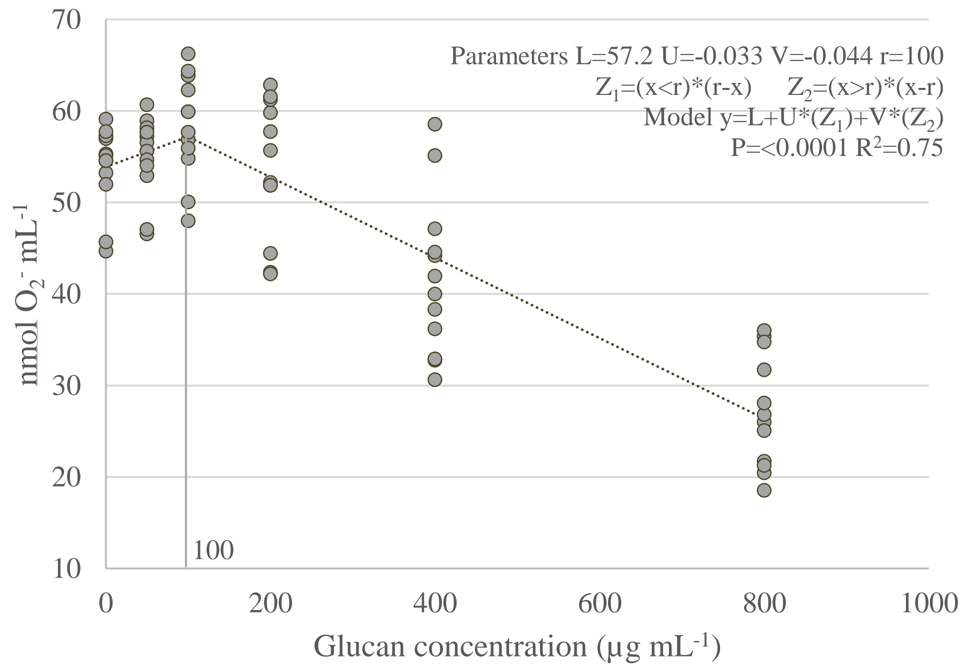


**Figure 9: Extracellular superoxide anion production by HSB isolated phagocytes incubated with graded doses of BG.**

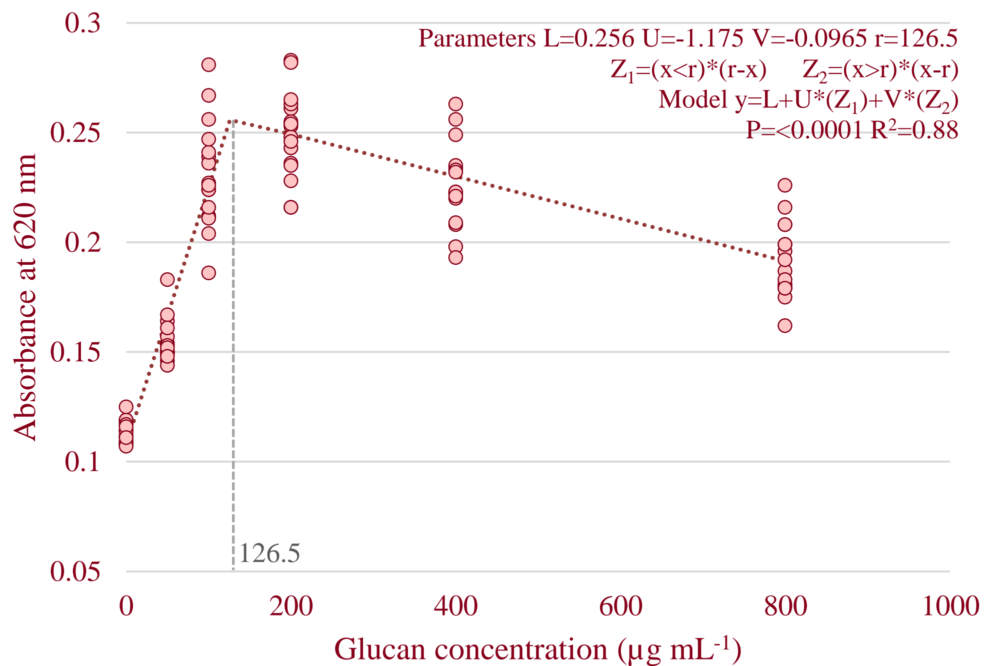


**Figure 10: Intracellular superoxide anion production by HSB isolated phagocytes incubated with graded doses of BG.**

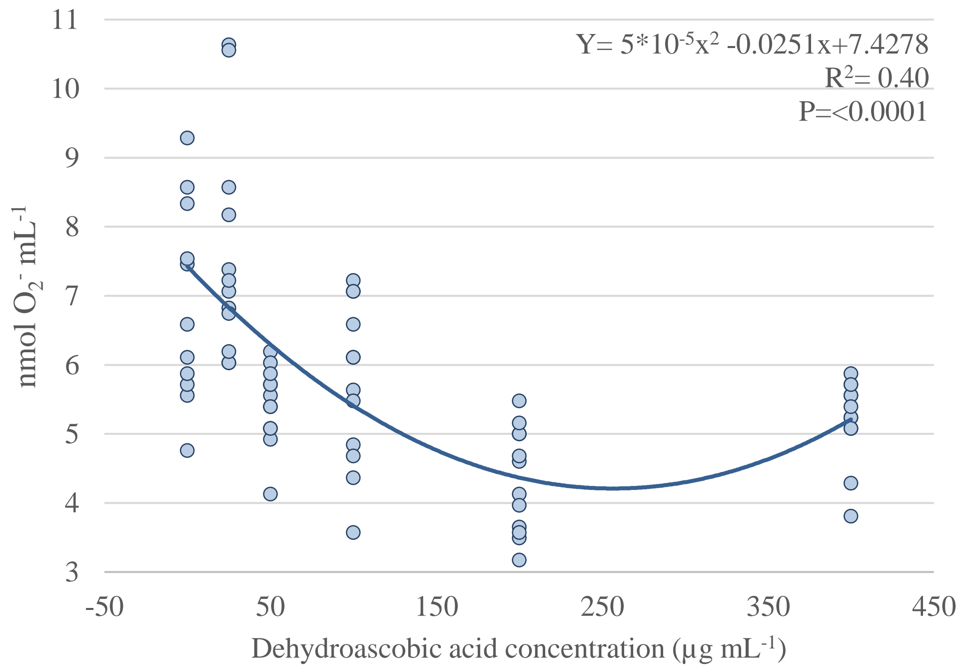


Figure 11: Extracellular superoxide anion production by HSB isolated phagocytes incubated with graded doses of vitamin C.

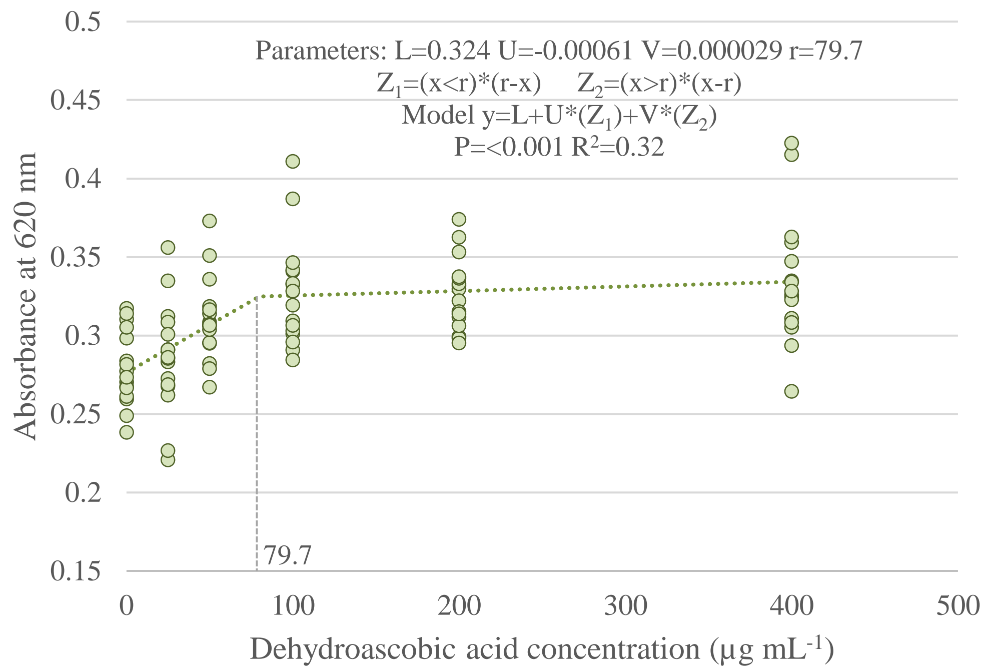


**Figure 12: Intracellular superoxide anion production by HSB isolated phagocytes incubated with graded doses of vitamin C**

The supplementation of BG and vitamin C in the blood affected ROS production of circulating leucocytes (NBT) (Table15). A synergistic effect between BG and vitamin C was detected ($P<0.05$) and cells incubated with both additives had the highest production of ROS when compared to the vitamin C and control groups. The same pattern for ECSA and ICSA could be detected by the isolated phagocytes incubated with BG and vitamin C, but instead of the control group, the supplementation of vitamin C presented the lowest ROS production values. Plasma lysozyme was significantly ($P=0.001$) affected by BG supplementation, with the BG-supplemented group having higher lysozyme activity than the non-supplemented group. Incubating blood with Vitamin C increased plasma protein and immunoglobulin significantly ($P<0.001$ and $P<0.01$, respectively) when compared to the non-supplemented group. No significant differences were detected for plasma antiprotease.

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Table 15: Immunological responses of whole blood, plasma, and isolated phagocytes from hybrid striped bass when incubated with beta-glucan (BG), Vitamin C (Vit C), or combination of both.

| | NBT | Antiprotease | Lysozyme | Total Protein | Total Ig | ICSA | ECSA |
|---|---|---|---|---|---|---|---|
| | (Abs. 545 nm) | (%) | (U $mL^{-1}$) | (mg $mL^{-1}$) | (mg $mL^{-1}$) | (Abs. 620 nm) | ($O_2^-$ nmol $mL^{-1}$) |
| Control | $0.280^{C}$ | 21.8 | 461.3 | 176.2 | 59.4 | $0.117^{C}$ | $10.2^{C}$ |
| Vit. C | $0.301^{B}$ | 16.9 | 557.9 | 189.3 | 75.1 | $0.093^{D}$ | $2.00^{D}$ |
| BG | $0.324^{A}$ | 17.9 | 663.6 | 180.8 | 54.9 | $0.229^{B}$ | $24.0^{B}$ |
| BG × Vit C | $0.326^{A}$ | 17.6 | 619.3 | 186.6 | 67.2 | $0.268^{A}$ | $28.8^{A}$ |
| PSE | 0.003 | 1.3 | 36.9 | 3.0 | 3.77 | 0.006 | 0.73 |
| *Main effects of* Vit C | | | | | | | |
| 0 μg $mL^{-1}$ | 0.302 | 19.4 | 562.5 | $178.5^{B}$ | $57.7^{B}$ | 0.173 | 17.13 |
| 100 μM $L^{-1}$ or 100 μg $mL^{-1}$ | 0.314 | 17.8 | 588.6 | $187.9^{A}$ | $70.9^{A}$ | 0.180 | 15.41 |
| *Main effects of* BG | | | | | | | |
| 0 μg $mL^{-1}$ | 0.291 | 19.9 | $509.6^{B}$ | 182.7 | 67.2 | 0.105 | 6.11 |
| 100 μg $mL^{-1}$ | 0.325 | 17.3 | $641.4^{A}$ | 183.7 | 61.3 | 0.248 | 26.42 |
| *Two-way ANOVA P value* | | | | | | | |
| Vit C | 0.0029 | 0.23 | 0.48 | <0.01 | 0.001 | 0.21 | <0.02 |
| BG | <0.0001 | 0.057 | 0.001 | 0.78 | 0.128 | <0.0001 | <0.0001 |
| BG × Vit C | 0.02 | 0.09 | 0.066 | 0.26 | 0.72 | <0.0001 | <0.0001 |
| Individual fish | <0.0001 | <0.0001 | 0.03 | <0.0001 | <0.0001 | - | - |

Abbreviations: NBT: Nitroblue tetrazolium; Total Ig: Immunoglobulin; ICSA: Intracellular superoxide anion; ECSA: Extracellular superoxide anion; PSE: Pooled Standard Error.

*5.3.2. Productive performance, proximate composition, immunological parameters, and microbiota of digestive tract contents from the in vivo trial*

Growth performance and feed efficiency of juvenile hybrid striped bass were slightly but significantly (P<0.05) impaired by the supplementation of BG in the diet (Table 16). For instance, the weight gain response of fish fed the BG-supplemented diet was reduced by approximately 18.9% compared to fish fed the diets without BG supplementation. No statistical differences were detected for HSI, IPF ratio, or survival. The moisture content of whole-body after 8 weeks of feeding the experimental diets was lower for fish fed diets supplemented with BG compared to fish fed the diets without BG (P<0.05) (Table 17). No differences were detected for whole-body protein, lipid, and ash, as well as protein retention efficiency. An interaction between dietary BG and vitamin C enhanced the ability of circulating phagocytes to produce ROS (P<0.05), with fish fed diets supplemented with BG and vitamin C having the highest values compared to fish fed diets supplemented with vitamin C only (Table 18). Fish fed diets supplemented with BG had higher concentrations of plasma immunoglobulin and ICSA production when compared to fish fed the basal diet (P<0.05). No differences were detected for ALKP regardless of which intestinal segment was sampled or which treatment the fish were fed (Table 19). However, a synergistic effect of BG and vitamin C was detected for the anterior part of the intestine, in which, fish fed BG and vitamin C diet had the highest ACDP when compared

Table 16: Growth performance, condition indexes, feed efficiency and survival of hybrid striped bass after 8 weeks of feeding the experimental diets

| | Initial weight (g) | Final weight (g) | PWG (%) | Feed efficiency | HSI (%) | IPF Ratio (%) | Survival (%) |
|---|---|---|---|---|---|---|---|
| Control | 9.27 | 56.4 | 508 | 0.79 | 1.41 | 5.04 | 100 |
| Vit. C | 9.28 | 55.7 | 505 | 0.77 | 1.32 | 4.78 | 100 |
| BG | 9.30 | 53.9 | 481 | 0.78 | 1.36 | 4.49 | 100 |
| BG × Vit. C | 9.33 | 55.5 | 495 | 0.77 | 1.30 | 4.72 | 100 |
| PSE | 0.05 | 0.9 | 8.6 | 0.006 | 0.04 | 0.18 | 0 |
| *Main effects* of Vit. C | | | | | | | |
| 0 g $kg^{-1}$ | | 55.1 | 495 | 0.78 | 1.39 | 4.77 | 100 |
| 500 mg $kg^{-1}$ | | 55.6 | 500 | 0.77 | 1.31 | 4.57 | 100 |
| *Main effects* of BG | | | | | | | |
| 0 g $kg^{-1}$ | | 56.0 | $507^{A}$ | $0.78^{A}$ | 1.37 | 4.91 | 100 |
| 100 mg $kg^{-1}$ | | 54.4 | $488^{B}$ | $0.77^{B}$ | 1.33 | 4.61 | 100 |
| *Two-way ANOVA P Values* | | | | | | | |
| Vit. C | | 0.64 | 0.56 | 0.44 | 0.44 | 0.12 | |
| BG | | 0.16 | 0.048 | 0.02 | 0.11 | 0.94 | |
| BG × Vit C | | 0.23 | 0.35 | 0.70 | 0.82 | 0.20 | |

Abbreviations: PWG: Percentage of weight gain; IPF ratio: intraperitoneal fat; Vit. C: Vitamin C; BG: Beta-glucan; PSE: Pooled Standard Error.

Table 17: Proximate composition of whole-body tissues of hybrid striped bass and protein conversion efficiency (PCE) after 8 weeks of feeding the experimental diets. Values are expressed as g $kg^{-1}$, unless stated otherwise

| | Moisture | Protein | Lipid | Ash | PRE (%) |
|---|---|---|---|---|---|
| Control | 688.7 | 175.0 | 94.9 | 41.0 | 43.5 |
| Vit. C | 689.3 | 179.8 | 92.2 | 41.7 | 45.8 |
| BG | 681.9 | 180.2 | 101.0 | 37.9 | 46.2 |
| BG × Vit. C | 673.9 | 185.8 | 98.5 | 40.9 | 46.7 |
| | | | | | |
| PSE | 4.45 | 3.94 | 3.66 | 1.4 | 1.27 |
| | | | | | |
| *Main effects* of Vit. C | | | | | |
| 0 g $kg^{-1}$ | 685.3 | 177.6 | 97.9 | 39.4 | 44.8 |
| 500 mg $kg^{-1}$ | 681.6 | 182.8 | 95.3 | 41.3 | 46.2 |
| | | | | | |
| *Main effects* of BG | | | | | |
| 0 g $kg^{-1}$ | $688.9^{A}$ | 177.4 | 93.5 | 41.3 | 44.6 |
| 100 mg $kg^{-1}$ | $677.9^{B}$ | 183 | 99.7 | 39.4 | 46.4 |
| | | | | | |
| *Two-way ANOVA P Values* | | | | | |
| Vit. C | 0.42 | 0.18 | 0.11 | 0.17 | 0.10 |
| BG | 0.03 | 0.21 | 0.48 | 0.21 | 0.16 |
| BG × Vit C | 0.35 | 0.91 | 0.98 | 0.43 | 0.71 |

Abbreviations: PRE: Protein retention efficiency; Vit. C: Vitamin C; BG: Beta-glucan; PSE: Pooled Standard Error;

Table 18: Immunological responses of whole blood, plasma and isolated phagocytes from hybrid striped bass fed the experimental diets for 8 weeks

| | NBT | Lysozyme | Antiprotease | Total protein | Total Ig | ICSA | ECSA |
|---|---|---|---|---|---|---|---|
| | (Abs. 545) | (U mL$^{-1}$) | (%) | (mg mL$^{-1}$) | (mg mL$^{-1}$) | (Abs. 620 nm) | ($O_2^-$ nmol mL$^{-1}$) |
| Control | 0.555$^{AB}$ | 266.6 | 23.3 | 180.5 | 96.6 | 0.220 | 14.3 |
| Vit. C | 0.534$^{B}$ | 283.0 | 20.7 | 180.5 | 99.9 | 0.248 | 18.4 |
| BG | 0.558$^{AB}$ | 416.0 | 18.7 | 187.4 | 101.4 | 0.269 | 16.0 |
| BG × Vit. C | 0.564$^{A}$ | 266.6 | 18.0 | 184.6 | 108.7 | 0.263 | 16.5 |
| PSE | 0.01 | 45.9 | 1.7 | 5.4 | 2.7 | 0.014 | 1.2 |
| *Main effects* of Vit. C | | | | | | | |
| 0 g kg$^{-1}$ | 0.556 | 341.0 | 21.3 | 183.9 | 99.9 | 0.245 | 15.2 |
| 500 mg kg$^{-1}$ | 0.549 | 275.0 | 19.3 | 184.4 | 104.3 | 0.255 | 17.4 |
| *Main effects* of BG | | | | | | | |
| 0 g kg$^{-1}$ | 0.545 | 275.0 | 22.0 | 180.5 | 98.3$^{B}$ | 0.234$^{B}$ | 16.4 |
| 100 mg kg$^{-1}$ | 0.561 | 341.0 | 18.4 | 187.4 | 105.1$^{A}$ | 0.266$^{A}$ | 16.2 |
| *Two-way ANOVA P Values* | | | | | | | |
| Vit. C | 0.01 | 0.18 | 0.06 | 0.14 | 0.09 | 0.44 | 0.10 |
| BG | 0.25 | 0.18 | 0.35 | 0.91 | 0.043 | 0.03 | 0.90 |
| BG × Vit C | 0.03 | 0.10 | 0.61 | 0.91 | 0.51 | 0.22 | 0.19 |

Abbreviations: NBT: Nitroblue tetrazolium; Total Ig: Immunoglobulin; ICSA: Intracellular superoxide anion; ECSA: Extracellular superoxide anion; Vit. C: Vitamin C BG: Beta-glucan; PSE: Pooled Standard Error.

Table 19: Immuno-related enzyme activity from different sections of the intestine of hybrid striped bass fed the experimental diets for 8 weeks

| | Alkaline phosphatase (U mg$^{-1}$ of tissue) | | | Acid phosphatase (U mg$^{-1}$ of tissue) | | | Lysozyme (U mL$^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Anterior | Medium | Posterior | Anterior | Medium | Posterior | Anterior | Medium | Posterior |
| Control | 127.6 | 104.5 | 74.3 | 299.1$^{C}$ | 250.4 | 189.1 | 141.6 | 150 | 166.6 |
| Vit. C | 106.7 | 105.9 | 89.4 | 278.0$^{C}$ | 262.1 | 189.4 | 125.0 | 150 | 116.6 |
| BG | 104.3 | 103.7 | 89.8 | 350.8$^{B}$ | 300.8 | 226.3 | 183.3 | 158.3 | 75.0 |
| BG × Vit. C | 107.1 | 82.2 | 81.7 | 398.1$^{A}$ | 281.8 | 275.1 | 266.6 | 158.3 | 100 |
| PSE | 11.4 | 8.9 | 9.4 | 10.5 | 32.5 | 14.5 | 38.9 | 22.4 | 28.9 |
| *Main effects* of Vit. C | | | | | | | | | |
| 0 g kg$^{-1}$ | 116.0 | 104.1 | 82.1 | 324.9 | 275.6 | 207.7 | 162.5 | 154.1 | 120.8 |
| 500 mg kg$^{-1}$ | 106.9 | 94 | 85.6 | 338.1 | 271.9 | 232.2 | 195.8 | 154.1 | 108.3 |
| *Main effects* of BG | | | | | | | | | |
| 0 g kg$^{-1}$ | 117.2 | 105.2 | 81.8 | 288.5 | 256.3 | 189.3$^{B}$ | 133.3$^{B}$ | 150 | 141.6 |
| 100 mg kg$^{-1}$ | 105.7 | 92.9 | 85.8 | 374.4 | 291.3 | 250.7$^{A}$ | 225.0$^{A}$ | 158.3 | 87.5 |
| *Two-way ANOVA P Values* | | | | | | | | | |
| Vit. C | 0.44 | 0.2 | 0.68 | 0.24 | 0.91 | 0.12 | 0.41 | 1.00 | 0.09 |
| BG | 0.32 | 0.29 | 0.72 | <0.0001 | 0.31 | 0.002 | 0.04 | 0.71 | 0.67 |
| BG × Vit C | 0.32 | 0.23 | 0.25 | 0.01 | 0.64 | 0.13 | 0.23 | 1.00 | 0.22 |

Abbreviations: Vit. C: Vitamin C; BG: Beta-glucan; PSE: Pooled Standard Error.

to fish fed the control and vitamin C-only diets ($P<0.001$). Supplementing BG to the diet also enhanced the ACDP in the posterior part of the intestine, and lysozyme activity in the anterior intestinal section, compared to fish fed the non-supplemented diet. No differences were observed for ACDP activity in the mid-intestine, nor for lysozyme activity in the mid or posterior intestinal sections. The dendrogram band patterns for the DGGE analysis showed that the dietary treatments did not affect the microbial community in the digestive tract with the various treatments having 100% identical populations (Fig. 13).

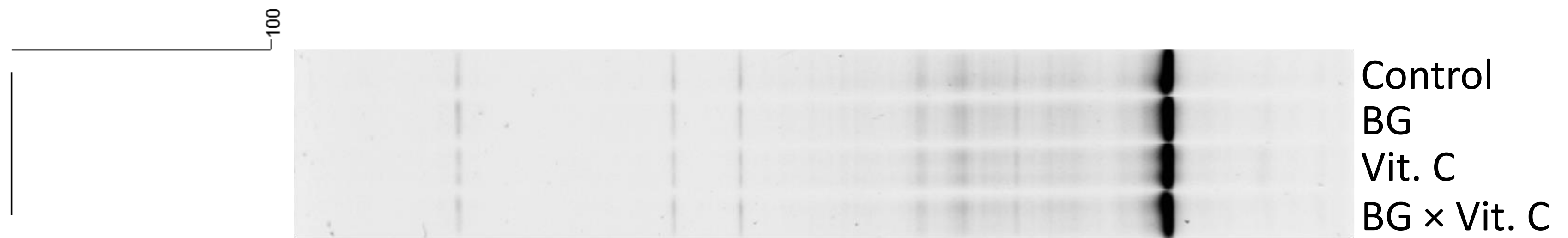


Figure 13: Dendrogram of HSB digesta microbiota after 8 weeks of feeding the experimental diets; showing identical populations (Dice percentage similarity coefficient > 95%).

5.4. Discussion

With regulations becoming more restrictive for animal production and the banishment by EU of antibiotics for use in farmed animals [16], prophylactic measures to reduce disease incidence and chemotherapeutics usage are needed [155]. Immunostimulants like BG have been examined for quite some time and applied in fish farming [14]. BG trigger fish leucocytes and prompt their immunological responses as if they were being instigated by a foreign pathogenic microorganism. However, immunostimulation can be metabolically costly and generate catabolites that cause deleterious effects to the animal. To circumvent these issues, nutrients related to health may be supplemented in the fish diet to alleviate oxidative stress or to further enhance their immunological responses [12, 156]. Data generated in the present study verified this hypothesis in a series of *in vitro* and *in vivo* assays evaluating the interaction between vitamin C and BG on the immunological responses of HSB.

The concept of BG eliciting the production of ROS by phagocytes has been very well established, regardless of the animal model or the BG source, and verified by many *ex vivo* studies [59-61, 128, 157]. The reduction of molecular oxygen to the superoxide anion by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase takes place in the phagosomal membrane of fish phagocytes, and the resulting $O_2^-$ and its derivatives products (*e.g.*, $H_2O_2$) can have bactericidal properties [57, 158]. As expected, the graded doses of BG applied to HSB isolated phagocytes increased the production of ICSA and ECSA, until a declining plateau was reached. In parallel, HSB phagocytes were incubated with graded doses of vitamin C, and an optimum concentration could only be established for ICSA. Interestingly, ECSA production diminished when graded levels of dehydroascorbic acid were added to the culture media. The pattern of the regression observed for this parameter resembled that of a previous study

conducted in our laboratory where graded doses of crude *Euglena gracilis* extract were incubated with isolated red drum (*Sciaenops ocellatus*) phagocytes [59]. In that study it was hypothesized that nutrients with antioxidant properties could have been resuspended in the cell culture medium after digesting the crude euglenoid extract, scavenging the ROS produced extracellularly. The present study utilized dehydroascorbic acid as a vitamin C source, which is the oxidized molecule of vitamin C, and a molecule that can be actively taken up by leucocytes without the mediation of membrane transporters [148]. It is possible that the layered phagocytic cells reduced the oxidized vitamin C, returning the molecule to its original state and releasing it to the culture medium, scavenging the ECSA in the same fashion as previously speculated.

A significant interaction between BG and vitamin C yielded a superior production of reactive oxygen species in the *ex vivo* studies regardless if it was incubated with the HSB isolated cells or with the leucocytes from the whole blood. Previous studies with HSB demonstrated that either the supplementation of vitamin C [34] or BG [74] could enhance the production of ROS with isolated phagocytes, and to our knowledge, this is the first time both supplements are tested *ex vivo* for an aquaculture species. Other parameters such as lysozyme activity were only affected by BG supplementation in the whole blood. When rainbow trout (*Oncorhynchus mykiss*) were fed diets with BG, increased gene expression of lysozyme genes could be observed in the head kidneys of the fish [39, 124]. Lysozyme is a mucolytic enzyme, produced by monocytes, macrophages and neutrophils, that acts on the linkage between the N-acetylmuramic acid and the N-acetyl-D-glucosamine of bacterial walls, disrupting it [125, 129]. This enzyme is an important marker for physiological stress and immune response, and it is not surprising that lysozyme secretion by circulating phagocytes was attenuated in response to a microbial component exposure, such as BGs [16]. Total plasma protein and total plasma

immunoglobulin concentrations increased when vitamin C was supplemented to HSB whole blood. The administration of a vitamin C *in vitro* enhanced the ability of human and rainbow trout lymphocytes to proliferate, which can be correlated to the findings of the present study of higher secretion of antibodies in the plasma of HSB [159, 160].

A plethora of studies reported increased growth responses when supplementing BG in the diet of farmed teleost fish [73, 75, 95, 124, 130]. In contrast to those reports, the present study showed reduced growth performance when HSB were given a diet containing 100 mg of BG derived from *Euglena gracilis* $kg^{-1}$. It is hypothesized that the immunostimulation, promoted by the pure BG used in this feeding trial, may have partitioned the dietary energy destined for growth to myelopoiesis or for leucocyte metabolism. Hence, fatty acid oxidation, amino acid, and glucose metabolism could have been directed to fuel the phagocytes for their increased metabolism or even for the proliferation of lymphocytes; therefore, limiting the accretion of these nutrients to support the growth of the fish, and ultimately impairing such. From another perspective, fish fed diets supplemented with BG had a lower moisture content in whole-body tissues when compared to the non-supplemented group, which could explain the reduced growth performance, although the mechanisms underlying this observation are not obvious. Reduced growth performance also was observed for rainbow trout fed diets with barley BG (10 g $kg^{-1}$ of diet) for 3 weeks [95], but not for 9 weeks where no differences were detected; therefore, the length of BG exposure could have an effect on this species. On the other hand, opposite results were observed by Dawood et al. [35] where red sea bream (*Pagrus major*) had higher growth performance when fed diets supplemented with vitamin C, BG, and BG + vitamin C, compared to those fed the unsupplemented control. It is noteworthy to mention that the dosages used in that trial were eight-fold higher for BG and two-fold higher for vitamin C when compared to the

present study. It remains unclear how dietary BG can be a growth promoter for fish. The reasons suggested for better performance in the previous studies included cleavage of the BGs either by microbiome-produced β-glucanases or, less likely, by endogenous synthesis of these enzymes by the fish, or by immunostimulating the intestine in a manner that would allow for clearance of enteric pathogens, which could be hampering growth performance [14]. A third hypothesis to be considered is that BG from these supplements are not actually playing a role, as the majority of the products used in these studies were manufactured from treated *Saccharomyces cerevisiae*. Perhaps, there are still remaining compounds from the yeast cell wall with growth-promoting properties, such as nucleotides or other prebiotics, that may benefit the fish and improve their growth performance.

Unfortunately, the immunological parameters collected from the *in vivo* trial in the present study did not reflect the results observed in the *ex vivo* trials. The high individual variability of the experimental units appeared to limit statistically significant differences among the dietary treatments and possible synergistic effects between them. However, it should be noted that reduced P-values were observed for all parameters that did not present statistical significance (*i.e.,* plasma antiprotease and lysozyme activities, total protein concentration, and ECSA production of isolated phagocytes). Nevertheless, reactive oxygen species produced by whole-blood circulating phagocytes of fish fed the BG plus vitamin C treatment were significantly superior when compared to the vitamin C supplemented group. Without being adequately stimulated by BG supplementation, it is predicted that fish fed the diet supplemented with vitamin C only may have had high concentrations of this nutrient in the circulating plasma, thus the reduction of phagocyted NBT particles could have been hampered by the antioxidant properties of vitamin C. This finding is in agreement with previous studies evaluating dietary

vitamin C supplementation and ROS production by teleost fish [161, 162]. Other immune parameters were affected only by the supplementation of BG in the present study. For instance, increased production of ICSA by isolated phagocytes and a higher concentration of immunoglobulins in the plasma were observed in fish fed diets supplemented with BG compared to the non-supplemented fish. An increased antibody titer also was observed in Asian catfish (*Clarias batrachus*), and rainbow trout fed BG-supplemented diets [95, 135, 163].

Acid phosphatase (ACDP) is a lysosomal hydrolase present within macrophages and other antigen-presenting cells' phagolysosomes, which can be an indicator of microbicidal activity within these phagocytes [16, 102]. In the present study, BG and vitamin C synergistically enhanced the activity of ACDP in the proximal intestine, and BG supplementation only promoted a higher ACDP activity in the posterior part of the intestine, which can suggest that either an increased number of phagocytes were present in these sections of the tissue, or the phagocytes were expressing enhanced bactericidal activity. It is possible that dietary BG supplementation may have increased the recruitment of neutrophils to these intestinal sites, hence increasing their numbers within the tissues. Macrophages harvested from mice treated with yeast and mushroom BG also had a higher acid phosphatase activity [164, 165]. Intestinal alkaline phosphatase (ALKP) can be a marker for mucous-secreting goblet cells, enterocyte maturation, and it can detoxify the endotoxin component of lipopolysaccharides from gram-negative bacteria of the gut microbiota [166-168]. However, no differences were observed for ALKP in the present study. The enhanced lysozyme activity in the anterior part of the intestine of the fish fed diets supplemented with BG may also be an indicator that an increased number of macrophages and neutrophils were recruited to this tissue.

The gut microbiota of HSB was not altered by the supplementation of BG and/or vitamin C in the present study and appeared to be essentially identical among the dietary treatments. It was anticipated that BG supplementation might affect the microbiota profile by either serving as a substrate for β-glucanase-producing bacteria, which could benefit from this carbohydrate [169], or by reducing pathogenic microorganisms by the immune enhanced cells in the gastrointestinal tissues [14]. By using the same molecular microbiota characterizing tool, similar results were observed when red drum were fed for 3 weeks diets supplemented with crude *Euglena gracilis* extract as a source of Paramylon BG [59]. On the other hand, when turbot larvae were fed rotifers and *Artemia* enriched with Macrogard®, the supplement promoted a faster development of the gut microbiota at 24 days post-hatch [170]. Alteration of the gut microbiome also was observed for *Cyprinus carpio* fed the same commercial feed supplement in three different studies [171-173]. Perhaps the method utilized in the present study had limited capacity to verify if BG derived from Paramylon truly affected the microbiota profile of HSB when it cannot detect changes in bacterial abundance. Further investigation into this topic is warranted.

In conclusion, supplementation of BG and vitamin C synergistically augmented some immunological parameters of HSB *ex vivo* and *in vivo*. However, the BG inclusion level and the length of exposure impaired growth performance of HSB, and vitamin C suplementation did not alleviate this issue. Therefore, intermittent administration of diets supplemented with this BG product and fortified with vitamin C is recommended to avoid exhausting the immune system, or to intervene by feeding such diets prior to stressful events when disease incidences are more likely to occur. Developing nutritional strategies to overcome the exorbitant use of chemotherapeutic agents in aquaculture is paramount, and more studies evaluating the potential interaction of immunostimulants with other nutrients related to health are suggested.

# CHAPTER VI

# SUMMARY AND CONCLUSIONS

## Summary

Four separate studies evaluated the immunostimulatory properties of paramylon, a β-glucan (BG) extracted from the microalgae *Euglena gracilis*, as an alternative to yeast *Saccharomyces cerevisiae* BG products derived from the brewer's industry. Results obtained with three different fish species of importance in aquaculture support the application of paramylon in short-term administration to enhance various immunological responses.

In the first study, isolated kidney phagocytes of Nile tilapia were incubated with the *Euglena gracilis* extract Algamune™ as well as with purified paramylon to gauge their ability to elicit the production of reactive oxygen species as non-specific immune responses. A linear response was observed for extracellular superoxide anion for both sources but only Algamune™ enhanced intracellular superoxide anion production. Tilapia fed diets with 200 mg of Algamune™ $kg^{-1}$ had increased extracellular superoxide anion production by isolated phagocytes. Even though the live disease challenge with *Streptococcus iniae* did not show statistical differences, it is worth mentioning that fish fed intermediate doses of Algamune™ had the lowest numerical mortality values. Therefore, Algamune™ was demonstrated to enhance some immunological responses of tilapia both in *ex vivo* and *in vivo* evaluations.

In the second study, isolated phagocytes from red drum had an increased bactericidal activity against *Streptococcus iniae*, and production of intracellular $O_2^-$ superoxide anion was stimulated by both BG sources. A reduced activity of extracellular superoxide anion was observed for phagocytes incubated with Algamune™. A comparative feeding trial evaluating

graded levels of Algamune™ in the diet for juvenile red drum was conducted, and no significant differences were detected for oxidative radical production from whole blood, or isolated phagocytes; whereas, plasma lysozyme activity and intestinal microbiota were significantly affected by the level of Algamune™. In addition, the total hemolytic activity of red drum plasma was significantly higher for fish fed 100 and 200 mg $kg^{-1}$ of dietary Algamune™ compared to fish fed the basal diet. Based on results from both *ex vivo* and *in vivo* trials, BG from Algamune™ had a moderate immunostimulatory effects on red drum.

In the third study, hybrid striped bass (HSB) fed diets containing 50 mg paramylon $kg^{-1}$ of diet had increased immunoglobulin levels in the plasma. In addition, fish fed diets supplemented with zymosan from *Saccharomyces cerevisiae* or paramylon had increased production of reactive oxygen species at two different collection points. The second part of this study evaluated the injection of BG solutions (10 mg of BG $kg^{-1}$ of body weight) with phosphate buffer solution serving as the control. Seven days after injection, blood samples were collected and immunological profiles from whole blood and plasma were significantly ($P<0.05$) affected by the different BGs. Results from this study indicated that both dietary and injected paramylon and zymosan could differently modulate the immunological responses of HSB.

In the fourth study, HSB isolated cells and tissues demonstrated a synergistic effect between BG plus vitamin C supplementation, as evidenced by enhancement of extracellular superoxide anion (ECSA) and intracellular superoxide ion (ICSA) production by isolated phagocytes and reactive oxygen species (ROS) production by circulating phagocytes in whole blood. The addition of vitamin C in the *ex vivo* experiment also increased protein and immunoglobulin concentrations in the plasma. On the other hand, BG supplementation increased lysozyme activity in the plasma. When evaluated in a comparative feeding trial, immune

responses were not as attenuated as in the *ex vivo* trial. Nevertheless, a synergistic effect between dietary BG and vitamin C was observed with regard to increasing the intestinal acid phosphatase activity. Thus, BG supplementation in the diet increased production of ICSA, immunoglobulins in the plasma, and lysozyme and acid phosphatase activities in the anterior and posterior part of the intestine, respectively. However, fish fed diets supplemented with BG had slightly but significantly impaired growth and reduced moisture in whole-body tissues compared to the non-supplemented group.

## Conclusions

In conclusion, this series of studies proved the efficacy of paramylon administration to enhance the immunological responses of different aquaculture species in short-term feeding trials. However, even though the parameters were immunostimulated by this BG, the various improved responses were likely species-specific. More research is warranted to further investigate possible interactions of BG with other nutrients related to health (*e.g.*, amino acids, vitamin E and A), with the ultimate goal of optimizing the immunological responses of fish upon BG stimulation. In addition, more studies also are suggested to clarify the administration strategies including frequency and duration of dietary BG exposure prior to or after experiencing potentially health-compromising events. In addition, there is a need to investigate the promising adjuvant ability of paramylon during vaccination to possibly decrease the mortality losses in aquaculture caused by various pathogens.

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