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Effect of β-Glucans in Diets on Growth, Survival, Digestive Enzyme Activity, and Immune System and Intestinal Barrier Gene Expression for Tropical Gar (*Atractosteus tropicus*) Juveniles

Karen N. Nieves-Rodríguez [1], Carlos Alfonso Álvarez-González [1,*], Emyr S. Peña-Marín [1,2], Fernando Vega-Villasante [3], Rafael Martínez-García [1], Susana Camarillo-Coop [1], Dariel Tovar-Ramírez [4], Laura T. Guzmán-Villanueva [4,5], Karl B. Andree [6] and Enric Gisbert [6]

1 Laboratorio de Acuicultura Tropical, División Académica de Ciencias Biológicas, Universidad Juárez Autónoma de Tabasco, Km 0.5 Carretera Villahermosa-Cárdenas Entronque a Bosques de Saloya, Centro 86039, Tabasco, Mexico; kammy526@hotmail.com (K.N.N.-R.); ocemyr@yahoo.com.mx (E.S.P.-M.); biologomartinez@hotmail.com (R.M.-G.); susana.camarillo.coop@gmail.com (S.C.-C.)
2 Cátedra Consejo Nacional de Ciencia y Tecnología, Universidad Juárez Autónoma de Tabasco, Ciudad de Mexico 03940, Mexico
3 Laboratorio de Calidad de Agua y Acuicultura Experimental, Centro de Investigaciones Costeras, Universidad de Guadalajara, Puerto Vallarta, Jalisco 48280, Mexico; fernandovega.villasante@gmail.com
4 Laboratorio de Fisiología Comparada y Genómica Funcional, Centro de Investigaciones Biológicas del Noroeste, Calle IPN 195, La Paz BCS 23096, Mexico; dtovar04@cibnor.mx (D.T.-R.); lauriddae@hotmail.com (L.T.G.-V.)
5 Cátedra Consejo Nacional de Ciencia y Tecnología, Centro de Investigaciones Biológicas del Noroeste, Ciudad de Mexico 03940, Mexico
6 Unitat de Cultius Aqüicoles, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Sant Carles de la Ràpita, Tarragona 43540, Spain; karl.Andree@irta.es (K.B.A.); enric.gisbert@irta.cat (E.G.)
* Correspondence: alvarez_alfonso@hotmail.com; Tel.: +52-993-358-1500

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Abstract: The application of β-1,3/1,6-glucan derived from yeast at five concentrations (0%, 0.5%, 1.0%, 1.5%, and 2.0%) in formulated diets was evaluated in juveniles for its effects on the growth, survival, digestive enzymatic activity, and expression of genes associated with the immune system (interlukin-10 (*IL-10*), transforming growth factor (*TGF*), occludin (*OCC*), mucin2 (*MUC2*), lysozyme (*LYS*), and nucleotide-binding and oligomerization domain 2 (*NOD2*)) in tropical gar (*Atractosteus tropicus*). For the experiment, three replicates of 30 fish per experimental unit (70 L) were cultivated for 62 days. The growth results showed no statistically significant differences in relation to weight and total length between treatments. The activity of digestive enzymes (alkaline proteases, trypsin, leucine aminopeptidase, and amylase) did not show significant differences between treatments, except for chymotrypsin activity, where fish fed 1.0% and 1.5% of β-glucans showed higher activities compared with the rest of the treatments. On the other hand, the analysis of gene expression did not show significant differences between treatments, although a tendency of increase in the expression of *IL-10*, *TGF*, *MUC2*, and *OCC* was observed with an addition of 1.5% of the prebiotic, but there was a decrease in the fish fed with 2% of the prebiotic. It is possible to include concentrations of between 0.5% and 1.5% of β-glucans in the diets for *A. tropicus*, with no detectable adverse effects on growth, survival, digestive enzyme activity, or specific gene expression. β-glucan 1,3/1,6 added at 1.0% and 1.5% in the diet significantly increases chymotrypsin activity.

Keywords: aquaculture; immune system; nutrition; prebiotics; *A. tropicus*

1. Introduction

Aquaculture in the southeast of Mexico has great potential, especially with the use of native freshwater species, significant among which is the tropical gar, or so-called pejelagarto (*Atractosteus tropicus*, Gill 1863). However, the production of this species has been limited by the lack of studies regarding the development of specific foods that allow maximizing their growth and survival and increasing production on a commercial scale [1]. For this reason, several studies on *A. tropicus* have focused on maximizing the production performance of the species [2], developing balanced foods based on digestive physiology [3], and improving the growth and utilization of nutrients [4,5]. On the other hand, health impacts due to outbreaks of diseases under culture conditions generate large economic losses [6]; therefore, to reduce this impact, the use of immunostimulants could enhance the immune response and increase resistance of the organism against infectious diseases [7]. In this regard, β-glucans are one of the prebiotics with greater interest in the area of aquaculture. Specifically, the β-1,3/1,6-glucan from the cell walls of yeast (*Saccharomyces cerevisiae*, Hansen 1883) is one the most studied prebiotics, and is composed of a heterogeneous group of glucose polymers consisting of a backbone of β-1,3-linked β–D–glucopyranosyl units with β-1,6-linked side chains of varying distribution and length. Such chemical structures are abundant in microbial communities and can be termed pathogen-associated molecular patterns (PAMP), which play a role as alarm molecules to activate the immune system [8] via their binding to pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), scavenger receptors (SR), and dectin-1, which allow the activation of the inflammatory response and the synthesis of cytokines [8–10]. These receptors activate leukocytes directly to stimulate phagocytosis, cytotoxic and antimicrobial activity, and also to modulate the production of cytokines that control networks responsible for innate immune responses [11], promote growth [12,13], improve survival [14,15], stimulate antibody production [16], and modulate expression of genes related to various immune system pathways [17].

Studies conducted on different fish species, such as Atlantic salmon (*Salmo salar*, Linnaeus 1758) [18], rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) [15,19,20], spotted rose snapper (*Lutjanus guttatus*, Steindachner 1869) [21], common carp (*Cyprinus carpio*, Linnaeus 1758) [16,22,23], roho labeo (*Labeo rohita*, Hamilton 1822) [13,24], gilthead sea bream (*Sparus aurata* Linnaeus 1758) [25], sea bass (*Dicentrarchus labrax*, Linnaeus 1758) [26,27], Nile tilapia (*Oreochromis niloticus*, Linnaeus 1758) [28], silver seabream (*Pagrus auratus*, Forster 1801) [12], large yellow croaker (*Pseudosciaena crocea*, Richardson 184) [29], and southern bluefin tuna (*Thunnus maccoyii*, Castelnau 1872) [30] have reported that β-glucans act by modulating different parameters of the immune response and increasing resistance to diseases [8,12]. Therefore, the objective of the present study was to determine the effect of different oral doses of β-1,3/1,6-glucan on growth, survival, digestive enzymatic activity, and the expression of several genes associated with the immune system and the intestinal barrier function in juveniles of the tropical gar (*A. tropicus*).

2. Results

The tropical gar were fed diets supplemented with β-1,3/1,6-glucans at different concentrations (0%, 0.5%, 1.0%, 1.5% and 2.0%) for 62 days. No significant differences were observed in survival or growth variables, such as final weight, total length, specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (K) values (Table 1).

Table 1. Survival and growth parameters of *A. tropicus* fed formulated diets with different concentrations of β-1,3/1,6 glucans (0%, 0.5%, 1.0%, 1.5%, and 2.0%).

Growth Parameters		Treatments (β-1,3/1,6 Glucans)				
		0%	0.5%	1.0%	1.5%	2.0%
Total length (mm)	Initial	4.60 ± 0.11	4.64 ± 0.06	4.62 ± 0.09	4.64 ± 0.09	4.65 ± 0.06
	Final	12.10 ± 0.19	12.13 ± 0.17	12.35 ± 0.73	12.52 ± 0.87	12.27 ± 0.29
Weight (g)	Initial	0.47 ± 0.03	0.46 ± 0.06	0.44 ± 0.07	0.52 ± 0.03	0.52 ± 0.09
	Final	5.64 ± 0.49	5.40 ± 0.17	5.91 ± 1.11	6.14 ± 1.49	5.76 ± 0.29
Survival (%) [1]		96.9 ± 2.69	93.1 ± 1.53	95.3 ± 3.52	91.3 ± 2.90	95.6 ± 5.09
SGR (% day^{-1}) [2]		4.75 ± 0.29	4.74 ± 0.28	4.99 ± 0.09	4.69 ± 0.33	4.79 ± 0.02
FCR [3]		0.31 ± 0.02	0.30 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	0.30 ± 0.01
K [4]		326.4 ± 27.0	302.6 ± 32.3	313.8 ± 30.4	312.9 ± 26.9	311.8 ± 10.9

[1] Survival (%): (final fish number/initial fish number) × 100; [2] Specific growth rate (SGR): [(ln final weight-ln initial weight)/days] × 100; [3] Feed conversion ratio (FCR): (feed intake, g dry matter)/(fish weight gain, g); [4] Condition factor (K): (final mean body weight/final mean body length^3) × 100.

The results of the enzymatic digestive activities showed a significant increase ($p < 0.05$) for chymotrypsin activity in fish fed with glucans at 1.0% and 1.5% (8.97 ± 2.40 and 8.30 ± 1.02 U mg protein^{-1}, respectively) as compared to the other diets. However, total alkaline proteases, trypsin, leucine aminopeptidase, lipase, and α-amylase did not show significant differences between the different treatments (Table 2).

Table 2. Digestive enzymatic activity in *A. tropicus* fed formulated diets supplemented with β-1,3/1,6 glucans at different concentrations (0%, 0.5%, 1.0%, 1.5%, and 2.0%).

Enzymatic Activity (U mg Protein^{-1})	Treatments (β-1,3/1,6 Glucans)				
	0%	0.5%	1.0%	1.5%	2.0%
Total alkaline proteases	1.26 ± 0.43	1.34 ± 0.23	0.86 ± 0.23	1.03 ± 0.23	0.71 ± 0.42
Trypsin	4.06 ± 0.32	3.76 ± 2.99	1.78 ± 1.13	2.90 ± 1.54	2.80 ± 1.05
Leucine peptidase	5.86 ± 3.47	8.38 ± 2.75	7.18 ± 3.08	5.56 ± 2.19	5.14 ± 1.63
Chymotrypsin *	1.14 ± 0.74 ^{b}	1.12 ± 0.65 ^{b}	8.97 ± 2.40 ^{a}	8.03 ± 1.02 ^{a}	1.21 ± 1.04 ^{b}
Lipase	2.52 ± 0.15	2.83 ± 0.08	2.78 ± 0.34	2.84 ± 0.25	2.50 ± 0.16
α-amylase	0.66 ± 0.17	3.90 ± 2.36	6.78 ± 3.30	9.00 ± 5.44	7.80 ± 1.03

* The superscripts a and b show the statistically significant differences between treatments ($p < 0.05$).

Regarding gene expression results, data analysis revealed that there were no statistically significant differences among groups ($p > 0.05$), which was mainly attributed to a high inter-individual variability. However, a trend was observed in the data related to the increase of dietary β-glucans. This trend is summarized as follows: in the case of interlukin-10 (*IL-10*), it was noted that the treatment of β-glucans at 2.0% was greater compared to the other treatments with lower concentrations of β-glucans. The expression of transforming growth factor (*TGF*) in the treatment with 0.5% of β-glucans was lower compared to the treatments with higher prebiotic inclusion. While the expression of occludin (*OCC*) showed a higher value in the fish fed with the lowest concentration (0.5%), decreasing expression was found in the treatments with greater amount of the prebiotic. Considering mucin2 (*MUC2*) expression, there was a clear tendency to increase its expression as the concentration of β-glucans increased up to 1.5%; nevertheless, higher concentration of β-glucans (2.0%) decreased the relative expression considerably (Figure 1).

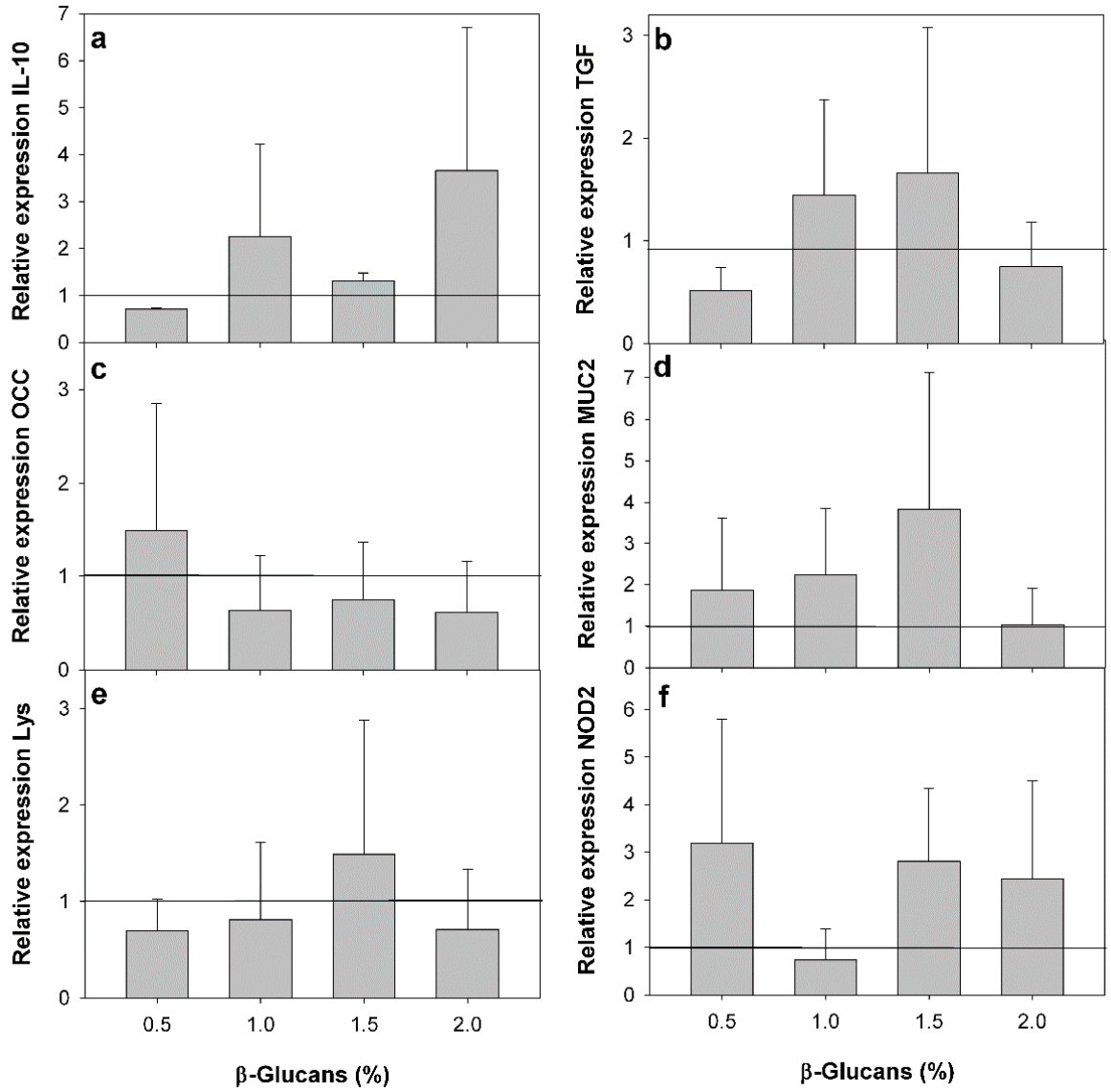

Figure 1. Box-plot of the relative expression different molecular markers from the intestinal tissue (median ± quartile 10%, n = 3) of *A. tropicus* juveniles normalized to the average of Elongation factor 1 (*EF1*) and β-actin expression. (**a**) Interlukin-10 (*IL-10*); (**b**) transforming growth factor (*TGF*); (**c**) Occludin (*OCC*); (**d**) Mucin2 (*MUC2*); (**e**) Lysozyme (*Lys*); (**f**) nucleotide-binding and oligomerization domain 2 (*NOD2*) (K-W, $p > 0.05$).

3. Discussion

Within fish culture systems of the aquaculture industry there are sources of stress (crowding, handling, transport) that put animals at higher risk of infectious disease as a consequence of the negative effects of increased cortisol levels resulting from the stressful conditions [16]. The ensuing diseases generate great economic losses during culture; therefore, the use of immunostimulants has increased in the aquaculture industry [21], with the prebiotic β-1,3/1,6-glucan being one of the most frequently used [31]. Under present experimental conditions, the incorporation of this prebiotic in balanced feeds designed for *A. tropicus* did not result in an improvement of the somatic growth parameters (body weight, length, SGR, FCR, and K). Many studies indicate that β-glucans promote the growth of aquatic animals in relation to the amount included in the feed, the duration of feeding, culture temperature, type of β-glucans, and species being raised [32]. However, the exact mechanism

of how β-glucans promote growth in aquatic animals is not clear. Some authors suggest that somatic growth is enhanced due to the production of glucanase that decomposes β-glucans to generate energy [33,34], while others by the improvement of the intestinal immune response and indirectly promoting growth [8]. Nevertheless, it is known that this type of prebiotic does not necessarily promote an increase in somatic growth, which is consistent with the results obtained in several studies in turbot (*Scophthalmus maximus*, Linnaeus 1758) [35], *O. mykiss* [36], and cod (*Gadus morhua*, Linnaeus 1758) [37], where no differences were reported in the growth parameters, but there were differences in parameters related to the improvement of the immune system (see review in Reference [38]). In addition, under current experimental conditions, the incorporation of the prebiotic did not increase the survival among any of the treatments when compared to the control without the compound, but it must be mentioned that the survival was very high (>90%), which agrees with results reported for Persian sturgeon (*Acipenser persicus*, Borodin 1897) [39] and *O. niloticus* [40].

Regarding the activity of digestive enzymes of the tropical gar fed with β-glucans, there was a decrease in chymotrypsin-like activity that correlated inversely with the concentration of β-glucan in the diet, whereas no differences in activity were found for the other assayed digestive enzymes. These results differed from data for the Pacific red snapper (*Lutjanus peru*, Nichols and Murphy 1922) [10], where the increase of β-glucans resulted in an increase in the activity of aminopeptidase, trypsin, and chymotrypsin, using concentrations between 0.1% and 0.2%. These differences between current data and those obtained in *L. peru* might be explained by differences in the doses of β-glucans tested in both species. In the former study, doses were lower in comparison to those used in this work; there are also differences in the digestive physiology between marine species and freshwater tropical fish. Therefore, it may be possible that the high amounts of β-glucans used in the present study (2.0%) could have contributed to a saturation of amino acid transporters that could promote a possible inhibition or inactivation of the digestive enzymes, as well as the possible alteration of the gut microbiota; however, there are few studies that evaluate what effects an excess of β-glucans has on the digestive capacity of fish [41]. The different effect β-glucans have on alkaline proteases, trypsin, and chymotrypsin ratio deserves further attention, as well as the potential role of this prebiotic on enhancing the digestive function in fish [42].

It is well known that β-glucans directly activate leukocytes, stimulate phagocytosis, cytotoxic, and antimicrobial activity, in addition to modulating the production of cytokines, which are responsible for controlling immune responses [11]. In relation to the cytokines evaluated in this work, *TGF* binds to its cognate receptor as part of inflammatory responses triggering a cascade of transcription that leads to cell differentiation, chemotaxis, proliferation, and activation of many immune cells [43] and *IL-10* is responsible for decreasing and regulating the inflammatory responses produced by cells, as well as reducing the adaptive responses of T cells [44]. Studies in fry [45] and juveniles of *O. mykiss* [46] have reported β-glucans increase cytokine levels and mRNA expression of the *IL-10* transcription factor, TGF-β, which was not in agreement with the results obtained in the present work. This may be due to the high interindividual variability and the administered concentration of glucans in the diet. Different studies show that concentrations higher than 0.5% of β-glucans have no effect on gene expression in different fish species [8]. Further, the effect of this immunostimulant is species-dependent and effects can vary with the route of administration (oral versus intraperitoneal injection).

The use of β-glucans has also been shown to improve physical barriers of the intestine, which are the first lines of immune defense of the host [47]. The mucus layer coating the intestinal mucosa is formed mainly by mucins, high molecular weight glycoproteins that help to form the composition of the mucus, determine its adhesiveness, viscoelasticity, transport, and protective capacity [48]. Herein, it was reported that the expression of *MUC2* increased directly with the increase of β-glucans in the diet, which might suggest that the ability to regulate the expression of mucin is a benefit for responding to pathogens. However, a decrease in expression was observed in *A. tropicus* fed 2.0% β-glucans in the diet, which indicated that high concentrations of this immunostimulant inhibited the expression of *MUC2* (a quadratic response), which is in accordance with reports in *C. carpio*, where the increase in

the mucus layer, rich in oligosaccharide-modified glycoproteins was observed with the administration of this immunostimulant [49]. Likewise, studies in mammals, evaluating galactooligosaccharides and fructooligosaccharides, reported a positive effect on the mucin content in the intestinal mucosa [50,51]. In this way, it is known that mucins have the ability to recognize foreign particles and bodies to give way to defensive responses, such as increased mucus lining the lumen of the intestine, which, in mice, leads to greater bacterial adhesion to the gut surface epithelium and, consequently, alterations in intestinal permeability [52,53].

As mentioned above, the intestine of fish principally performs hydrolysis of macromolecules by the action of digestive enzymes and the absorption of nutrients. In addition, the intestine functions as an immunological barrier through humoral immune factors [54]. In this way, the intestinal epithelium is characterized by a high permeability to paracellular ions, which is determined by properties of tight junctions [55]. Thus, paracellular permeability is controlled by membrane proteins, such as claudins, occludins, and tricellulines that support the exchange of chloride ions and maintain integrity of membrane proteins, among other factors [56]. This is consistent with results of the present study, where an overexpression of the *OCC* gene with 0.5% β-glucans was observed. However, the expression decreased as the percentage of β-glucans in the feed increased, which suggests a negative consequence on the permeability of the cell membrane under high doses of beta glucans. These results were also reported in the herbivorous carp (*Ctenopharyngodon idella*, Valenciennes 1844) [57] and *Scophthalmus maximus* [58]. Therefore, expression of *OCC* in the intestine can be modified via feed formulations to improve the function of the cellular barrier against possible pathogens. In studies conducted on goldfish (*Carassius auratus*, Linnaeus 1758) [59], it has been reported that a low expression of *OCC* can affect the permeability of transmembrane junctions, causing a decrease in the intestinal epithelium that potentially lowers the resistance to pathogens by the redistribution of tight junction proteins, thereby altering the correct functioning of the intestinal barrier. However, the concentrations used in this study did not show an apparent negative effect on the health of the fish.

4. Materials and Methods

The present study was carried out in the facilities of the Tropical Aquaculture Laboratory in the Academic Division of Biological Sciences of the Universidad Juárez Autónoma de Tabasco (DACBIOL-UJAT). A total of 450 juveniles of *A. tropicus* (average total length 4.63 ± 0.07 mm and weight 0.49 ± 0.04 g) were obtained from the induced spawning of one female (3.5 kg) and three males (1.5 kg) by means of an intramuscular injection of luteinizing hormone-releasing hormone analogue (Ovaprim, Syndel, Ferndale, WA, USA, 35 µg) kg of fish^{-1} in the pelvic fin area. After egg hatching (day 3 post-fertilization), the eleutheroembryos were placed in circular plastic tanks of 70 L and a feeding scheme was followed according to previous reports [60], which started after the absorption of the yolk sack (two days after hatching [DAH] to 17 DAH), where *Artemia* nauplii were provided (2–5 nauplii mL^{-1}) and from 18 to 20 DAH co-feeding was performed with trout feed (TD, Silver Cup, 45% protein and 16% lipids) and frozen *Artemia nauplii* and finally from 21 DAH were provided with formulated feed using the appropriate granulometry in relation to the size of the mouth of the larvae.

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (NOM-062-ZOO-1999) on 18 June 2001.

4.1. Experimental Design and Diets

Nutritional supplementation of β-1,3/1,6-glucan (Macrogard, derived from yeast *S. cerevisiae*) in the following levels was evaluated and designed as: D1, 0%; D2, 0.5%; D3, 1.0%; D4, 1.5%; and D5, 2.0%. The treatments were carried out in triplicate with 30 fish per experimental unit distributed in 15 circular plastic tanks of 70 L coupled to a recirculation system that consisted of a 1500 L reservoir that functions as a solidifier and a biological filter. The fish were fed five times a day (7:00, 10:00, 12:00, 14:00, and 16:00 h) and partial water replacement (10%) was made by siphoning daily.

The diets were designed as described by Frías-Quintana [4] with the support of package MIXIT-WIN v5.0 (Agricultural Software Consultants, Inc. San Diego, CA, USA) for diet formulation (Table 3). The ingredients were sieved (<150 microns) and mixed for 30 min in a rotating blender (CRT Global® Mix-B30GA, Nuevo León, Monterrey, Mexico), then added to the mixture of 35%–40% water and then pelletized (5 mm diameter) in a meat grinder (TORREY® Model M-22R1, León, Guanajuato, Mexico). The pellets were cut manually and dried in an oven (CORIAT® Model HC-35-D, Ciudad de Mexico, Mexico) at 37 °C for 12 h. Subsequently, they were milled and sieved to homogenize the particle size of the diets. All feeds were kept frozen at −20 °C until use.

Table 3. Chemical composition of the formulated diet for juveniles of *A. tropicus*.

Ingredients (g 100 g Dry Matter)	
Feed grade Poultry by-products meal [a]	42.00
Pork meal [a]	11.60
Soybean meal [b]	4.60
Corn starch [c]	15.40
Sardine oil [a]	3.00
Fish protein hydrolizate [a]	10.00
Soy lecithin [d]	3.60
Shrimp meal [a]	3.00
Bovine blood meal [a]	0.00
Grenetin [e]	2.00
Vitaminic premix [f]	1.00
Mineral premix [f]	0.50
Vitamin C [g]	0.08
Sorghum meal 8%–10% [b]	3.09
Chemical composition (% dry matter, except moisture. Mean ± SD)	
Energy (KJ g^{-1})	21.549 ± 0.04
Crude protein (%)	40.6 ± 0.20
Ether extract (%)	16.1 ± 0.00
Fiber (%)	1.1 ± 0.10
Ash (%)	10.8 ± 0.00
NFE (%) *	31.50
Moisture (%)	4.0 ± 0.10

[a] Proteínas Marinas y Agropecuarias, Guadalajara, Jalisco, México; [b] GALMEX Comercializadora de Insumos Agrícolas, Villahermosa, Tabasco, México; [c] MSA Industrializadora de Maíz, Guadalajara, Jalisco, México; [d] Pronat Ultra, Mérida, Yucatán, México; [e] D'gari Productos alimenticios y dietéticos Relámpago, Tlalpan, Edomex, México; [f] Consorcio Súper Sociedad Anónima de Capital Variable. (for trout by courtesy), Guadalajara, Jalisco, México; [g] DSM (Heerlen, Netherlands, active agent 35%). * NFE = Nitrogen free extract, calculated as 100 − (%Protein + %Ether extract + %Ash + %Fiber).

4.2. Sampling and Preparation of Multienzymatic Extracts

At the end of the experiment, the anterior intestine of six juveniles was removed (two fish per tank) in order to perform digestive enzyme activity tests; tissue samples were homogenized in a volume 1:10 with Tris-HCl 50 mmol L^{-1} buffer solution, pH 7.5. Subsequently, samples were centrifuged (16,000× *g* for 15 min at 4 °C), the supernatant was recovered, distributed in aliquots of 500 µL and frozen at −80 °C until use. The concentration of soluble protein was evaluated by means of the Bradford method [61] using as standard a bovine serum albumin curve.

Total alkaline protease activity was measured using 1% casein as a substrate in 100 mmol L^{-1} Tris-HCl buffer, 10 mmol L^{-1} $CaCl_2$ (pH = 9.0); the reaction was stopped with trichloroacetic acid (TCA, 10%). The unit of enzymatic activity was defined as 1 µg of tyrosine released per minute, based on the molar extinction coefficient (MEC) of 0.005 mL μg^{-1} cm^{-1} [62]. The chymotrypsin activity was determined using as substrate BTEE (*N*-benzoyl-L-tyrosine ethyl ester) 5 mmol L^{-1} in buffer Tris-HCl 44.4 mmol L^{-1} + $CaCl_2$ 55.5 mmol L^{-1} (pH = 7.8). One unit of enzymatic activity was defined

as 1 mole of BTEE hydrolyzed per minute with an MEC of 964 cm^{-1} M^{-1} [63]. The trypsin-like activity was determined using BAPNA (*N*α-Benzoyl-DL-Arginine-*P*-nitroanilide) as substrate, where a unit of enzymatic activity was defined as 1 µmol of p-nitroanilide released per minute with an MEC of 8800 cm^{-1} M^{-1} [64]. The leucine aminopeptidase activity was determined by using 1 mmol L^{-1} L-leucine p-nitroanilide (0.01 mmol L^{-1}) in Dimethyl sulfoxide (DMSO) as a substrate in 50 mmol L^{-1} sodium phosphate buffer at pH 7.2. A unit of enzymatic activity was defined as 1 µmol of p-nitroanilide released per minute with a MEC of 8800 cm^{-1} M^{-1} [65]. The α-amylase activity was determined using starch as substrate in citrate-phosphate buffer 100 mmol L^{-1}, 50 mmol L^{-1} NaCl. A unit was defined as the amount of enzyme that releases 1 µg of maltose per minute [66]. The lipase activity was determined using as substrate β-naphthylcaprylate (200 mmol L^{-1}) dissolved in a 50 mmol L^{-1} Tris-HCl buffer solution (pH = 7.2) [67]. The lipolytic activity is defined as 1 µg of naphthol released per minute with a MEC of 0.02 cm^{-1} M^{-1}.

4.3. Growth, Survival and Food Quality Indexes

For the evaluation of β-glucan supplementation among treatments, at the end of the experiment growth, mean weight (g) and total length (mm), survival (%), SGR, FCR, and K were calculated for each replicate.

4.4. Gene Expression

For the assessment of the impact of β-glucan on the expression of selected genes involved in gut immunity and condition, the anterior intestine of five juveniles of *A. tropicus* was obtained from experimental tanks from each treatment (0%, 0.5%, 1.0%, 1.5%, and 2.0% of β-1,3/1,6-glucan). The samples were collected after a 24 h fasting period and preserved in RNAlater (volume 1:10) then stored at −80 °C until use. The total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of the RNA was determined by spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain) by measuring the absorbance at λ = 260 and 280 nm. RNA quality was verified by visualization of the 28S and 18S ribosomal RNA bands by 2% agarose gel electrophoresis. For the preparation of cDNA, the total RNA was treated with DNase 1, (AMPD1-1KT, Sigma-Aldrich, Broendby, Denmark), according to the manufacturer's instructions to eliminate possible contaminating genomic DNA. In a 20 µL volume, 2 µg of total RNA was reverse transcribed in a reaction containing SuperScript II™ reverse transcriptase (Invitrogen), oligo (dT) (0.5 µg μL^{-1}) and random hexamer primers (50 ng μL^{-1}). The reverse transcription reactions were prepared in 0.2 mL tubes following the SuperScript II First Strand cDNA Synthesis kit protocol (Invitrogen), then placed in a thermal cycler (Mastercycle® nexus GSX1, Eppendorf AG, Hamburg, Germany) and run for 5 min at 65 °C, 10 min at 25 °C (primer annealing), 50 min at 42 °C (cDNA strand extension), 15 min at 70 °C (Reverse Transcrptase inactivation), and finally 20 min at 37 °C (RNase H treatment). Negative controls (without RT enzyme) were included to confirm the absence of genomic DNA contamination. The samples were diluted 1:10 in water of molecular biological grade for analysis. To confirm the quality of all cDNA samples, duplicates of each sample were amplified using the endogenous control gene Elongation factor 1 (*EF1*) to establish that all samples had similar C_t values. The cDNA was stored at −20 °C until further analysis.

The target gene sequences of *A. tropicus* were obtained from a previous study [68] in which pertinent genes related to gut health had been identified. The specific genes tested were the cytokines *IL-10* chosen for their role in specific immune cell stimulation and *TGF* chosen as a proinflammatory gene involved in triggering cellular differentiation. Nucleotide oligomerization domain 2 (*NOD2*) was chosen as a first responder in the innate immune response for pathogen antigen recognition, while *LYS* is a functional effector of cell lysis for the innate immune response. Occludin (*OCC*) was chosen as a marker of intercellular junctions that affect gut epithelial permeability and *MUC2* as a marker of mucus production and mucosal layer development. The design of specific primers for the amplification from

cDNA for gene expression analysis was done manually using BioEdit (Ibis Therapeutics, Carlsbad, CA, USA) [69].

4.5. Real Time Chain Polymerase Reaction (RT-qPCR)

The qPCR relative expression analyses were carried out in triplicate using a CFX96® Real-Time System (BioRad, Hercules, CA, USA). For each sample a master mix was prepared containing: 9.25 µL SYBR Green Supermix (Applied Biosystems, Foster City, CA, USA), 0.625 µmol L^{-1} of each primer and 2 µL of cDNA in a final volume of 25 µL. The q-PCR cycle was performed as follows: 10 min at 95 °C, 45 cycles of 95 °C for 25 s, followed by a recognition stage of 65 °C for 20 s (the annealing temperatures were adjusted to each pair of primers), followed by 72 °C for 15 s, with a final melting curve stage of increments of 0.5 °C to 75 °C to 95 °C. The amplification efficiency (E%) for each pair of primers was evaluated to confirm the utility of each assay using five sets of ten-fold dilutions of tissue cDNA. The E% calculation followed the equation: $E\% = [10^{(-1/\text{slope})}] - 1$, where the "slope" is calculated from the regression line of the standard curve. *EF1* and β-actin (*BAC*) were used for the endogenous control reference genes.

4.6. Statistical Analysis

For the evaluation of fish growth and for gene expression analysis, a nonparametric test of Kruskal–Wallis and a posteriori Nemenyi tests were used. Survival data, food quality indexes, and enzymatic activity complied normality (K-S) and homoscedasticity (Levene) postulates, therefore were analyzed by one-way Analysis of Variance (ANOVA). For all tests, a significance of 0.05 was used, using the statistical program Statgraphics Centurion XVI® (Statgraphics Technologies, Inc., The Plains, VA, USA).

5. Conclusions

In our study, according to the data obtained from *A. tropicus* juveniles, the treatments tested that included β-glucans in the diet seem to improve growth, survival, digestive enzyme activities, some immune system responses, and the integrity of the intestinal barrier. In this sense, the effect of β-glucans in different fish species depends on the dosage, duration of feeding with the additive, and the stage of life of the organism. For this reason, we should consider to evaluate a lower dose of β-glucan (<0.5%), and to determine changes in cellular morphology through histological techniques and perform challenges with pathogenic bacteria with the objective of increasing knowledge about the effect of β-glucans prebiotics on ancestral teleosts.

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