



Addition of β -glucans in diets for tropical gar (*Atractosteus tropicus*) larvae: effects on growth, digestive enzymes and gene expression of intestinal epithelial integrity and immune system

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Received: 9 January 2023 / Accepted: 24 May 2023 / Published online: 13 June 2023
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Abstract The effect of β -glucans 1,3/1,6 from *Saccharomyces cerevisiae* yeast at different inclusion percentages (0.0, 0.2, 0.4, 0.6, and 0.8%) in the diet for tropical gar (*Atractosteus tropicus*) larvae was evaluated on growth, digestive enzyme activity and, relative expression of the immune system genes. The bioassay started on the third day after hatching (DAH) and lasted 21 days, using a total of 1500 larvae of 0.055 ± 0.008 g and, a total length of 2.46 ± 0.26 cm. Larviculture was carried out in a recirculation system with 15 tanks of 70 L using a density of 100 organisms per experimental unit. No significant differences in larval growth were observed by the inclusion of β -glucans ($p > 0.05$). Digestive enzymes showed changes in lipase and trypsin

activities, presenting higher values in fish fed 0.6% and 0.8% β -glucans diets compared to the other treatments ($p < 0.05$). Leucine-aminopeptidase, chymotrypsin, acid phosphatase, and alkaline phosphatase activity showed higher activities in larvae fed with a 0.4% β -glucan diet compared to the control group. The relative expression of intestinal membrane integrity (mucin 2) *muc-2*, (occludins) *occ*, (nucleotide-binding oligomerization domain) *nod-2*, and immune system *lys* (lysosome) genes showed over-expression in larvae fed the 0.4% β -glucan diet to the rest of the treatments ($p < 0.05$). The inclusion of β -glucans at 0.4–0.6% in diets for *A. tropicus* larvae could improve larviculture, as effects on the increase in the activity of several digestive enzymes and the expression of genes of the immune system.

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Keywords β -glucans · Prebiotic · Tropical gar · Digestive enzymes · Gene expression

Introduction

Currently, there is a market focused on improving the health and survival of fish by adding various types of prebiotics to the formulations. In fish, the most commonly used prebiotics have been GOS (galactooligosaccharides), MOS (mannan oligosaccharides), FOS (fructooligosaccharides), scFOS (short-chain fructooligosaccharides), XOS (xylooligosaccharides), AXOS (arabinooligosaccharides) and inulin, as well as their mixes (Guerreiro et al. 2017). In addition, depending on the sources of the prebiotics, we find different beneficial attributes, such as fat and sugar substitution, improved palatability through tongue lubrication, and even improved immune response (Singla and Chakkaravarthi 2017; Khangwal and Shukla 2019). The polysaccharide β -glucan (1,3/1,6) is one of aquaculture's most widely used immunostimulants (Miest et al. 2016). This prebiotic is classified as a dietary fiber with particularities to improve the innate immune response; at the cytokine level, it promotes better growth and tolerance to stress, improving survival rates (Wood 2007; Pizarro et al. 2014; Dos Santos-Voloski et al. 2019; Revina et al. 2020).

Thus, β -glucans are a valuable and functional ingredient with essential properties at the physiological level, one of the main characteristics of β -glucans from the yeast *Saccharomyces cerevisiae* is the immunostimulant functions (Wilson et al. 2015). The effects of β -glucans have been evaluated in fish such as *Danio rerio* inoculated with the pathogen *Aeromonas hydrophila*, where their effect decreased mortality (Rodríguez et al. 2009), by stimulating β -glucans proteins such as chemokines and cytokines, which are involved in the process of attraction and migration of leukocytes involved as antigens (Nunes et al. 2016). Likewise, the addition of β -glucans increases the expression of the IFN- γ (Interferon-gamma) gene, which plays an important role in the innate immune response (Xu et al. 2019). On the other hand, the *muc-2* gene is a mucin releaser to allow the maintenance of the gastric system regarding its lubrication by creating a barrier of protection and permeability against pathogens (Pelaseyed et al. 2014; Xue et al. 2015). Also, the occludins are membrane proteins with a hermetic function at the cytoplasmic level and allow the cellular reorganization of actin, which enables movement functions, cell division, phagocytosis,

and external activities (García & Jay 2006; Edelblum & Turner 2015; Kuo et al. 2019). The *nod-2* gene (Nucleotide-binding Oligomerization Domain Protein) is part of the intracellular family, expressed in the epithelial region, specifically in Paneth cells involved in stem cell function, intestinal morphogenesis, and mainly for being antimicrobial agents in the microbiota region (Caruso et al. 2014; Gassler 2017). On the other hand, lysozyme is part of the community of proteins associated with the innate immune system, produced from neutrophils and macrophages, which act as receptors of the NOD family for the degradation of the bacterial cell wall (Ragland and Criss 2017; Kawai et al. 2018).

On another note, in southeastern Mexico, there are native fish with the potential for aquaculture. Among them, the tropical gar (*Atractosteus tropicus*) is one of the essential freshwater species in the State of Tabasco. This species inhabits freshwater bodies with abundant vegetation (Miller et al. 2005). It is a culturally valued specimen, has a high productive potential in the region, and is an important biological model (Márquez-Couturier et al. 2006; Méndez-Marin et al. 2012; Vázquez-Navarrete 2015). Most of the work has focused on nutrition to develop specific feeds, such as the determination of protein and lipid requirements, the design of micro-encapsulated diets, live food substitution, and digestive physiology, among others (Frías-Quintana et al. 2016; Sáenz de Rodrigáñez et al. 2018; Huerta-Ortiz et al. 2018; Jiménez-Martínez et al. 2020). Recently, we began to explore the evaluation of functional additives in diets for *A. tropicus* juvenile by including β -glucans (1,3/1,6), where the inclusion between 1.0 and 1.5% improves growth, the response of immune system genes and increases the activity of alkaline proteases, trypsin, leucine aminopeptidase, and α -amylase (Nieves-Rodríguez et al. 2018). Also, FOS as another of the selected additives in aquaculture, improving enzymatic activity (acid proteases, chymotrypsin, and leucine aminopeptidase), the expression of intestinal barrier genes (*occ*, *muc2*, *nod2*, *β -actin*, *efl-a*) and the modification of morphology in the intestinal barrier, from 5 g kg⁻¹ for the juvenile stage (Sepúlveda-Quiroz et al. 2020). Therefore, due to the potentiating effects it has achieved for the species, some others, such as MOS, have been tested with favorable results in growth rates, digestive enzymatic activity, and immune markers (Nájera-Arzola et al. 2018). Accordingly, the aim of the present work was to evaluate the effects on

growth, digestive enzyme activity, and the expression of membrane integrity and immune system genes on *A. tropicus* larvae by the inclusion of different concentrations of dietary β -glucans (1,3/1,6).

Materials and methods

Obtaining organisms

The experiment was carried out in the Laboratory of Physiology in Aquatic Resources (LAFIRA) from the División Académica de Ciencias Biológicas (DACBIOL) at Universidad Juárez Autónoma de Tabasco (UJAT). The organisms for the experiment were obtained from a batch of *A. tropicus* broodstock available in the laboratory (one 3.8 kg female and five 1.5 kg males), spawning occurred by inducing it with the synthetic hormone GnRH (Synchrophorte) applied intramuscularly (using 35 $\mu\text{g}\cdot\text{kg fish}^{-1}$).

Experimental design

In this experiment, the effect of β -glucans at inclusion percentages of 0%, 0.2%, 0.4%, 0.6%, and 0.8% (control diet; diet 0.2, diet 0.4, diet 0.6 and diet 0.8, respectively) on *A. tropicus* larvae was evaluated. The bioassay was carried out for 21 days (24 DAH) from hatching (3 DAH), and a total of 1500 organisms (100 fish per experimental unit by triplicate) were used. Larval feeding was carried out five times a day with apparent satiety (8:00, 10:30, 12:30, 15:30, and 18:30 h), according to the feeding scheme proposed by Frías-Quintana et al. (2015). The experiment was carried out in a recirculation system connected to 15 tanks of 70 L with a constant flow of 10 L min^{-1} and a biological filter. Water quality was monitored every day with a HANNA multiparameter probe (HI9829-00041), measuring temperature (30.6 ± 0.14 °C), pH (7.1 ± 0.04) and dissolved oxygen (5.4 ± 0.34 mg L^{-1}). In addition, the surplus feed was siphoned off at the end of the day, and every 48 h, the system was replaced with 20% water.

Formulation and elaboration of experimental diets

Table 1 shows the formulation of the diets with the addition of the different percentages of β -glucans made from

the base diet proposed by Frías-Quintana et al. (2015). Practical diets were prepared by the protocol proposed by Álvarez-González et al. (2001). Proximate analyses were determined according to the official methods of the Official Association of Analytical Chemistry (AOAC 1990). Ash was determined by heating the diet residue in a muffle at 550 °C for 24 h. Protein was determined by the Kjeldahl method using hydrochloric acid as a trapping medium and sodium hydroxide for quantification. Lipids were determined using the technique described by Bligh & Dyer (1959) and the nitrogen-free extract (NFE) described by Brett & Groves (1979).

Growth indexes

At the end of the bioassay, growth was evaluated using a digital scale (Ohaus HH120) for wet weight, and total length was measured with imageJ software (NIH, Bethesda, MD, USA). Likewise, the following

Table 1 Formulation and composition of experimental diets with different concentrations of β -glucan for *A. tropicus* larvae

Ingredients	β -glucan concentrations (%)				
	0.0	0.2	0.4	0.6	0.8
Fish meal ^a	29.8	29.8	29.8	29.8	29.8
Poultry meal ^a	15.00	15.00	15.00	15.00	15.00
Pork meal ^a	15.00	15.00	15.00	15.00	15.00
Soybean meal 44% ^a	10.00	10.00	10.00	10.00	10.00
Corn starch ^b	10.00	10.00	10.00	10.00	10.00
Wheat bran ^a	8.66	8.46	8.26	8.06	7.86
Fish Oil ^a	4.1	4.1	4.1	4.1	4.1
Soybean lecithin ^b	4.00	4.00	4.00	4.00	4.00
β -Glucan 1,3/1,6	0.00	0.20	0.40	0.60	0.80
Vit-Min Premix ^c	1.00	1.00	1.00	1.00	1.00
Grenetin ^d	2.00	2.00	2.00	2.00	2.00
Vitamin C ^e	0.50	0.50	0.50	0.50	0.50
Chemical composition (%)	Energy (KJ/g) 17.40				
	Crude Protein 44.0				
	Ether Extract 15.01				
	Fiber 1.01				
	Ash 15.09				
	NFE 25.4				

^aMarine and agricultural proteins S.A. de C.V., Guadalajara, Jalisco

^bPronat Ultra, Merida, Yucatan, Mexico

^cVit-min premix DSM

^dD'gari, food and diet products relámpago S.A. de C.V

^eROVIMIX® STAY-C® 35–DSM, Guadalajara, Mexico

zootechnical indexes were calculated according to the proposal of Tomás-Almenar et al. (2020).

$$\text{Weight gain (WG)} = \frac{[\text{final weight} - \text{initial weight}]}{\text{initial weight}} \times 100$$

$$\text{Specific growth rate (SGR)} = \frac{[\ln \text{ final weight} - \ln \text{ initial weight}]}{\text{days}} \times 100$$

$$\text{Feed conversion rate (FCR)} = \frac{\text{consumed feed (g)}}{\text{weight gain (g)}}$$

$$\text{Survival (\%)} = \frac{\text{final number of organisms}}{\text{initial number of organisms}} \times 100$$

Digestive enzyme activities

At the end of the experiment, three larvae per experimental unit were taken to determine the activity of digestive enzymes. The larvae were euthanized after a thermal shock ($-2\text{ }^{\circ}\text{C}$) and then dissected, the stomach and intestine were extracted. From the organs samples, the multi-enzyme extracts were prepared, for which the organs were homogenized in cold ($4\text{ }^{\circ}\text{C}$) in a 1:10 weight/volume ratio in distilled water with an Ultra-Turrax homogenizer. The homogenized samples were centrifuged (12,000 g for 15 min at $4\text{ }^{\circ}\text{C}$), and the supernatant was separated into 60 μL aliquots in Eppendorf tubes to be frozen at $-80\text{ }^{\circ}\text{C}$ until further use. The Bradford technique (1976) determined protein concentration using a bovine serum albumin curve as a standard. Acid protease activity was measured by the technique of Anson (1938), using hemoglobin (0.25%) as a substrate dissolved with buffer in 100 mmol L^{-1} glycine-HCl buffer at pH 2 and $37\text{ }^{\circ}\text{C}$. Alkaline protease activity by the technique described by Kunitz (1947) modified by Walter (1984) using casein (1%) as a substrate in 100 mmol L^{-1} tris-HCl buffer, 10 mmol L^{-1} CaCl_2 (pH=9.0) at $37\text{ }^{\circ}\text{C}$. In both techniques, 15 μL of extract, 15 min of reaction and reaction was stopped with 0.5 mL of 20% trichloroacetic. A unit of enzymatic activity was defined as 1 μg of tyrosine released by minute, a molar extinction coefficient (MEC) of $0.008\text{ }\mu\text{M}^{-1}\text{ cm}^{-1}$ and reading at an absorbance of 280 nm were used. Trypsin activity by the technique of Erlanger et al. (1961) using 20 μL of enzyme extract in BAPNA (N- α -Benzoyl-DL-Arginine P-nitroanilide) 2 mmol L^{-1} substrate diluted in 50 mmol L^{-1} tris-HCl + CaCl_2 buffer pH 8. Chymotrypsin activity was assayed by the technique described by Del Mar et al. (1979) using 15 μL of extract on SAPNA (Succinyl-(Ala)2-Pro-Phe-p-nitroanilide) substrate 1.25 mmol L^{-1} with 50 mmol L^{-1} tris-HCl buffer (pH 8.0) with a reaction time of 30 min. Leucine aminopeptidase activity was measured with the

technique described by Maraux et al. (1973) using L-leucine-p-nitroanilide 1 mmol L^{-1} as substrate in sodium phosphate buffer 50 mmol L^{-1} at pH 7.8 and $37\text{ }^{\circ}\text{C}$ for the techniques described above with 15 μL of extract with a reaction time 30 min. The measurement of these techniques was performed at an absorbance of 410 nm, using a molar extinction coefficient (MEC) of $9.64\text{ }\mu\text{M}^{-1}\text{ cm}^{-1}$ for chymotrypsin and $0.0088\text{ }\mu\text{M}^{-1}\text{ cm}^{-1}$ for trypsin and L-aminopeptidase.

Lipase activity was measured by the technique described by Versaw et al. (1989) using 10 μL of enzyme extract with β -naphthyl caprylate 100 mmol L^{-1} as substrate in tris-HCl buffer 50 mmol L^{-1} with sodium cholate 100 mmol L^{-1} at pH 7.5 at $37\text{ }^{\circ}\text{C}$ performed with an incubation time of 15 min, MEC of $0.02\text{ }\mu\text{M}^{-1}\text{ cm}^{-1}$ and absorbance 540 nm was used. The α -amylase activity was calculated with the Somoyi-Nelson technique described by Robyt & Whelan (1968), using 15 μL of extract on starch (2%) as substrate in phosphate-sodium buffer 100 mmol L^{-1} , NaCl 50 mmol L^{-1} at pH 7.5 with an incubation time of 30 min, an MEC $0.0034\text{ }\mu\text{M}^{-1}\text{ cm}^{-1}$ and an absorbance of 600 nm. Finally, to measure acid and alkaline phosphatase activities, the technique of Bergmeyer et al. (1974) was applied using 2.04% 4-nitrophenylphosphate for acid phosphatases in citric acid and sodium citrate buffer (1:1 w/w) 0.1 mmol L^{-1} at pH 5.5, in glycine NaOH buffer 0.1 mmol L^{-1} at pH 10 for alkaline phosphatases, with an incubation time of 15 min, 10 μL of extract, an MEC $0.0185\text{ }\mu\text{M}^{-1}\text{ cm}^{-1}$ and absorbance of 405 nm. The activities of all enzymes were calculated with the following equations: (1) Units per mL = $(\Delta\text{abs} \times \text{final volume of the reaction (mL)}) \times (\text{MEC} \times \text{time (min)}) \times \text{enzymatic extract volume (mL)}$; (2) Units per mg of protein = Units per mL/mg of soluble protein.

Gene expression analysis

In the evaluation of the expression of genes involved in the barrier protection response *nod-2* (Nucleotide-Binding and Oligomerization Domain), *muc2* (Mucin2), and *occ* (Occludin) and of the immune system *lys* (Lysosima), primers were designed from the transcriptome of *A. tropicus* (Martínez-Burguete et al. 2021) and *ef1a* (Elongation factor 1) and β -actin (Beta-Actin) were used as reference genes (Table 2), where the relative expression was calculated from the average of both reference genes compared to the control (Livak & Schmittgen

2001). For this purpose, three larvae (24 h of starvation) per experimental unit were sampled and euthanized with cold shock ($-2\text{ }^{\circ}\text{C}$) and preserved in RNA later (volume 1:10 volume:weight) for 24 h and then stored at $\sim 80\text{ }^{\circ}\text{C}$ until use. Total RNA was extracted with trizol reagent (Invitrogen® reagent code) according to the manufacturer's instructions and total RNA was quantified spectrophotometrically (A260/280) using a nanodrop Jenway®. Subsequently, cDNA synthesis was performed from 1.5 μg of total RNA from the samples, under the specifications of the iScript™ Reverse Transcription Supermix for RT-PCR kit (BIO-RAD®), using oligo dT and/or random primers in a final reaction volume of 20 μL per sample. The cDNA was stored at $\sim 80\text{ }^{\circ}\text{C}$ until further analysis. Quantification of relative gene expression was performed by qPCR analysis, using a CFX96 Touch™ Real-Time Thermal Cycler CFX96 (Bio-Rad) in a total volume reaction of 10 μL per sample, where 5 μL of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 4.5 μL cDNA, 0.5 μL of primers (10 μmol) were used. All reactions were performed using the following conditions: 1 cycle of $50\text{ }^{\circ}\text{C}$ for 2 min, followed by 1 cycle $95\text{ }^{\circ}\text{C}$ for 10 min, 40 cycles $95\text{ }^{\circ}\text{C}$ for 15 s and $60\text{ }^{\circ}\text{C}$ for 1 min, and ended with a melting curve under standard 60-cycle program conditions to confirm amplification of a single product in each reaction.

Statistical analysis

All data were analyzed using the normality test (Kolmogorov–Smirnov) and homoscedasticity (Levene). Growth data were analyzed by one-way analysis of

variance (ANOVA), and the Tukey test was used as a post hoc test. Enzyme activity and gene expression analyses were performed using the Kruskal–Wallis nonparametric and Nemenyi test a posteriori. All analyses were performed in the Statistica 7 statistical program and a significance of $p < 0.05$ was used.

Results

Growth indexes

Table 3 shows the growth indexes *A. tropicus* larvae fed with the different inclusion percentages of β -glucans. *A. tropicus* larvae fed for 21 days with the different inclusion percentages of β -glucans showed no significant differences in growth (wet weight g and total length cm) with respect to larvae fed with control diet ($p > 0.05$). The other growth indexes also showed no significant differences compared to larvae fed with control treatment. Only in survival percentage of larvae fed with the control, 0.2% and 0.6% diets presented statistically significant higher values than larvae fed with the 0.4% and 0.8% β -glucan diets ($p < 0.05$).

Digestive enzyme activities

The activity of digestive enzymes in larvae fed with different percentages of β -glucan inclusion is shown in Table 4. The activity of acid and alkaline proteases was not modified by the inclusion of β -glucans ($p > 0.05$). On the other hand, the highest

Table 2 Oligonucleotide reference sequence for intestinal barrier defense genes for qPCR of *A. tropicus*

Gen name	Symbols	Oligo	Primer sequence (5'-3')	Amplified sequence (pb)	Temperature ($^{\circ}\text{C}$)
Nucleotide-binding and oligome Domain-2	<i>nod-2</i>	-F	GTAGTGAACAAGGAGGCGGAC	150	66
		-R	TGAGCTCATCCAGGCCATCG		
Occludin	<i>occ</i>	-F	TGACGAATACCACAGACTGAAG	66	64
		-R	CGATCATAGTCGCTGACCATC		
Mucin-2	<i>muc-2</i>	-F	GGCTCCTCAAGAGCAGCACGGTG	70	70
		-R	TCTGCACGTGGAGCACTCAATG		
Lisozyme	<i>lys</i>	-F	CACTGCAGCCATCAATCACAAAC	66	66
		-R	ATTAGTCAGCAGCTTGCTGCAG		
Elogation Factor 1	<i>efl</i>	-F	ACGCTGAAGGCCGGCATGGTG	70	70
		-R	GGATGTCCTTCACCGACACGTTT		
β -actin	<i>bac</i>	-F	GTCATCACCATTGGCAATGAGAG	68	68
		-R	GACAGTACAGTGTGGCATAACAG		

Table 3 Growth indexes and survival rates of *A. tropicus* larvae fed with different concentrations of β -glucans

Days	Growth indexes	β -glucan concentrations (%)				
		0	0.2	0.4	0.6	0.8
0	TIL (cm)	2.50 \pm 0.18	2.48 \pm 0.09	2.53 \pm 0.13	2.36 \pm 0.12	2.41 \pm 0.11
	IBW (g)	0.054 \pm 0.23	0.055 \pm 0.20	0.058 \pm 0.28	0.051 \pm 0.16	0.057 \pm 0.26
21	TFL (cm)	2.63 \pm 0.23	2.66 \pm 0.20	2.76 \pm 0.28	2.58 \pm 0.16	2.64 \pm 0.26
	FBW (g)	0.59 \pm 0.02	0.58 \pm 0.01	0.63 \pm 0.02	0.56 \pm 0.01	0.59 \pm 0.02
	Survival (%)	33.66 \pm 4.93 ^a	34.66 \pm 3.05 ^a	27.33 \pm 1.41 ^b	32.99 \pm 7.79 ^a	25.99 \pm 1.73 ^b
	SGR (% days)	5.18 \pm 0.06	5.17 \pm 0.14	5.21 \pm 0.14	5.26 \pm 0.18	5.08 \pm 0.23
	WG (%)	13.37 \pm 0.4	13.34 \pm 0.6	13.80 \pm 0.9	13.22 \pm 0.8	13.19 \pm 0.7
	FCR	1.30 \pm 0.82	1.15 \pm 0.91	1.20 \pm 0.25	1.83 \pm 0.44	1.43 \pm 0.61

ITL, initial total length; *IBW*, initial body weight; *FTL*, final total length; *FBW*, final body weight; *WG*, weight gain; *SGR*, specific growth rate; *FCR*, feed conversion ratio

trypsin activity was detected in larvae fed with the 0.8% diet (608.67 \pm 63.45 U mg protein⁻¹) compared to larvae fed the control diet (189.13 \pm 33 U mg protein⁻¹; $p < 0.05$). The highest chymotrypsin (26.33 \pm 3.32 U mg protein⁻¹) and L-aminopeptidase (552.63 \pm 38.06 U mg protein⁻¹) activities were recorded in fish fed the 0.6% β -glucan diet relative to larvae fed the control diet (16.64 \pm 1.05 U mg protein⁻¹ and 214.64 \pm 6.68 U mg protein⁻¹, respectively; $p < 0.05$). On the other hand, the highest lipase activities were recorded in larvae fed the 0.6% and 0.8% β -glucan diet (3.56 \pm 0.21 U mg protein⁻¹ and 4.0 \pm 0.46 U mg protein⁻¹, respectively; $p < 0.05$). The highest acid and alkaline phosphatase activities were obtained in larvae fed the diet containing 0.4% β -glucans (0.71 \pm 0.04 and 0.33 \pm 0.04 U mg

protein⁻¹, respectively; $p < 0.05$). Finally, α -amylase activity did not show significant differences between treatments ($p > 0.05$).

Gene expression analysis

The relative expression of genes analyzed in *A. tropicus* larvae fed with different percentages of β -glucans is shown in Fig. 1. The expressions of *muc-2* and *occ* showed the highest values for larvae fed with the inclusion of 0.4% β -glucans (14.75 \pm 1.93 and 3.97 \pm 0.30, respectively; $p > 0.05$; Fig. 1A and B). The highest *lys* expression (9.34 \pm 0.39; $p > 0.05$) was detected for larvae fed with the inclusion of 0.4% β -glucans (Fig. 1C). The *nod-2* gene had the highest

Table 4 Digestive enzyme activities in *A. tropicus* larvae fed with different concentrations of β -glucans

Enzymatic activity (U mg protein ⁻¹)	β -glucan concentrations (%)				
	Control	0.2	0.4	0.6	0.8
Acid protease	8.02 \pm 0.89	7.35 \pm 0.86	9.84 \pm 0.11	8.80 \pm 2.06	8.12 \pm 1.72
Alkaline protease	13.56 \pm 3.08	8.05 \pm 0.95	9.43 \pm 1.09	13.12 \pm 4.10	8.61 \pm 2.86
Trypsin	189.13 \pm 33 ^c	212.44 \pm 13.64 ^c	282.51 \pm 44.42 ^b	323.93 \pm 9.48 ^b	608.67 \pm 63.45 ^a
Chymotrypsin	16.64 \pm 1.05 ^b	19.18 \pm 0.21 ^b	26.33 \pm 3.32 ^a	21.61 \pm 1.90 ^b	23.30 \pm 1.97 ^b
L-aminopeptidase	214.64 \pm 6.68 ^c	355.25 \pm 61.49 ^b	552.63 \pm 38.06 ^a	376.76 \pm 52.73 ^b	365.77 \pm 47.13 ^b
Lipase	1.47 \pm 0.24 ^b	0.18 \pm 0.11 ^c	1.86 \pm 0.48 ^b	3.56 \pm 0.21 ^a	4.0 \pm 0.46 ^a
α -amylase	0.23 \pm 0.05	0.14 \pm 0.02	0.16 \pm 0.02	0.22 \pm 0.07	0.14 \pm 0.05
Acid phosphatase	0.31 \pm 0.05 ^c	0.44 \pm 0.03 ^b	0.71 \pm 0.04 ^a	0.53 \pm 0.04 ^b	0.45 \pm 0.04 ^b
Alkaline phosphatase	0.21 \pm 0.01 ^c	0.24 \pm 0.002 ^c	0.33 \pm 0.04 ^a	0.27 \pm 0.02 ^b	0.29 \pm 0.02 ^b

Letters in superscript indicate significant differences between columns treatments

expression (4.65 ± 1.05 ; $p > 0.05$) in larvae fed with the inclusion of 0.6% β -glucans (Fig. 1D).

Discussion

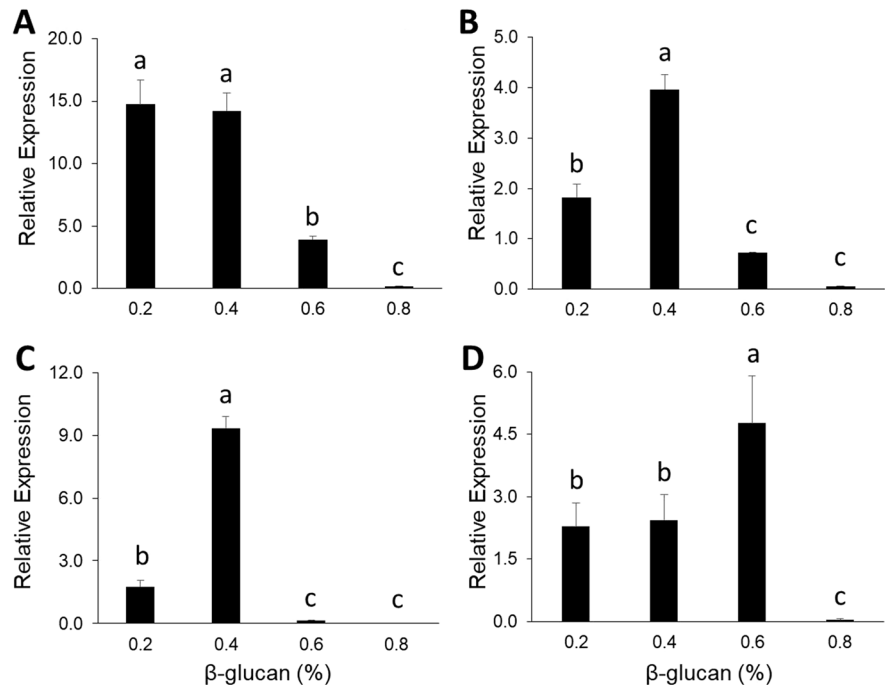
The use of β -glucans in *A. tropicus* larvae as a growth promoter did not show a direct effect; however, our results regarding SGR, FCR and survival are like those described by several authors when studying larviculture (Frías-Quintana et al. 2015; Palma-Cancino et al. 2019; Maytorena-Verdugo et al. 2022). In this regard, the study of Nieves-Rodríguez et al. (2018) with *A. tropicus* juveniles did not increased growth when using β -glucans as well as in other species, such as *Oreochromis niloticus* (Pilarski et al. 2017), *Cyprinus carpio* (Pionnier et al. 2013), and *Morone chrysops* \times *Morone saxatilis* (Li et al. 2009). Low prebiotic concentrations typically will not directly increase growth (Dalmo and Børgwald 2008). Also, the metabolism of β -glucans to hydrolyze them from complex oligosaccharides to simple monosaccharides could only be performed by the action of some intestinal and pancreatic enzymes of the β -glucanase type mainly that are generally not present in carnivorous fish (Chen & Seviour 2007; González et al. 2011). Under this

scenario, fish must have bacteria (microbiome) that can digest complex cell walls to simpler oligosaccharides, such as specific Bacteroides that use glycans as a substrate to be hydrolyzed by glycoside hydrolase (Temple et al. 2017). This capacity promotes the stability of the microbiome, thus linking the improvement of digestion and nutrient assimilation, as well as decreasing the damage caused by pathogens by improving the immune system (Ghanbari et al. 2015).

Considering the above-mentioned, β -glucans are polysaccharide chains and are structural components of the cell wall of various species such as yeast, cereals, fungi, bacteria, and some algae (Vetvicka et al. 2013). The types of bonds may vary according to the ownership of the sources, for example, the β -(1 \rightarrow 3), (1 \rightarrow 6)-glucan is specifically from yeast (*Saccharomyces cerevisiae*) and are activators of macrophage-mediated protective response, antioxidant effects, wound healing, and receptor-mediated immune cells (monocytes, lymphocytes, neutrophils, Langerhans cells) and non-immune cells (epithelial, endothelial and fibroblasts; Petravić-Tominac et al. 2010).

In addition, β -glucans from yeast form part of additives that promote beneficial effects in aquatic organisms (Dharsono et al. 2018). Thus, they have been evaluated in species such as *Lutjanus peru* by exposure of

Fig. 1 Relative expression (mean \pm SD) of a) Mucin-2 (*muc-2*); b) Occludin (*occ*); c) Lysozyme (*lys*) and d) Nucleotide binding domain and oligomerization domain 2 (*nod-2*) in *A. tropicus* larvae fed different inclusion percentages of β -glucans in diets



nanoparticles of 5 mg mL⁻¹ of saline solution added to 100 mg of zinc hydrochloride; the results showed an improved cellular immune response, measuring levels of the enzyme superoxide dismutase, catalase, and peroxidase (Velazquez-Carriles et al. 2018). Likewise, its addition improved the viability of ZT4 cells (10 µg mL⁻¹ for 24 h) that are targeted to glycoproteins to inhibit SVCV (carp virus) replication in a challenge tested with *Danio rerio* (Medina-Gali et al. 2018). Another effect when β-glucans are used was related as an agent against enteritis disorder in *Oncorhynchus mykiss* and *Pagrus major* at concentrations of 0.0%, 0.1%, and 0.2% over a 30-day period, which improved blood parameters, antioxidant capacity, stress tolerance, and growth rate performance (Ji et al. 2019; Shadrack et al. 2022). In crustaceans such as *Litopenaeus vannamei* (Álvarez-Sánchez et al. 2018), *Portunus pelagicus* (Anjugam et al. 2016), and *Cherax tenuimanus* (Sang and Fotedar 2010) its use has been successful with concentrations higher than 0.1% with beneficial results on the immune system.

One aspect to highlight is the increase in chymotrypsin-like and L-aminopeptidase activities, which showed the highest values for *A. tropicus* larvae fed with 0.4% β-glucans. This enhancement in digestive enzyme activities (proteases, lipases, α-amylase, and phosphatases) allows the larvae to take better advantage of the protein during the feeding changes; from the moment of the first feeding with live prey (*Artemia* sp. nauplii), the mixing of *Artemia* sp. biomass with the balanced feed and the weaning process of *A. tropicus* larvae, which present feeding habits typical of a carnivore in its early life stage (Frías-Quintana et al. 2015). In this aspect, the activity of digestive enzymes has been evaluated in several species, such as *Lutjanus peru* (Guzmán-Villanueva et al. 2014), *Scophthalmus maximus* (Miest et al. 2016) and *Cyprinus carpio* (Mohammadian et al. 2019). It should be noted that incremental effects of digestive enzyme activities are shown by including β-glucans, which is related to a more diverse microbial composition and improves gastric functions, absorption, intestinal digestion, and intestinal cell integrity (Dimitroglou et al. 2011; Santos et al. 2020). A study reported in *Carassius auratus var. Pengze* by Cao et al. (2019) shows increased lipase, trypsin, acid phosphatase, and alkaline phosphatase activities, indicating increased functionality of enterocytes (Dimitroglou et al. 2011). In addition, phosphatase activities are related to the transphosphorylation of phosphate mono esters and are considered essential for metabolic regulatory capacity,

energy metabolism, and translation of cellular signaling pathways (Arias-Moscoso and Ezquerro-Brauer 2015).

As for α-amylase activity, it does not show a significant difference with the inclusion of β-glucans. In this sense, Liranço et al. (2013) reported a decrease in activity in juveniles of *Oreochromis niloticus* since this enzyme degrades starches and glycogen from the cleavage of the α-glucosidic bond (Singh et al. 2012). Hence, the presence of β-glucan is a non-digestible carbohydrate, which has a different effect by reaching into the microbiota region and stimulating the secretion of short-chain fatty acids from bacterial fermentation (Wang et al. 2015). Regarding lipase in *A. tropicus* larvae, an increase in its activity was observed, which would be related to the greater capacity to break the ester bond of fatty acids and promote an improvement in lipid metabolism (Dawood et al. 2020). A trial with *Pagellus acarne* coincides that the implementation of prebiotics is related to greater availability of nutrients from the increase of digestive enzymatic activities, which promotes an increase in feed efficiency (Zaineldin et al. 2018). Furthermore, Sarao & Arora (2017) relate this effect to the modification of colonic bacteria and increased fermentation by microbial metabolism. In this same sense, it is mentioned that the use of prebiotics, such as β-glucans, can contribute to the increase of enzymatic activity of lipases, trypsin, proteases, and cellulases, this is due to the bacterial community by the production of extracellular enzymes as demonstrated in *Lutjanus peru* (Guzmán-Villanueva et al. 2014). However, the benefits of prebiotics may vary in each species, from stimulation of enzymatic activity, improvement of intestinal morphology, greater resistance to infections, increased growth, improved body composition, and a better balance of microbial communities (Merrifield et al. 2010).

On the other hand, β-glucans can stimulate humoral factors by recognizing damage-associated molecular patterns (DAMPs), which causes an energetic response against bacterial infections (Reis et al. 2021). Thus, an immunostimulant's effects are to increase the protective response mechanisms, which reduces the effects of immune suppressors (stress, bacteria, contaminants, parasites, among others) through phagocytic activities, releasing nutrients, activating proteins and antioxidants (Kiron 2012). The first line of defense of the innate immune response is the mucus in fish, and it is known that in the case of β-glucans, they can increase the expression of mucins, which have the purpose of increasing mucus production (Anadón et al. 2019).

The *muc-2* (Mucin-2) gene expresses a glycoprotein from epithelial cells, which creates a defense network from cell–matrix signaling and cytokines (Wang & El-Bahrawy 2014). Thus, by including 0.2 and 0.4% β -glucans in *A. tropicus* diets, the *muc-2* expression increased significantly. In this aspect, Shelby et al. (2009) argue that in *Oreochromis niloticus* juvenile trial, the prolonged use of β -glucans can cause only a transitory effect, just as high doses reach to inhibit the immune effects, which response to the discrepancies to establish specific benefits in other teleosts. Similarly, the expression of *occ* (Occludin) and *lys* (Lysozyme) genes in *A. tropicus* larvae fed 0.4% β -glucan increases the expression of both genes. This increase in expressions upon additional β -glucan has been reported in other fish species, such as *Cyprinus carpio*, *Oreochromis niloticus*, and *Danio rerio*, as this prebiotic can stimulate immune activity with the action of lysozyme protein (Wang et al. 2017; Carballo et al. 2018; Nguyen et al. 2019). These proteins have a bactericidal effect, associated with the monocyte and macrophage system located in the mucus and lymphoid organs (kidney, gills, skin, thymus) and plasma, which is reflected by inhibition of the growth and invasion of pathogens (Dawood et al. 2015; Nayak et al. 2018). In teleosts, the claudin family is broad due to physiological plasticity; these molecules are found in higher amounts in epithelial tissues of the skin and gills, as these organs are exposed to water and are vulnerable to environmental changes (Kolosov et al. 2013). Unlike claudins, which are essential for macromolecular cohesion, the Occludin protein transcends in the assembly of such junctions, i.e., they maintain the stability of the intercellular barrier function of the plasma membrane, as it maintains its cohesion, which prevents cell diffusion and prevents the passage of harmful substances through intestinal cells in organisms (Campbell et al. 2017).

Relatedly, the *nod-2* gene was overexpressed in *A. tropicus* larvae fed 0.6% β -glucans. The expression of *nod-2* is known to synthesize a cytoplasmic sensor protein which has been previously reported in several teleost species associated with the gut microbiota (Azad et al. 2012). This molecule participates in the innate detection of potential pathogens, promotes the release of bacterial peptidoglycans, and regulates proinflammatory responses and intramacrophage survival (Zou et al. 2016). Likewise, its activity has been observed in enterocytes in *Danio rerio*, where its overexpression

potentiated the recognition of inflammatory intestinal diseases (Salinas and Parra 2015). Likewise, it has been demonstrated that β -glucans increase the immune and protective capacity against SVCV (Spring Viremia of Carp Virus) infection from the action of cytokines (Medina-Gali et al. 2018). Two trials conducted with *Cyprinus carpio* fed with β -glucans (6 mg per kg body weight) for 14 days and subsequently an intraperitoneal injection with *Aeromonas salmonicida* showed interesting results, where the highest level of cytokine expression was recorded after 6 h of sampling and subsequently reporting a decrease in gene transcript levels in intestine and kidney (Falco et al. 2012). The second assay showed peaks in the expression of C-reactive protein (CRP) at 6 h and 12 h for a subsequent decrease (Pionnier et al. 2013); both authors conclude that β -glucans promote resistance against bacterial infections. Similarly, in *Pseudosciaena crocease*, an experiment was conducted with diets supplemented with 0%, 0.09%, and 0.18% β -glucan for 4 weeks, where the 0.09% dose showed an increase in the phagocytic burst, respiratory and a lower mortality rate (Ai et al. 2007).

When comparing our results with that of Nieves-Rodríguez et al. (2018) in *A. tropicus* juveniles, similarities are observed by detecting an increase in the expression of immune response genes (*il-10*, *tgf*, *muc2* and *occ*) with the intermediate supplementation of 1.5% of β -glucan, while the higher dose of 2% decreases the level of expression. Thus, overexpression in *A. tropicus* larvae was very clear at lower concentrations (0.4–0.6% of β -glucan) than with juveniles. However, physiological responses are not always clear since this depends on the dose and type of prebiotic administered, the developmental stage of the fish, and the infectious agent, among others, so that when the prebiotic is properly applied, an inflammatory response is enhanced as mentioned by Anderson (1992), who indicate that if prebiotic doses are exceeded, it can cause immunosuppression due to interference in cytokines (Dung et al. 2021).

Conclusion

The inclusion of β -glucans in feed for *A. tropicus* larvae did not show a significant improvement in growth rates; however, an increase in lipase and trypsin activity was detected with the inclusion of 0.4% and 0.6% of β -glucans. Likewise, L-aminopeptidase, chymotrypsin,

acid, and alkaline phosphatase activities increased with the addition of 0.4% of the prebiotic. The overexpression patterns of *muc*, *lys*, and *occ* may be associated with intestinal barrier maintenance, lubrication, and defense when using 0.2% and 0.4% of β -glucan, while the *nod-2* gene increased its expression with supplementation of 0.6% of the prebiotic. Therefore, including doses between 0.4 and 0.6% of β -glucan in the diet for *A. tropicus* larvae improves physiological functions associated with the activity of digestive enzymes and the expression of genes associated with the intestinal barrier and immune system. Therefore, further studies are required to evaluate the microbiome and antioxidant capacity modification by means of stress tests and/or bacterial infections.

Author contribution Conceptualization, L.A.C-R., F.J.T-S., and C.A.A-G.; methodology, S.A.F-G., C.S.A-V., R.G-Z.; formal analysis, L.A.C-R., F.J.T-S., S.C-C., R.M-G.; writing—original draft preparation: L.A.C-R.; writing—review and editing, all authors of the manuscript.

Funding This research was funded by the National Council on Science and Technology (CONACYT) in Mexico, grant number CB-2016-01-282765. This study was carried out with the collaboration of Red CYTED LARVApplus (117RT0521). L.A.C-R. acknowledges the National Council on Science and Technology (CONACYT, Mexico) for the master fellowship No. 1078719.

Data availability The authors declare that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval Animals were handled in compliance with the Norma Oficial Mexicana NOM-062-ZOO-1999 from Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación, the Mexican standards for good welfare practices of laboratory animals.

Competing interests The authors declare no competing interests.

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