Acta Ichthyologica et Piscatoria 51(3), 2021, 311–320 | DOI 10.3897/aiep.51.67095

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ACTA ICHTHYOLOGICA ET PISCATORIA

Dietary live yeast (*Debaryomyces hansenii*) provides no advantages in tropical gar, *Atractosteus tropicus* (Actinopterygii: Lepisosteiformes: Lepisosteidae), juvenile aquaculture

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Academic editor: Jolanta Kiełpińska ♦ **Received** 28 August 2020 ♦ **Accepted** 6 March 2021 ♦ **Published** 13 September 2021

Citation: Hernández-López IA, Tovar-Ramírez D, De La Rosa-García S, Álvarez-Villagómez CS, Asencio-Alcudia GG, Martínez-Burguete T, Alberto Galaviz M, Guerrero-Zárate R, Martínez-García R, Peña-Marín ES, Álvarez-González CA (2021) Dietary live yeast (*Debaryomyces hansenii*) provides no advantages in tropical gar, *Atractosteus tropicus* (Actinopterygii: Lepisosteiformes: Lepisosteidae), juvenile aquaculture. Acta Ichthyologica et Piscatoria 51(3): 311–320. https://doi.org/10.3897/aiep.51.67095

Abstract

Tropical gar, *Atractosteus tropicus* Gill, 1863, is an ancient freshwater fish that is commercially cultivated in southern Mexico. Currently, there is a specific diet for its culture; however, the addition of probiotics has not been investigated. The objective of this study was to evaluate the supplementation of live yeast *Debaryomyces hansenii* for *A. tropicus* juveniles on growth, productive parameters, survival, somatic index, digestive enzyme activity, and immune system gene expressions (interleukin 10, *il-10*, Transforming growth factor β1, *tgf-β1*, and β2 microglobulin, *b2m*). Three experimental diets increased the dose of live yeast (0.5, 1.0, and 1.5%; 10^{14}, 10^{15}, and 10^{16} CFU g diet^{-1}, respectively) and a control diet (CD; without yeast) were designed. Daily weight gain and specific growth rate were higher in fish fed with CD and 0.5% *D. hansenii*. High activities of trypsin, chymotrypsin LAP, and α-amylase, as well as overexpression of *il-10* in the spleen, were detected in fish feed 0.5% *D. hansenii*. The inclusion of *D. hansenii* had no positive effect on aquaculture for *A. tropicus*, lower doses should be tested to optimize the diet.

Keywords

digestive physiology, enzymes, gar, immune system, nutrition, probiotics

* Both authors contributed equally.

Introduction

Fish production worldwide is facing challenges related to disease control and nutrition improvement through food optimization, where probiotics show beneficial effects for the host, showing several advantages in the aquaculture production (Akhter et al. 2015), including the immune system, maturation of the digestive system, and host metabolism (Hai 2015; Angulo et al. 2017) and antagonistic capacity to possible pathogenic organisms (Navarrete and Tovar-Ramírez 2014). Yeast *Debaryomyces hansenii* is a halotolerant, non-pathogenic ubiquitous yeast capable of growing and proliferating in a variety of environments, including marine fish (Raggi et al. 2014) and freshwater fish gut (Andlid et al. 1995) and several studies demonstrate positive effects of *D. hansenii* on digestive maturation, increase in survival, the activity of pancreatic and luminal digestive enzymes, increase in resistance to infection by the improvement of immunity and resistance as well to generate a positive effect on the antioxidative status in several fish species fed yeast such as European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758); gilthead seabream, *Sparus aurata* Linnaeus, 1758; leopard grouper, *Mycteroperca rosacea* (Streets, 1877), to name just a few (Tovar-Ramírez et al. 2004, 2010; Reyes-Becerril et al. 2008; Reyes-Becerril et al. 2011). Moreover, the inclusion of probiotics has been evaluated through the immune system gene expression such as interleukins, where Il-10 stands out, which is an immunosuppressive cytokine that prevents immune-mediated damage to the host by dampening inflammatory responses of the immune response (Howes et al. 2014), the β2 microglobulin (*b2m*) is classified as part of the set of the immunoglobulin superfamily (IgSF) and play important roles in the adaptive immune system (Chen et al. 2010). The immunological function of *b2m* is to assist classical major histocompatibility complex (MHC) class I (i.e., MHC-I) molecules in assembling endogenous antigen peptides, forming a trimolecular complex (i.e., pMHC-I) and then presenting them to the surface of antigen-presenting cells (APCs); through interaction with T cell receptors (TCRs), specific cytotoxic T lymphocyte (CTL) immunity is thus induced (Flajnik and Kasahara 2001; Li et al. 2020), and the transforming growth factor β1 (*tgf-β1*), which is an important multifunctional cytokine involved in the regulation of cell proliferation, differentiation, survival, migration, and apoptosis under physiological and pathological conditions (Qi et al. 2016). For example, *tgf-β1* acts as an immunosuppressive mediator and inhibits nitric oxide production in TNF-γ-activated macrophages in fish (Wang et al. 2017). For instance, the positive effects and benefits of *D. hansenii* are well known; however, beneficial effects depend on the supplemented concentration (Hai 2015), where overdosing or underdosing results in lower efficacy and unnecessary costs, with low efficiency of the probiotic (Sharifuzzaman and Austin 2017). Therefore, the evaluation of the possible impact of probiotic inclusion through the understanding of physiological and metabolic changes is part of a compressive understanding of the new emerging aquaculture species (Angulo et al. 2020).

Tropical gar, *Atractosteus tropicus* Gill, 1863, is an ancestral, carnivorous, freshwater fish species native to the south-eastern Mexico and Central America that possess ecological, biological, and economic importance (Márquez-Couturier and Vázquez-Navarrete 2015). Recently, this species has been used as a nutritional fish model, with several nutritional studies focused on the characterization of digestive enzymes during larval and juvenile stages (Guerrero- Zárate et al. 2014; Frías Quintana et al. 2015), development of microparticulate and microencapsulated diets for larvae (Saenz de Rodrigáñez et al. 2018), the inclusion of starch sources (Frías-Quintana et al. 2016, 2017), lipogenic metabolism characterization (Jiménez-Martínez et al. 2019), among others. Recently, Nieves-Rodríguez et al. (2018) and Nájera-Arzola et al. (2018) evaluated β-glucans and mannan oligosaccharides during the juvenile stage in *A. tropicus*, finding positive effects on growth performance, digestive enzymes activity, and immune system. The objective of this study was to evaluate the supplementation of live yeast *D. hansenii* for *A. tropicus* juveniles on growth, productive parameters, survival, somatic index, digestive enzyme activity, and gene expressions of *il-10*, *tgf-β1*, and *b2m*.

Material and methods

Juveniles rearing

Tropical gar juveniles were obtained from the División Académica de Ciencias Biológicas (DACBiol) from Universidad Juárez Autónoma de Tabasco (UJAT). Spawning was induced using a female and three tropical gar males. The larval development was carried out according to previously described methodologies by Saenz-Rodrigáñez et al. (2018). At the required size (0.18 ± 0.01 g), the organisms were used for the experimental.

Probiotic yeast and cell viability

Yeast *D. hansenii* strain CBS 8339 was provided by CIBNOR, S.C. This strain was isolated from the trout intestine (Andlid et al. 1995) and was produced according to the protocol of Tovar et al. (2002). The strain was cultured in yeast peptone dextrose (YPD, Sigma-Aldrich) at 25°C with constant aeration until the early stationary phase (24 h). The cell suspension was centrifuged (1000 *g* for 5 min at 4°C) and the recovered pellet was immediately incorporated into the diet ingredients in different concentrations as shown in Table 1. The viability of the yeast biomass was determined as colony forming units (CFU)

Table 1. Ingredient content, proximate analysis, and gross energy content of the experimental diets supplemented with *Debaryomyces hansenii*.

Ingredient [%]	Treatment (Diet)			
	CD	D1	D2	D3
Fish meal[a]	40.7	40.7	40.7	40.7
Renderer meal[a]	30.0	29.5	29.0	28.5
Corn starch[b]	15.4	15.4	15.4	15.4
Fish oil[a]	6.9	6.9	6.9	6.9
Soybean lecithin[c]	4.0	4.0	4.0	4.0
Grenetin[f]	2.0	2.0	2.0	2.0
Vitamin c[d]	0.5	0.5	0.5	0.5
Vitamin and mineral premix[e]	0.5	0.5	0.5	0.5
D. hansenii concentration % and CFU g of diet^{-1}	0.0	0.5 6.3×10^{14}	1.0 1.2×10^{15}	1.5 1.9×10^{16}
Proximate composition [%]				
Energy [kj g^{-1}]	17.7	17.7	17.7	17.7
Protein	43.6	44.2	43.3	43.0
Ether extract	15.0	14.8	14.9	15.1
Ash	15.0	14.9	14.6	15.2
NFE[1]	26.4	26.1	27.1	26.7

CD = control diet, D1, D2, and D3 are experimental diets featuring increasing content of *D. hansenii*; Renderer meal is a poultry by-product; [a]Marine and agricultural proteins S.A. de C.V., Guadalajara, Jalisco; [b]IMSA Corn Industrializer S.A de C.V. Guadalajara, Jalisco, México; [c]Pronat Ultra, Mérida, Yucatán, México; [d]ROVIMIX® STAY-C® 35 – Vitamins – Products – DSM México, Guadalajara, Jalisco, México and [e]Trouw Nutrition México S.A. de C.V. (by courtesy). [f]D'gari Productos alimenticios y dietéticos Relámpago, Tlalpan, Edomex, Mexico. NFE[1] = Nitrogen-free extract: 100 – (%protein – % ether extract – % ash); CFU = colony forming units.

by plating on YPD plates supplemented with antibiotics (chloramphenicol, 1 mg L^{-1}; polymyxin sulfate B, 1.6 mg L^{-1}; amoxicillin, 2.5 mg L^{-1}, Sigma-Aldrich). The plates were incubated for 48 h at 30°C before colony counting.

After 35 days of culture, yeast cell counts were taken using the trypan blue dye exclusion test to also measure the cell viability, and percent survival was calculated using the total viable yeast cell. A 1:10 dilution (w/v) was performed and mix 500 µL of 0.4% (w/v) trypan blue (Sigma, Aldrich) and 500 µL of dilution. Allow mixture to incubate for 3 min at room temperature. 10 µL of the dilution was taken to load the Neubauer chamber, and the microscope was observed in 400× magnification, where live cells (unstained) and dead cells (stained blue) were counted and the viable cell [%] was determined by mL with the following equation:

$$\text{Viable cells [\%]} = \frac{\text{Total number of viable cells per aliquot [mL]}}{\text{Total number of cells per aliquot [mL]}} \times 100$$

Experimental design and diet manufacture

The experiment designed consisted of four formulated diets, using diet reported by Frías-Quintana et al. (2016) a control diet (CD, without *D. hansenii*) and three diets supplemented with high increasing levels of yeast *D. hansenii* (D1 with 0.5%; D2 with 1.0 and D3 with 1.5% of *D. hansenii*) as shown in Table 1, being isocaloric and isolipidic. The experimental diets were manufactured following the protocol proposed by Alvarez-González et al. (2001). The diets obtained were dried at 40°C for 12 h. The experiment was performed in 70 L plastic tanks, connected to a recirculation system with a settler and biofilter, randomizing 20 juveniles per tank. All treatments were performed in triplicate. Water quality was monitored daily (mean ± standard deviation, SD), with mean values of temperature (26.9 ± 0.4°C) and dissolved oxygen (5.1 ± 0.4 mg L^{-1}) determined by an oximeter (YSI 85; OH), and pH (7.2 ± 0.1) with a potentiometer (HANNA HI991001, Romania).

Growth, somatic index, and sampling

Biometrics was performed every 15 days during 45 days of experimentation, recording wet weights and total length. At the end of the experiment, three juveniles per tank (nine per treatment) were euthanized with an overdose of clove oil dissolved in ethyl alcohol in 1:1 ratio and then dissected to record individual organ weight (stomach, intestine, and liver); additionally, mesenteric fat for each fish was removed and intestine length (from the pylorus until the anus) was measured. For digestive enzyme activity analysis (from the same fishes), the stomach and intestine were removed, rinsed with distilled water, and frozen at –80°C until the enzymatic process. For gene expression analysis, two fish per tank (six per treatment) were sacrificed. The liver, intestine, and spleen samples were fixed in RNA Later® (Thermo Fisher Scientific, Waltham, MA, USA) and frozen at –80°C for future treatment.

Based on the data obtained from feed consumption, growth, weight, and survival the following parameters and somatic indexes were calculated:

$$\text{Daily weight gain } \left(\text{DWG [g Day}^{-1}\text{]}\right) = \frac{\text{Final biomass [g]} - \text{Initial biomass [g]}}{\text{Number of days}}$$

$$\text{Specific growth rate } \left(\text{SGR [\%Day}^{-1}\text{]}\right) = \frac{\text{Ln final mean weight} - \text{Ln initial mean weight}}{\text{Number of days}} \times 100$$

$$\text{Feed conversion rate (FCR)} = \frac{\text{Total food consumed [g]}}{\text{Final biomass [g]} - \text{Initial biomass [g]}}$$

$$\text{Protein conversion rate (PER)} = \frac{\text{Final biomass [g]} - \text{initial biomass [g]}}{\text{Protein ingested [g]}}$$

$$\text{Survival (S [\%]} = \frac{\text{Final number of organisms}}{\text{Initial number of organisms}} \times 100$$

$$\text{Hepatosomatic index (HSI)} = \frac{\text{Liver weight [g]} \times 100}{\text{Body weight [g]}}$$

$$\text{Viscerosomatic index (VSI)} = \frac{\text{Viscera weight [g]} \times 100}{\text{Body weight [g]}}$$

$$\text{Mesenteric fat index (MSI)} = \frac{\text{Mesenteric fat weight [g]} \times 100}{\text{Body weight [g]}}$$

$$\text{Condition factor (CF)} = \frac{\text{Body weight [g]}}{\text{Body length [cm]}^3} \times 100$$

$$\text{Relative intestine size (RIL)} = \frac{\text{Intestine length [cm]} \times 100}{\text{Body length [cm]}}$$

Digestive enzymes techniques

The stomach and intestine were homogenized separately in distilled water in 1:5 ratio (w:v) with an Ultra Turrax (IKA T18 basic, Wilmington, USA), under cold conditions (4°C), then centrifuged at 16 000 *g* by 15 min at 4°C and the supernatant was recovered to be stored at –80°C until the analyzes were performed. Soluble protein concentration in the stomach and intestine multienzymatic extracts were determined with Bradford (1976) technique using bovine serum albumin as the standard protein.

Acid protease activity (stomach homogenate) was determined according to Anson (1938) using hemoglobin (1%) as a substrate in 100 mmol L^{-1} glycine-HCl buffer at pH 2, where the absorbance of the reaction was measured at 280 nm. Alkaline protease activity (intestine homogenate) was determined according to Walter (1984) using casein (1%) as substrate in buffer Tris-HCl 100 mmol L^{-1}, 10 mmol L^{-1} $CaCl_2$ at pH 9, where the absorbance of the reaction was measured at 280 nm. Trypsin activity was determined according to Erlanger et al. (1961) using 1 mmol L^{-1} BAPNA (Nα-Benzoyl-DL-arginine-p-nitroanilide) as substrate, dissolved in 50 mmol L^{-1} Tris-HCl buffer, 10 mmol L^{-1} $CaCl_2$ at pH 8.2, and the absorbance of the reaction was measured at 410 nm. The chymotrypsin activity was determined according to Hummel (1959) using N-benzoyl-L-tyrosine ethyl ester (BTEE) 5 mmol L^{-1} as the substrate dissolved in Dimethyl sulfoxide (DMSO) in 100 mmol L^{-1} Tris buffer, 100 mmol L^{-1} $CaCl_2$ at pH 7.8, and the absorbance was determined at 256 nm. The leucine aminopeptidase (LAP) activity was determined according to Maroux et al. (1973) using leucine p-nitroanilide (1.2 mmol L^{-1}) as substrate in 50 mmol L^{-1} sodium phosphate buffer at pH 7.2, and the absorbance was measured at 405 nm. The α-amylase activity was determined according to Robyt and Whelan (1968), using potato starch (2%) as substrate, dissolved in 100 mmol L^{-1} citrate-phosphate buffer and 50 mmol L^{-1} sodium chloride at pH 7.5, and reducing sugars were measured at 600 nm. The lipase activity was determined according to Versaw et al. (1989), using β-naphthyl caprylate (200 mmol L^{-1}) as a substrate dissolved in buffer 50 mmol L^{-1} Tris-HCl and 100 mmol L^{-1} sodium cholate at pH 7.2, and the absorbance was measured at 540 nm. The alkaline phosphatase activity was determined according to Bergmeyer (1974), 4-nitrophenyl phosphate (2.4%) as substrate, dissolved in buffer 100 mmol L^{-1} glycine-NaOH at pH 10.1, and the absorbance was measured at 405 nm. All the techniques were performed at 37°C.

One unit (U) of enzymatic activity was defined as the amount of enzyme that produced 1 µmol of product released per minute. Total activity was calculated applying equation

$$\text{Total activity [Units mL}^{-1}\text{]} = \frac{\Delta\text{abs} \times \text{reaction final volume [mL]}}{\text{MEC} \times \text{time [min]} \times \text{extract volume [mL]}}$$

where Δabs represent the increase in absorbance, and MEC represents the molar extinction coefficient.

Specific digestive enzyme activity was calculated using equation

$$\text{U mg protein}^{-1} = \frac{\text{Units mL}^{-1}}{\text{mg protein mL}^{-1}}$$

where mg $protein^{-1}$ is determined by Bradford method (1976).

RNA isolation and Quantitative Reverse Transcription PCR (RT-qPCR)

Total RNA from each tissue (liver, intestine, and spleen) was extracted individually using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration and purity of the RNA samples were assessed by the ratio of the absorbance at 260/280 nm using a spectrophotometer (NanoDrop 2000). The RNA integrity of the samples was verified by visualization of 28S and 18S rRNAs after 1% agarose gel electrophoresis. The cDNA synthesis was performed using the Improm II Reverse Transcription System (Promega, Madison, WI, USA) following the manufacturer´s recommendations. On the ice, 0.5 µg of experimental RNA was combined with 1 µL of Oligo dT in nuclease-free water for a final volume of 5 µL. The primer/template mix was thermally denatured at 70°C for 5 min and chilled on ice. Subsequently, 15 µL of the reverse transcription reaction mix (5 × reaction buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, 1 µL reverse transcriptase, and 20 u ribonuclease inhibitor) was added in a final volume of 20 µL. The reaction mix was incubated at 25°C for 5 minutes following at 42°C for 60 min. The synthesized cDNA was diluted 1:3 (v/v) and stored at –80°C until later use.

The RT-qPCR was performed in a CFX96 Real-Time System (BioRad, Hercules, CA, USA) using 10 µL of IQ™ SYBR Green Supermix (BioRad), 1 µL primers mix, and 9 µL of diluted cDNA for a final volume of 20 µL. The cycles in the RT-qPCR program used was the following: 50°C for 2 min, 95°C 10 s, followed by 40

cycles at 95°C 15 s, and 62°C 1 min. As a reference, the gene the elongation factor (*ef1*) was used. Relative gene expression was calculated as fold-change compared with control and calculated by means of the formula $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen 2001). Design of specific oligonucleotides for Interleukin 10 (*il-10*), β2 microglobulin (*b2m*), Transforming growth factor β1 (*tgf-β1*) were obtained from the *A. tropicus* transcriptome (Martínez-Burguete et al. 2021, Accession: PRJNA395289), shown in Table 2, and Elongation factor (*ef1*) was obtained as previously reported (Jiménez-Martinez et al. 2019).

Table 2. Primers designed for Interleukin 10, β2 microglobulin, Transforming growth factor β1 and Elongation factor genes for qPCR of *Atractosteus tropicus*.

Gene name	Symbols	Oligo	Primers sequence (5′–3′)	Temp. [°C]
Interleukin 10	*il-10*	–F	GCTGCCGAAGGTACTTCTCTT	60.03
		–R	GTCTGATAATGGGGAAATCCTG	59.67
β2 microglobulin	*b2m*	–F	AAGAACAAGCAGCAGATGGAG	59.63
		–R	TTTACATGTCAGGTTCCCAGGT	60.64
Transforming growth factor β1	*tgf-β1*	–F	TTCGATAAGACCAGAGGGGATA	59.92
		–R	CACACAGCAGTTTTCCATCTTC	59.78
Elongation factor	*ef1*	–F	CCTGCAGGACGTCTACAAGATCG	62.86
		–R	GACCTCAGTGGTCACGTTGGA	61.97

Statistical analysis

Data of growth, productive performance, enzymatic activities, and gene expression were analyzed for postulates of normality (KS) and homoscedasticity (Levene). One way (ANOVA) was performed and a posteriori Tukey test, if required. All tests were carried out using a level of significance of 95% in the Sigma Plot program (analytical software, AZ, USA).

Results

Cell viability in all experimental diets was 95.24 ± 8.90% at the end of the experiment without differences between treatments ($P > 0.05$). Fish fed with the CD and D1 obtained higher DWG and SGR compared to those fed D2 and D3 ($P < 0.05$). Feeding intake (FI) and survival did not show significant differences between treatments ($P > 0.05$); additionally, FCR showed a higher value for fish fed D3, while PER showed a lower value for fish fed D3 ($P < 0.05$) (Table 3). The HSI, VSI, and CF did not show significant differences between treatments ($P > 0.05$), while MSI and RIL showed lower value for fish fed D3 (Table 3).

The digestive enzymatic activity showed that acid proteases, total alkaline proteases, and lipase did not present significant differences between treatments ($P > 0.05$), while trypsin and chymotrypsin activities showed the lowest values for fish fed D3. LAP showed lower activity in fish fed D2 and D3 compared with fish fed CD and D1 treatments, α-amylase showed the lowest activity in fish feed D2, while alkaline phosphatases showed higher activity in fish fed CD ($P < 0.05$) (Table 4).

Table 3. Productive values, survival and somatic indexes of *Atractosteus tropicus* juveniles fed with experimental diets supplemented with *Debaryomyces hansenii*.

Parameter	Treatment (Diet)			
	CD	D1	D2	D3
DWG	0.070 ± 0.002^{a}	0.053 ± 0.003^{ab}	0.047 ± 0.003^{b}	0.037 ± 0.001^{c}
SGR	5.19 ± 0.14^{a}	4.82 ± 0.14^{ab}	4.85 ± 0.08^{b}	4.25 ± 0.28^{b}
FCR	1.83 ± 0.13^{a}	1.85 ± 0.16^{a}	1.98 ± 0.14^{a}	2.58 ± 0.26^{b}
PER	1.72 ± 0.18^{a}	1.25 ± 0.14^{a}	1.10 ± 0.07^{ab}	0.96 ± 0.11^{b}
S	90.5 ± 4.5	81.6 ± 5.8	86.7 ± 7.6	76.7 ± 5.8
FI	4.28 ± 0.47	4.57 ± 0.32	4.59 ± 0.36	5.29 ± 0.97
HSI	3.36 ± 0.52	3.75 ± 0.47	3.51 ± 0.52	3.11 ± 0.45
VSI	7.49 ± 2.24	7.83 ± 0.89	7.46 ± 0.42	7.17 ± 1.75
MSI	2.17 ± 0.34^{a}	$1.52 \pm .0.38^{ab}$	1.85 ± 0.49^{ab}	1.34 ± 0.65^{b}
CF	0.33 ± 0.06	0.29 ± 0.02	0.29 ± 0.01	0.30 ± 0.05
RIL	33.58 ± 5.99^{a}	29.91 ± 4.64^{ab}	28.89 ± 2.32^{ab}	25.56 ± 5.55^{b}

All weights expressed in grams [g], all lengths in centimeters [cm]; DWG = daily weight gain; SGR = specific growth rate; FCR = feed conversion rate; PER = protein conversion rate; S = survival; HSI = hepatosomatic index; VSI = viscerosomatic index; MSI = mesenteric fat index; CF = condition factor; RIL = relative intestine size. Values are mean ± standard deviation ($n = 9$). Different superscript letters within rows indicate significant ($P < 0.05$).

Table 4. Digestive enzyme activities of *Atractosteus tropicus* juveniles fed with experimental diets supplemented with *Debaryomyces hansenii*.

Total activity [U mg protein^{-1}]	Treatment (Diet)			
	CD	D1	D2	D3
Acid proteases	299.46 ± 31.10	403.59 ± 57.79	305.71 ± 38.40	305.71 ± 38.40
Alkaline proteases	38.80 ± 2.66	44.69 ± 3.28	30.80 ± 2.52	29.10 ± 3.39
Trypsin	32.40 ± 1.76a	38.24 ± 0.51a	28.76 ± 0.41a	21.42 ± 0.30b
Chymotrypsin	111.3 ± 19.8a	158.4 ± 13.0a	86.1 ± 14.8ab	62.6 ± 23.2b
LAP*	68.43 ± 4.08a	75.79 ± 4.57a	46.27 ± 0.82b	44.84 ± 1.22b
Lipase	31.69 ± 2.11	47.66 ± 8.17	29.59 ± 3.05	34.92 ± 2.92
α-amylase	27.69 ± 4.71a	30.72 ± 5.11a	11.76 ± 4.20b	20.21 ± 4.07ab
Alkaline phosphatases	2335.9 ± 98.0a	2118.1 ± 213.0b	1124.2 ± 34.1.0c	1495.0 ± 110.70b

*Leucine aminopeptidase. Values are mean ± standard deviation ($n = 6$). Different superscript letters within rows indicate significant ($P < 0.05$) differences.

On the other hand, gene expression of *il-10* showed significant differences between all treatments ($P < 0.05$), where all yeast supplemented treatments showed down-regulation in the liver (Fig. 1A) and intestine (Fig. 1B) with increasing yeast level dose; however, spleen showed up-regulation only in fish fed D1 (Fig. 1C). The *b2m* gene expression showed significant differences between all treatments ($P < 0.05$), where fish fed yeast supplemented diets treatments had down-regulation in the liver (Fig. 2A) and intestine (Fig. 2B), but in the spleen, the increment of yeast dose showed up-regulation in fish fed D3 (Fig. 2C). Finally, gene expression of *tgf-β1* showed that fish fed yeast supplemented treatments showed down-regulation in the liver (Fig. 3A) and intestine (Fig. 3B) ($P < 0.05$); nevertheless, when increasing yeast dose, the spleen (Fig. 3C) did not show significant differences between treatments ($P > 0.05$).

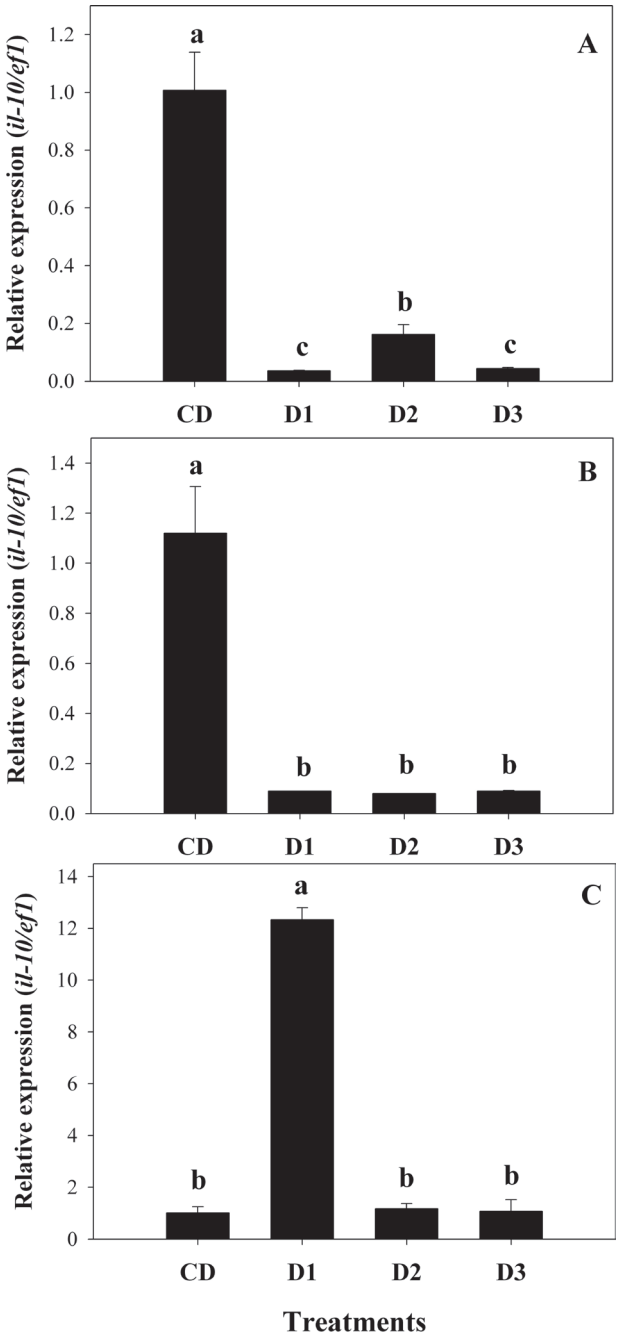

Figure 1. Relative expression of *il-10* in liver (1A) intestine (1B) and spleen (1C) of *Atractosteus tropicus* juveniles fed with experimental diets supplemented with *Debaryomyces hansenii*. Values are mean ± standard deviation (n = 4). Different superscript letters indicate significant ($P < 0.05$).

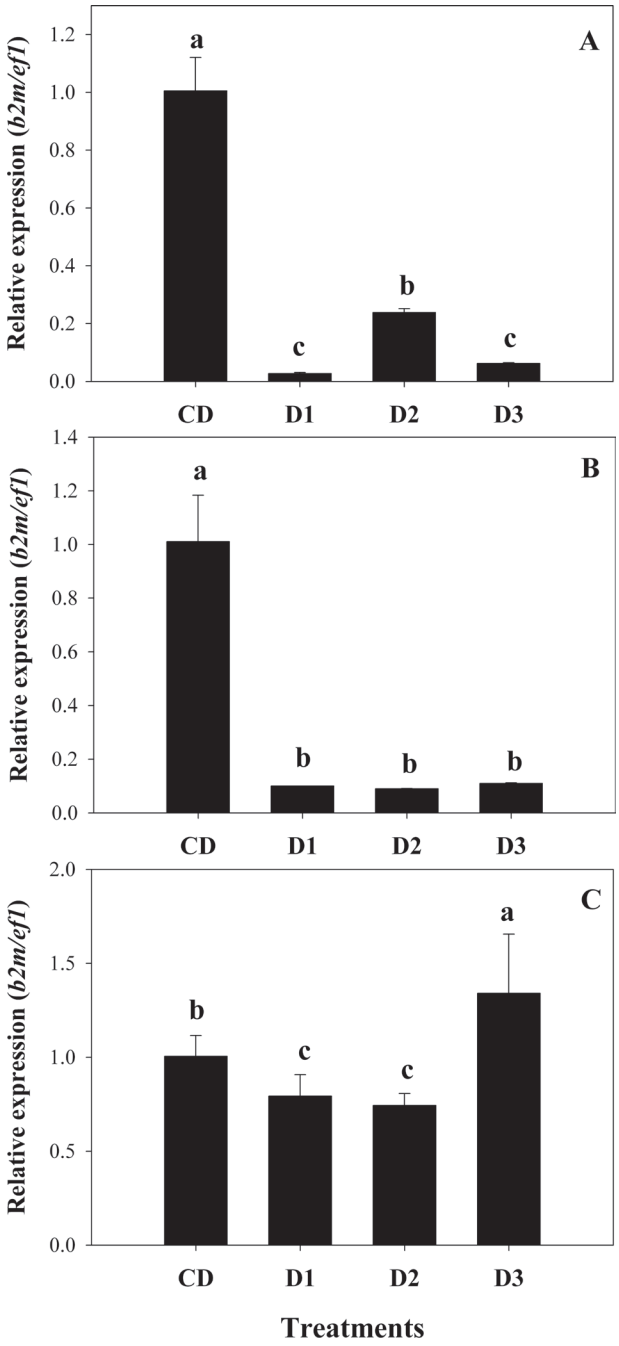

Figure 2. Relative expression of *b2m* in liver (2A) intestine (2B) and spleen (2C) of *Atractosteus tropicus* juveniles fed with experimental diets supplemented with *Debaryomyces hansenii*. Values are mean ± standard deviation (n = 4). Different superscript letters indicate significant ($P < 0.05$).

Discussion

During the feeding trial, *A. tropicus* juveniles fed CD, and D1 showed the same DWG and SGR. In this regard, the incorporation of yeast *D. hansenii* had no positive effect on growth, including the dose of 0.5% (10^{14} CFU g diet^{-1}). Meanwhile, higher doses of *D. hansenii* (1.0 and 1.5%, 10^{15} and 10^{16} CFU g diet^{-1}) could be highly excessive than 0.5%, which was reflected in some parameters such as productive values (FCR and PER), somatic indexes (HSI, VSI, CF, and MSI) and digestive enzymes (acid and alkaline proteases, trypsin and chymotrypsin). It is well known that probiotics' positive effect depends on the concentration (Tovar-Ramírez et al. 2004). However, the appropriate concentration of probiotic levels depends on the probiotic type, fish species, physiological status, rearing conditions, and the specific goal of the applications, where 10^5 CFU mL^{-1} is commonly accepted probiotic density (Hai 2015). Nevertheless, reports in rainbow trout, *Oncorhynchus mykiss*

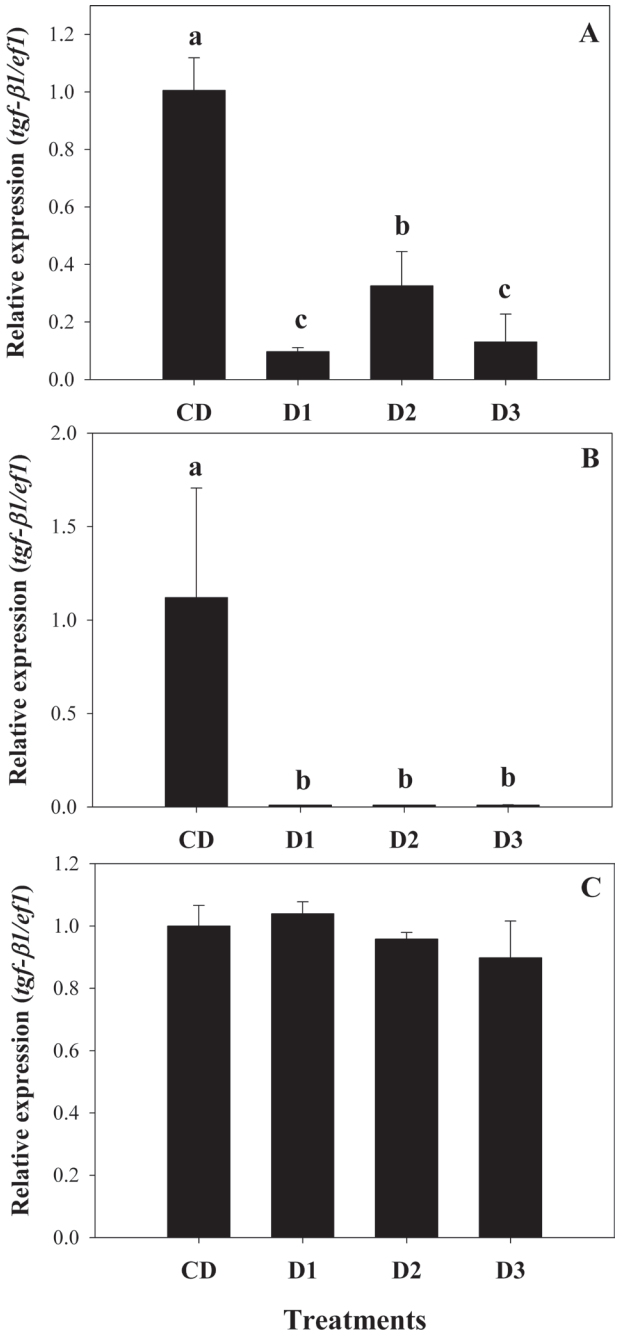

Figure 3. Relative expression of *tgf-β1* in liver (3A) intestine (3B) and spleen (3C) of *Atractosteus tropicus* juveniles fed with experimental diets supplemented with *Debaryomyces hansenii*. Values are mean ± standard deviation (n = 4). Different superscript letters indicate significant ($P < 0.05$).

(Walbaum, 1792), fed a probiotic (*Lactobacillus rhamnosus*) diet in a range of 10^9 or 10^{11} CFU g^{-1}, that can be considered as high doses, showed benefits on head kidney leukocyte phagocytic activity and improved serum lysozyme and alternative complement activity in a group that received the probiotic at 10^{11} CFU g $diet^{-1}$, as well to (Panigrahi et al. 2004). Nevertheless, to our knowledge, doses of live yeast as a probiotic over 10^{14} CFU g $diet^{-1}$ are not still reported.

As characteristics, *D. hansenii* shows high adherence to fish gut mucosa (Tovar et al. 2002), being part of the two main microbes of the microbiome core of wild and reared carnivore marine fishes, regardless of host species, domestication status, geographic location, and water salinity (Raggi et al. 2014). Our study showed that trypsin, chymotrypsin, LAP, and α-amylase had high activities in *A. tropicus* feed control diet and 0.5% *D. hansenii*, compared with higher inclusion (1.0 and 1.5%). Although it should be noted that the inclusion of 0.5% yeast was not statistically different from fish fed the control diet, it is observed that the digestive enzyme activities in fish supplemented with 0.5% *D. hansenii* are slightly higher than the control treatment. Furthermore, *D. hansenii* is associated with high production of polyamines (spermine, spermidine, and putrescine), considered as natural growth factors (Bardócz et al. 1993). Even polyamines play a role in promoting intestinal maturation and increasing the ability of enterocytes to absorb nutrients, in high concentrations decrease the absorption of nutrients (Sousadias and Smith 1995; Tovar et al. 2002, Tovar-Ramírez et al. 2004), where polyamines enter to enterocytes and induce a hormonal cascade that affects pancreas and liver (Peulen et al. 2000). As previously reported in *D. labrax* larvae, a low performance by feed high dose inclusion of *D. hansenii* is related to the high release of polyamines in the intestinal lumen (Tovar-Ramírez et al. 2004).

The beneficial effects of probiotics are consequences of several microbe properties, associated with the immune stimulation by providing molecules such as β-glucans, chitins, mannans, polyamines, among others (Akhter et al. 2015; Angulo et al. 2020), the production of inhibitory compounds that compete against pathogens bacteria for nutrients and adhesion sites and improving the microbial balance (Reyes-Becerril et al. 2017). These properties have been demonstrated in fish aquaculture, for example, cytokines as protein mediators contribute to cell growth, differentiation, and defense mechanisms of the host, where probiotics can modulate pro-inflammatory cytokines such as IL-10 and TGF-β1 in many species (Munir et al. 2016; Román et al. 2013). Accordingly, the liver and intestine showed a down-regulated effect of *il-10* and *tgf-β1* with yeast's inclusion; however, *il-10* expression was up-regulated in the spleen for *A. tropicus* feed 0.5% live yeast. Therefore, down-regulation of *il-10* and *tgf-β1* for fish feed 1.0 and 1.5% of live yeast corresponds to a negative effect to protect cells from an eventual inflammatory response (Kokou et al. 2015). On the other hand, *b2m* is involved in antigen presentation for the production of antibodies and has been reported to be regulated by diet composition (Murray et al. 2010). In our experiment, this gene was down-regulated in the liver and intestine and up-regulated in the spleen in *A. tropicus* feed 0.5%. Therefore, down-regulation could indicate the immune system's low ability to act against antigens (Kokou et al. 2015); however, this aspect should be verified by pathogen challenge in *A. tropicus*.

We hypothesize that incorporation of high dietary doses of *D. hansenii* (10^{14}, 10^{15}, and 10^{16} UFC g $diet^{-1}$) in diets for *A. tropicus* juveniles promote hyper colonization in the digestive tract with the concomitant high production of polyamines and the adverse effects on

growth, pancreatic and intestinal enzyme activities, as well as an immune-suppression of the immune systems (Tovar-Ramírez et al. 2004; Hai 2015). In this sense, fish feed at 0.5% (10^{14} UFC g diet^{-1}) of *D. hansenii* had better performance than fish feed higher doses; however, this dose of yeast cannot be recommended for the culture of *A. tropicus* either. Other studies, such as adherence bioassays and histological analyses of target tissues, clarify possible organ alterations or damage, as well as consider more immune-related gene targets.

Conclusions

Our results provide new evidence that the high inclusion of yeast *D. hansenii* (strain CBS 8339) (10^{14}, 10^{15}, and 10^{16} CFU g diet^{-1}) is not suitable for *A. tropicus* juveniles diet. These yeast concentrations affect growth, digestive enzymatic activity, and gene expression. For this reason, it is necessary to explore lower doses to optimize the inclusion of this probiotic and improve the growth and survival of this species.

Acknowledgments

This study was financially supported by the National Council for Science and Technology (CONACyT) by project CB-2016-01-282765 named "Study of the digestive physiology in larvae and juveniles of tropical gar (*Atractosteus tropicus*) based on histological, biochemical, and molecular techniques."

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