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Research Article

Inclusion of yeast *Candida tropicalis* and *Debaryomyces hansenii* in diets for tropical gar (*Atractosteus tropicus*) juveniles: effect on growth, digestive enzymatic activity, intestinal barrier gene expressions, and gut microbiota

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ABSTRACT. Including probiotic yeasts, such as *Debaryomyces hansenii*, in fish diets improves aquaculture production and fish health. Some fish-isolated yeasts, like *Candida tropicalis*, produce antimicrobial metabolites. However, there have been no reports on their *in vivo* effects. We evaluated the effects of dietary administration of both yeasts on growth, survival, digestive enzyme activity, expression of intestinal barrier genes, and intestinal microbiota composition in *Atractosteus tropicus* juveniles. We administered diets with three concentrations of *C. tropicalis* (10^4, 10^6, and 10^8 CFU g^{-1}), three with *D. hansenii* (10^3, 10^5, and 10^7 CFU g^{-1}), and a control diet without yeast for 45 days. We also evaluated the antagonistic capacity of both yeasts against fish pathogens *in vitro*. Results showed that lower doses of C. *tropicalis* (10^4 CFU g^{-1}) and *D. hansenii* (10^3 CFU g^{-1}) improved growth, survival, and lipase activity. However, *C. tropicalis* compromised intestinal barrier integrity by reducing *zo-1* expression and increasing *il-8* expression while altering the microbiota to favor Desulfovibrionaceae. Conversely, *D. hansenii* enhanced intestinal barrier integrity by increasing the expression of *muc-2*, *zo-1*, and *il-10* genes and decreasing *il-8* expression without altering the microbiota, where *Mycoplasma* predominated. Both yeasts showed antagonistic activity against pathogens. In conclusion, the dietary inclusion of *C. tropicalis* did not favor the intestinal health of the fish. Conversely, *D. hansenii* at a concentration of 10^3 CFU g^{-1} is recommended to improve the growth, digestive function, and health of *A. tropicus* juveniles.

Keywords: *Candida tropicalis*; *Debaryomyces hansenii*; yeast probiotic; intestinal microbiota; tropical gar

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INTRODUCTION

The use of probiotics as food additives that exert positive effects at the nutritional, physiological, and immunological levels of aquatic organisms has generated interest in the aquaculture sector (El-Saadony et al. 2021, Rohani et al. 2022), intending to meet consumer demands and provide well-being in culture conditions. Probiotics are those live microorganisms that, when supplemented in food, have beneficial effects on a host by contributing to the balance of the intestinal microbiota (Gram et al. 1999). Probiotic administration's effects are diverse, including cell proliferation and differentiation, immune system enhancement, improved digestive activity, growth, survival, and modulation of the intestinal microbiota. The intestinal microbiota plays a vital role in fish development and is subject to modulation by various factors such as environmental stimuli, diseases, diet type, and developmental stage (Miyake et al. 2015, Talwar et al. 2018). However, the effects of probiotics are strain-specific and depend on the administered concentration and the host (Merrifield et al. 2010). Many probiotics of great interest are primarily Gram-positive and lactic acid bacteria (Verschuere et al. 2000). However, yeasts can be good probiotics (Caruffo et al. 2015) since they are part of the normal microbiota of fish, with physiological effects attributed to the production of various enzymes, polyamines, and the components of their wall such as β-glucans and mannan-oligosaccharides (MOS), which promote the immune response (Lokesh et al. 2012, Navarrete & Tovar-Ramírez 2014). Also, they can modulate the intestinal microbiota's composition and influence the organisms' health status (Vargas-Albores et al. 2021). Yeasts of the genera *Pichia*, *Saccharomyces*, *Candida*, and *Debaryomyces* are most frequently isolated from the intestinal system of fish (Navarrete & Tovar-Ramírez 2014). These yeasts can produce killer toxins that act antagonistically against pathogens, thereby protecting the host from infections (Wang et al. 2018). Several studies have shown that feeding *Debaryomyces hansenii* to fish, including rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), European bass (*Dicentrarchus labrax*), and gilthead seabream (*Sparus aurata*), can enhance their growth, survival rate, intestinal condition, and function, and also regulate their intestinal microbiota (Tovar-Ramírez et al. 2004, Angulo et al. 2020, Telles et al. 2022, Sanahuja et al. 2023). On the other hand, *Candida tropicalis*, which has been isolated from healthy specimens of Nile tilapia (*Oreochromis niloticus*), produces secondary metabolites with antimicrobial activity (Siangpro et al. 2023), making it a potential probiotic. However, it is essential to analyze the effects of its *in vivo* administration on the physiological and immunological status and the composition of the fish microbiota.

Tropical gar (*Atractosteus tropicus*) is a species native to the southeast region of Mexico and is of ecological and biological importance. *A. tropicus* aquaculture for local and commercial consumption represents a growing economic activity (Márquez-Couturier & Vázquez-Navarrete 2015). However, the use of probiotics as an additive has been scarcely studied, representing a field of potential improvements in the cultivation of this species. This study investigated the impact of including *C. tropicalis* and *D. hansenii* yeasts in diets for *A. tropicus* juveniles on growth, digestive enzyme activity, immune system gene expression, intestinal barrier gene expression, and intestinal microbiota composition. Additionally, we evaluated the antagonistic capacity of both yeasts against fish pathogens *in vitro*.

MATERIALS AND METHODS

Yeast biomass

The *C. tropicalis* strain (PH-04) was provided by the Applied Microbiology Laboratory (DACBiol-UJAT), which was isolated from the intestine of an adult female of *A. tropicus*, and *D. hansenii* (CBS 8339), provided by CIBNOR, SC. The yeast biomass was obtained by stepwise cultivation for 48 h in dextrose yeast peptone broth (yeast extract 10 g, peptone 20 g, dextrose 20 g, distilled water 1,000 mL) in a rotary shaker (Thermo scientific MaxQ 600) at 140 rpm for 48 h at 32°C. The cultures were centrifuged at 2,500 *g* for 40 min, and the cell package was washed twice with sterile solution saline (85%). The colony-forming units (CFU) of the biomasses obtained were quantified (Hoben & Somasegaran 1982), and the number of CFU per gram of wet biomass was reported.

Pathogen antagonism test

The *in vitro* antagonistic capacity of the yeasts *C. tropicalis* and *D. hansenii* against different fish pathogenic bacteria was evaluated. The pathogenic bacteria used were *Aeromona hydrophila* (NCIBM 1134), *A. dhake*nsis (Caim, 1873), *A. ichthiosmia* (Caim, 1876), *Staphylococcus arlettae* (CAIM 1658 and UJAT-02), *Vibrio harveyi* (Caim, 1622), *V. campbelli* (Gibgen, 002) and *Photobacterium damselae* (Caim, 192). The overnight cultures of the bacteria were adjusted to an optical density of 0.6-0.9 at 520 nm

(approximately 1×10^8 CFU mL^{-1}). The adjusted suspension was diluted 1:10 in sterile saline solution (0.85%) to obtain an inoculum of 1×10^7 CFU mL^{-1}, 3.5 mL of the bacterial suspension was taken and placed in 31.5 mL of Mueller-Hinton Agar (Difco) (45°C), it was homogenized and poured into square Petri dishes (Greiner, Bio-one). After the agar had solidified, the yeast cultures from the 2-day-old culture were punctually inoculated with a calibrated loop (1 µL). The plates were incubated at 30°C for 24 h, and the antagonistic effect of each yeast strain against the specific bacterial strains was quantified by measuring the diameter of the inhibition zones in mm with the standard caliper. The experiments were performed in triplicate (Pantelides et al. 2015).

Preparation of the experimental diets

Seven experimental diets were formulated following the protocol of Frías-Quintana et al. (2016). Three diets were supplemented with *C. tropicalis* 10^4, 10^5 and 10^6 CFU g^{-1} and *D. hansenii* at 10^3, 10^5 and 10^7 CFU g^{-1} concentrations. A control diet without yeast (CD) was also included (Table 1). Yeast incorporation into the diets was achieved by suspending the biomass adjusted in 200 mL of sterile water. The diets were pelleted (3-5 mm), dried at 40°C for 15 h in a convection oven, and stored at 4°C until use. All diets' proximal analysis (ash, lipid, and protein) was conducted according to the Official Methods of Analysis of AOAC International (2000).

Viability of yeasts in experimental diets

At the experiment's beginning and end, the yeast concentration and viability in each experimental diet were confirmed by quantifying CFU per gram. A representative sample from each diet was obtained using the quartering method (Campos & Campos 2017). One gram of the diet was weighed and suspended in 9 mL of saline solution (0.85%), and exponential dilutions from 10^{-1} to 10^{-10} were prepared. One hundred microliters of the various dilutions were inoculated on Potato Dextrose Agar (PDA, Difco) supplemented with antibiotics (amikacin and chloramphenicol) to reduce bacterial contamination in the formulated foods. The inoculum was evenly spread using a sterile Digralsky loop, and the plates were incubated for 24-48 h at 32°C. Colonies in dilution boxes with 10-30 colonies were counted, and the CFU per gram of diet was calculated.

Experimental design

Three hundred seventy-eight *A. tropicus* juveniles at 38 days post-hatching (DPH) were used, with an average weight of 0.22 ± 0.08 g and a total length of 3.67 ± 0.29 cm, obtained from the aquaculture farm "Otot Ibam", Comalcalco, Tabasco, México. Twenty-one tanks (59 cm in diameter and 49 cm in height) were connected to a recirculation system powered by a 1 HP water pump linked to a 1,500 L reservoir with a biological and automated sand filter and an ultraviolet light lamp to minimize bacterial load. Eighteen fish were placed in each tank. The average temperature was 27.1 ± 0.8°C, dissolved oxygen was 5.7 ± 0.2 mg L^{-1}, and pH was 7.3-7.5, monitored daily using an oximeter (YSI 85, USA) and a potentiometer (HANNA HI 991001, Romania).

During the initial five days, the organisms were acclimated to the experimental diets with commercial feed: 80% commercial feed and 20% experimental diet. Gradually, each day, a higher proportion of the experimental diet was provided (70/30, 60/40, 50/50, and 40/60). Only the experimental and CD, starting from day six, were administered for 45 days. The treatments were conducted in triplicate. Fish were fed four times daily (07:00, 11:00, 15:00, and 19:00 h) until apparent satiety. Cleaning each tank and partial water replacement (10%) after each feeding using a siphoning method.

At the end of the bioassay, all fish were weighed and measured. Subsequently, they were anesthetized with clove oil (0.1 mL L^{-1}) and euthanized by decapitation. An incision was made in the ventral area for the extraction of the liver, visceral package, stomach, and intestine for somatic index determination. Additionally, three stomachs and three intestines per replicate were frozen for enzymatic activity evaluation. Only the organisms from treatments with the concentrations of each yeast showing the highest growth and survival were used to quantify the expression of genes related to the intestinal barrier function and determine the intestinal microbiota composition. For this purpose, three more intestines per replicate were preserved in RNAlater™ solution (Ambion) for gene expression analyses. Additionally, three intestines per replicate were taken and rinsed with RNase-free water (Sigma), then preserved in RNase-free tubes for metagenomic analyses. All samples were preserved at -80°C.

The study was conducted under the technical specifications for the care and use of laboratory animals, as stated in the Official Mexican Standard NOM-062-ZOO-1999 from the Ministry of Agriculture, Livestock, Rural Development, Fisheries, and Food, and we followed the Declarations of Helsinki recommendations.

Table 1. Composition of experimental diets supplemented with the yeasts and the control diet. [a]Marine and agricultural proteins S.A. de C.V., Guadalajara, Jalisco; [b]Pronat Ultra, Merida, Yucatan, Mexico; [c]Ragasa Industries S.A. de C.V.; [d]vitamin premix composition g mg^{-1} or international units per kg of diet: vitamin A, 10,000,000 IU; vitamin D3, 2,000,000 IU; vitamin E, 100,000 IU; vitamin K3, 4.0 g; thiamine B1, 8.0 g; riboflavin B2, 8.7 g; pyridoxine B6, 7.3 g; vitamin B12, 20.0 mg; niacin, 50.0 g; pantothenic acid, 22.2 g; inositol, 0.15 mg; nicotinic acid, 0.16 mg; folic acid, 4.0 g; biotin, 500 mg; vitamin C, 10.0 g; choline 0.3 mg, excipient q.s. 2 g; manganese, 10 g; magnesium, 4.5 g; zinc, 1.6 g; iron, 0.2 g; copper, 0.2 g; iodine, 0.5 g; selenium, 40 mg; cobalt 60 mg. Excipient q.s. 1.5 g; [e]ROVIMIX® STAY-C® 35 -DSM, Guadalajara, Mexico; [f]D´gari, food and diet products relámpago, S.A. de C.V.

Ingredients (g kg^{-1})	Control diet	*Candida tropicalis*			*Debaryomyces hansenii*		
		10^4	10^5	10^6	10^3	10^5	10^7
Fish meal[a]	305.4	305.4	305.4	305.4	305.4	305.4	305.4
Poultry meal[a]	150	150	150	150	150	150	150
Pork meal[a]	150	150	150	150	150	150	150
Soybean meal[a]	150	150	150	150	150	150	150
Fish oil[a]	34.95	34.95	34.95	34.95	34.95	34.95	34.95
Starch[b]	124.7	124.7	124.62	117.2	124.7	124.55	109.7
Soy lecithin[b]	10	10	10	10	10	10	10
Soybean oil[c]	34.95	34.95	34.95	34.95	34.95	34.95	34.95
Candida tropicalis	0	0.00075	0.075	7.5	0	0	0
Debaryomyces hansenii	0	0	0	0	0.0015	0.15	15
Mineral premix[d]	5	5	5	5	5	5	5
Vitaminic premix[d]	10	10	10	10	10	10	10
Vitamin C[e]	5	5	5	5	5	5	5
Grenetin[f]	20	20	20	20	20	20	20
Proximate composition (g kg^{-1} dry matter)							
Protein	45	45	45	45	45	45	45
Ether extract	13.82	15.00	15.25	16.17	15.76	16.41	13.08
Ash	10.80	11.04	11.10	11.51	11.76	10.68	11.17

Evaluation of growth indexes, survival, and somatic indexes

Every 15 days and at the end of the experiment, weight was determined using an analytical balance (Ohaus HH120, precision 120 ± 0.01 g, Shenzhen, China), and total length was measured using photographs analyzed with Image 1.5 software for all specimens.

At the end of the bioassay, the following productive parameters were determined: feed intake (FI): g dry matter / number of fish / day); absolute weight gain (AWG): final weight (g) - initial weight (g); specific growth rate SGR: [(Ln final weight - Ln initial weight) / days] × 100; feed conversion rate (FCR): (feed intake, g dry matter) / (fish wet weight gain, g); protein efficiency ratio (PER): wet weight gain (g) / protein intake (g); and survival (S): (number of final fish / number of initial fish) × 100.

Before tissues were preserved, the liver, visceral package, and intestines were weighed to calculate the following indexes: hepatosomatic indexes (HSI): (liver weight (g) × 100) / total body weight (g); viscerosomatic indexes (VSI): (viscera weight (g) × 100) / total body weight (g); and the condition factor (K): (final mean body weight / final mean body length^3) × 100).

Enzyme activities quantification

The multi-enzymatic extract was obtained from a pool of three stomachs and three intestines homogenized by replication in 50 mM TRIS-HCl, pH 7, and subsequently centrifuged at 14,000 *g* at 4°C for 15 min. The supernatant was stored at -80°C until use. Quantification of soluble protein was performed using the Bradford method (1976). The activity of trypsin was quantified using the Erlanger et al. (1961) technique with 1 mM BAPNA as substrate (Nα-benzoyl-DL-arginine-P-nitroanilide) in 50 mM Tris-HCl, 10 mM $CaCl_2$, pH 8.2 and the absorbance was measured at 410 nm. Chymotrypsin activity was quantified using 1.25 mM SAAPNA as substrate with 50 mM Tris-HCl, pH 8.2, and the absorbance was measured at 410 nm, following the technique of Delmar et al. (1979). Leucine aminopeptidase activity was determined using 0.1 M leucine p-nitroanilide as substrate, dissolved in

DMSO and diluted with 50 mM sodium phosphate, pH 7.2, incubated at 37°C and measured at 405 nm, according to the method of Maroux et al. (1973). α-amylase activity was quantified using 1% starch as substrate in buffer sodium citrate, 0.05 M NaCl, pH 7.5, and the absorbance was measured at 600 nm (Robyt & Whelan 1968). Lipase activity was carried out with a modified method by Gjellesvik et al. (1992), using 4-nitrophenyl palmitate as substrate and 0.5 M Tris-HCl, pH 7.4, 6 mM of sodium taurocholate and 10 µL of extract and the absorbance was measured at 540 nm. All data obtained are shown as unit per milligram of protein (U mg protein^{-1}) according to the following equations: units by mL (U mL^{-1}) = [Δabs × final reaction volume (mL)] × [ε × time (min) × extract volume (mL)]$^{-1}$; specific activity (U mg protein^{-1}) = U mL mg^{-1} of soluble protein.

RNA extraction and reverse transcription

Total RNA was extracted from three intestines per triplicate (nine intestines per treatment), using Trizol (Invitrogen, Waltham, MA) and following the manufacturer's protocol. RNA concentration and purity were assessed using a spectrophotometer (Jenway GenovaNano, Cole-Parmer, Staffordshire, UK). Subsequently, 1 µg of RNA was reverse-transcribed into cDNA within a thermocycler (Mastercycle nexus GSX1, Eppendorf, Hamburg, Germany) using the high-capacity cDNA reverse transcription kit (Maxima First Strand cDNA Synthesis Kit for RT-qPCR, Thermo Scientific, Waltham, MA) in a final volume of 20 µL, following the manufacturer's guidelines.

Gene expression analysis

Five genes were selected for evaluation of gene expression related to the intestinal barrier integrity such as mucus layer protein (*muc-2*) and tight junctions (*zo-1*), and the immune system as cytokine proinflammatory (*il-8*), cytokine anti-inflammatory (*il-10*), and lysozyme (bacteriolytic). The primers used are shown in Table 2. The qPCR reactions were carried out using 10 µL of Eva Green supermix (BioRad, Hercules, CA), 9 µL of cDNA (5 ng µL^{-1}), and 1 µL primers mix (3 mM) in a final volume of 20 µL. The β-actin gene (Jiménez-Martínez et al. 2021) was used as the reference gene. Subsequently, the qPCR was conducted using a CFX96™ Real-Time Thermocycler (BioRad, Hercules, CA). The relative gene expression changes compared to the untreated control were calculated using the ΔΔCt method (Livak & Schmittgen 2001).

DNA isolation from gut microbiota

Genomic DNA extractions were performed using the QIAGEN DNeasy PowerLyzer PowerSoil kit. Three gut tissues were pooled and placed in Lysing Matrix A (MP Biomedicals TM Santa Ana, CA, USA) with lysis buffer from the kit and homogenized per replicate using the FastPrep-24TM 5G Instrument (MP Biomedicals™, Santa Ana, CA, USA). Subsequent steps were carried out according to the manufacturer's protocol (QIAGEN). The DNA concentration was quantified using the Qubit 3.0 Fluorometer and the ds DNA BR Assay kit (Invitrogen by Thermo Fisher Scientific). DNA integrity was verified by 1% agarose gel electrophoresis.

Library preparation and bacterial 16S rRNA gene sequencing

The V4 hypervariable region of the bacterial 16s rRNA gene was amplified by PCR for the library preparation, using gene-specific primers V4-515f: 5′-GTGCC AGCMGCCGCGGTAA-3′ and V4-806r: 5′-GGACTA CHVGGGTWTCTAA T-3′ described by Caporaso et al. (2011). PCR amplification consisted of an initial denaturation step for 3 min at 98°C, followed by 25 cycles of denaturation for 15 s at 94°C, annealing for 15 s at 51°C, an extension step at 72°C and a final extension for 5 min at 72°C. A second PCR was performed using Nextera XT index (Illumina, San Diego, CA, USA) under the following conditions: 30 s at -95°C, 30 s at 61°C, and 5 min at 72°C. All PCR products were followed by an optimized Clean-Up step utilizing Agencourt AMPure XP beads following Illumina's published protocol. Finally, V4 libraries were sequenced with a MiSeq Reagent Kit v3 (300 cycles) using the MiniSeq Platform (Illumina, San Diego, CA, USA), and 2×150 cycles of paired-end sequencing were performed.

Bioinformatics analyses

All the raw sequencing reads of the V4 16S rRNA gene were processed with Quantitative Insights Into Microbial Ecology 2 (QIIME2) v.2022 (Bolyen et al. 2019). Six hundred five thousand five hundred eighty-eight raw reads were denoised using the "Deblur denoise-16S" plug-in of QIIME2, reads were trimmed at 150 bases, filtered based on quality scores, and chimeras were removed using Deblur. The taxonomic assignment of 201 amplicon sequence variants (ASVs) was performed using the classifier SILVA 132 database set at 99% of identity. Unassigned reads and ASVs with frequency <4 reads were eliminated from further analyses.

Table 2. Primers used for qPCR analysis.

Target gene	Gene function	Primer sequence (5'- 3')	Amplification efficiency (%)	Amplicon size (bp)	Reference
muc-2	mucus layer protein (mucina 2)	FW: GGCCTCCTCAAGAGCACGGTG RV:TCTGCACGCTGGAGCACTCAATG	90.94	100	Nieves-Rodríguez et al. (2018)
zo-1	tight junction protein	FW: TGTGCCTCAGATCACTCCAC RV: AAAGGCAGAGGGTTGGCTTC	98.58	123	Pérez-Jiménez et al. (2022)
lyz	bacteriolytic	FW: CACTGCAGCCATCAATCACAAC RV: ATTAGTCAGCAGCTTGCTGCAG	89.91	100	Nieves-Rodríguez et al. (2018)
il-8	proinflammatory cytokine	FW: ATATTCACTGGTGGGCGGAG RV: GTGCGGCCTGAGATTGTTT	94.18	369	Pérez-Jiménez et al. (2022)
il-10	anti-inflam-matory cytokine	FW: TTATAAAGCCATGGGGGAGCTG RV: CTGCACAGTCTGCCTCTAGT	94.47	91	This study
β-actin	cytoskeletic actin	FW:GAGCTATGAGCTGCCTGAGTGG RV:GTGGTCTCATGAATGCCACAGG	97.10	119	Jiménez-Martínez et al. (2021)

The "qiime diversity core-metrics-phylogenetic" function was set to analyze alpha and beta diversity among sample types, which requires rarefaction to a user-specified sampling depth before computing diversity metrics. Library sizes were adjusted, rarefying the number of reads with a minimum depth of 26,962 to avoid unequal sample sizes (n = 9, three replicas per treatment). A rarefaction curve was generated using ASVs to estimate species richness (alpha diversity) with the qiime diversity alpha-rarefaction plug-in.

Alpha diversity indices (ASV) of microbial community composition were analyzed with Shannon-Weaver for the biodiversity and the abundance estimator using Chao 1 and ACE; all indices were calculated in QIIME2 with q2-diversity. Pairwise comparisons of alpha diversity indices values were performed using one-way ANOVA ($P < 0.05$). To compare overall microbial community structures between different sample types (beta diversity), dissimilarity and similarity matrices were calculated based on the Bray-Curtis and Jaccard distances and the phylogenetic distance matrix based on the Weighted Unifrac index. Principal coordinate analysis (PCoA) was conducted on the calculated matrixes, performed by QIIME2, and visualized using EMPeror (Vázquez-Baeza et al. 2013). A pairwise comparison of the digestive tract beta diversity distances was performed using permutation multivariate analysis of variance (PERMANOVA) through 4,999 permutations with a *P*-value of 0.05 to the beta diversity analysis of QIIME2.

Data analysis and statistics

The statistical analyses were conducted independently for each yeast compared to the CD. Normality (Kolmogorov-Smirnov) and homoscedasticity (Bartlett) were tested for all treatments. Differences in growth indexes, survival, and digestive enzyme activities between diets were assessed using one-way ANOVA, followed by Tukey's test. As the gene expression results did not follow a normal distribution, differences in gene expression were determined by the Kruskal-Wallis and Nemenyi methods. All data were statistically analyzed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA) software with a significance value 0.05.

RESULTS

Yeast antagonism against pathogens

The antagonistic properties of the yeasts *C. tropicalis* and *D. hansenii* were evaluated against eight fish pathogens, including both Gram-positive (*Staphylococcus arlettae* strains CAIM 1658 and UJAT-02) and Gram-negative *(Aeromona hydrophila, A. dhakensis, A. ichistiomia*, *Vibrio campbelli*, *V. harveyi*, and *Photobacterium damselae*) bacteria. Table 3 shows the antagonistic capacity of the yeast *C. tropicalis* in inhibiting the growth of *A. ichistiomia* with inhibition zones of 13.0 ± 1.5 mm, *S. arlettae* (strain CAIM 1658) with inhibition zones of 14.7 ± 1.3 mm, and *V. harveyi* with inhibition zones of 13.5 ± 1.3 mm. Meanwhile, *D. hansenii* only inhibited the growth of *P. damselae*, with inhibition zones of 15.4 ± 1.8 mm.

Viability of yeasts in experimental diets

The CFU number after diet formulation and at the end of the experiment (50 days of storage) indicates that both yeasts were present in concentrations similar to the initial ones throughout the experiment (data not shown).

Table 3. Antagonistic activity of *Candida tropicalis* and *Debaryomyces hansenii* against pathogenic fish strains. The data is expressed as mean ± standard deviation. ND: antibacterial activity not detected.

Bacterial test	Zone of inhibition in diameter (mm)	
	Candida tropicalis	*Debaryomyces hansenii*
Aeromona hydrophila (NCIBM1134)	ND	ND
Aeromona dhakensis (CAIM 1873)	ND	ND
Aeromona ichtiosmia (CAIM 1876)	13.0 ± 1.5	ND
Staphylococcus arlettae (CAIM 1658)	14.7 ± 1.3	ND
Staphylococcus arlettae (UJAT-02)	ND	ND
Vibrio harveyi (CAIM 1622)	13.5 ± 1.3	ND
Vibrio campbelli (CAIM 159)	ND	ND
Photobacterium damselae (CAIM 192)	ND	15.4 ± 1.8

Growth indexes, survival, and somatic indexes

After 45 days of feeding fish with diets supplemented with *C. tropicalis* yeast at a concentration of 10^4 CFU g^{-1}, the fish exhibited significantly higher weight (3.16 ± 0.48 g) and total length (9.47 ± 0.45 cm) compared to the other two concentrations and the CD ($P < 0.02$) (Fig. 1a-b). On the other hand, fish-fed diets containing *D. hansenii* yeast at a concentration of 10^3 CFU g^{-1} showed the highest weight (3.29 ± 0.12 g) and total length (9.93 ± 0.37 cm), also displaying significant differences compared to the other two concentrations and the CD ($P < 0.02$) (Fig. 1c-d).

The determination of growth indices revealed that FI was lower in fish fed with all three inclusions of *C. tropicalis* compared to the CD ($P < 0.02$). On the other hand, no differences in FI were observed between fish fed with *D. hansenii* at 10^3, 10^5 CFU g^{-1}, and the CD. However, FI was lower in the diet with *D. hansenii* at 10^7 CFU g^{-1} compared to the other treatments ($P < 0.05$). Fish-fed diets supplemented with *C. tropicalis* at 10^4 CFU g^{-1} and *D. hansenii* at 10^3 CFU g^{-1} exhibited the highest AWG and SGR compared to the other treatments and the CD ($P < 0.02$). In this regard, higher survival rates (20.37 ± 3.20 and 25.92 ± 3.20%) were recorded in fish receiving the diet supplemented at the lower concentrations of *C. tropicalis* (10^4 CFU g^{-1}) and *D. hansenii* (10^3 CFU g^{-1}), respectively (Table 4). However, incorporating *C. tropicalis* yeast at different concentrations showed no significant effect ($P < 0.05$). Contrarily, significant differences in survival were observed in fish fed with *D. hansenii*, with a concentration of 10^3 CFU g^{-1} compared to the CD ($P < 0.01$) (Table 4). In contrast, the highest FCR was observed in fish fed with the CD and the higher concentration of *C. tropicalis* (10^5 CFU g^{-1}). The PER values did not show significant treatment differences.

The determination of somatic indices indicated that fish fed with the CD had higher VSI than those supplemented with *D. hansenii* ($P < 0.03$). Fish fed with *C. tropicalis* at 10^5 CFU g^{-1} also showed higher VSI, although this was not significantly different from the CD. The highest HSI value was observed in juveniles treated with *D. hansenii* at 10^3 CFU g^{-1}, although without significant differences (Table 4). The K value of fish fed with the CD was higher compared to fish fed with treatments supplemented with *D. hansenii* ($P < 0.05$). In contrast, no differences in K were observed between fish fed with the CD and those fed with treatments supplemented with *C. tropicalis* (Table 4).

Digestive enzyme activities

Juveniles fed with the *D. hansenii* diet at 10^5 CFU g^{-1} showed the highest specific activity of all evaluated enzymes, including trypsin, chymotrypsin, leucine aminopeptidase, amylase, and lipase, with significant differences compared to the other treatments and the CD ($P < 0.05$) (Table 5).

Regarding supplementation with *C. tropicalis*, it was observed that amylase activity was higher in juveniles fed at 10^5 CFU g^{-1}, while lipase activity was higher in those fed with 10^6 CFU g^{-1}. Significant differences were observed in both cases compared to the remaining treatments and the CD ($P < 0.05$). However, trypsin, chymotrypsin, and leucine amino-peptidase activities were significantly higher in fish fed with the CD compared to those supplemented with yeast ($P < 0.05$) (Table 5).

Intestinal barrier gene expressions

The relative expression of *muc-2*, *zo-1*, *il-8*, *il-10*, and *lyz* in juveniles treated with *C. tropicalis* at 10^4 CFU g^{-1} and *D. hansenii* at 10^3 CFU g^{-1} is shown (Fig. 2). Fish

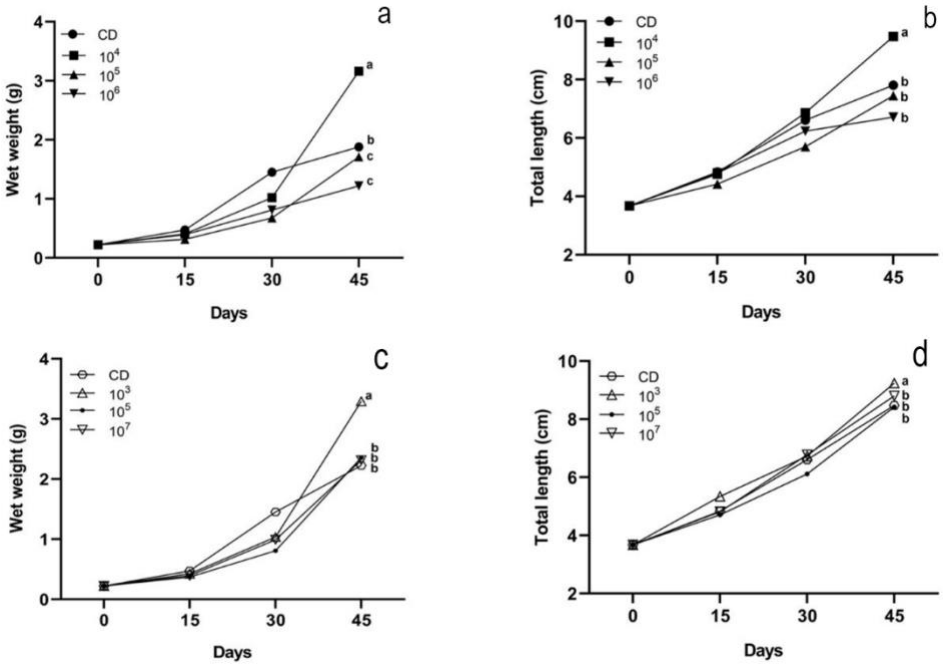

Figure 1. Growth in weight (g) and total length (cm) of *A. tropicus* juveniles fed with a-b) *C. tropicalis* (10_4, 10_5, 10_6 CFU g^{-1}), c-d) *Debaryomyces hansenii* (10_3, 10_5, 10_7 CFU g^{-1}), and the control diet (CD). Values are expressed as mean ± standard deviation. Significant differences among diets are indicated by different letters ($P < 0.05$). Values are expressed as mean ± standard deviation. Significant differences among diets are indicated by different letters ($P < 0.05$).

fed with *C. tropicalis* 10^4 CFU g^{-1} showed no changes in the expression of the *muc-2* gene but exhibited a notable decrease in *zo-1* expression ($P < 0.0422$). Although the *lyz* and *il-10* genes did not show changes in their expression levels compared to the control group, a significant increase in the *il-8* gene expression was observed compared to the CD ($P < 0.008$) (Fig. 2a). Furthermore, *D. hansenii* at 10^3 CFU g^{-1} significantly enhanced the expression of genes related to intestinal barrier function, *muc-2*, and *zo-1*, compared to the CD ($P < 0.02$). The evaluation of gene expression associated with the immune system revealed no significant differences in *lyz* expression compared to the CD. On the other hand, the expression of the *il-8* gene decreased significantly compared to the CD ($P < 0.001$), in contrast to the expression of *il-10*, which increased significantly compared to the CD ($P < 0.0001$) (Fig. 2b).

Intestinal microbial diversity

Table 6 shows the alpha-diversity values of the intestinal microbiota of juveniles treated with *C. tropicalis* at 10^4 CFU g^{-1} and *D. hansenii* at 10^3 CFU g^{-1}, along with the CD. The obtained values indicate no significant differences in the diversity of the microbiota present in the intestines of fish fed with *C. tropicalis* and *D. hansenii* compared to those fed with the CD. However, there is a trend toward higher values with *C. tropicalis* (Table 6).

The intestinal microbiota of *A. tropicus* juveniles fed with *C. tropicalis*, *D. hansenii*, and the CD comprised two dominant phyla: Tenericutes and Proteobacteria (Fig. 3). In fish fed with the CD and *D. hansenii*, the phylum Tenericutes predominated (82.43 and 78.80%, respectively), whereas in fish fed with *C. tropicalis*, it was found at a lower percentage of only 23.19%. In contrast, the phylum Proteobacteria was predominant in fish fed with *C. tropicalis* (76.22%), while in fish fed with the CD and *D. hansenii*, it was only found at 17.14 and 20.86%, respectively. Additionally, less than 1% of the microbiota in fish fed with both yeast treatments and the CD belonged to the phylum Firmicutes (CD 0.366%, *C. tropicalis* 0.397%, and *D. hansenii* 0.981%) (Fig. 3).

Eight families were identified, including Mycoplasmataceae, Desulfovibrionaceae, Moraxellaceae, and Neisseriaceae, which were found in the fish of the three treatments (Fig. 4). In fish fed with the CD and *D. hansenii*, the Mycoplasmataceae family predominated with 82 and 77%, respectively, while in fish fed with *C. tropicalis*, it was found at a lower percentage of only 23.19%. In contrast, the Desulfovibrionaceae family was predominantly found in fish fed with the yeast *C.*

Table 4. Growth performance, survival rate, and somatic indexes of *A. tropicus* juveniles fed diets supplemented with different concentrations of *C. tropicalis* and *D. hansenii* compared with the control diet. Values are mean ± standard deviation. Significant differences within the treatments were compared separately for *C. tropicalis* and *D. hansenii* are indicated by different letters between experimental and control diets ($P < 0.05$). FI: feed intake; AWG: absolute weight gain; SGR: specific growth rate; FCR: feed conversion ratio; PER: protein efficiency ratio; S: survival; HSI: hepatosomatic index; VSI: viscerosomatic index; K: condition factor.

	Control diet	*Candida tropicalis* (CFU g^{-1})			Control diet	*Debaryomyces hansenii* (CFU g^{-1})		
		10^4	10^5	10^6		10^3	10^5	10^7
FI (g d^{-1})	10.67 ± 0.33^{a}	09.48 ± 0.40^{b}	08.49 ± 0.23^{c}	09.39 ± 0.23^{b}	10.67 ± 0.33^{a}	10.60 ± 0.33^{a}	10.71 ± 0.21^{a}	09.42 ± 0.21^{b}
AWG (g $fish^{-1}$)	02.00 ± 0.34^{b}	03.07 ± 0.20^{a}	01.48 ± 0.13^{bc}	01.20 ± 0.21^{c}	02.00 ± 0.34^{b}	03.07 ± 0.08^{a}	02.13 ± 0.04^{b}	01.89 ± 0.01^{b}
SGR (% d^{-1})	05.07 ± 0.34^{b}	05.97 ± 0.13^{a}	04.50 ± 0.16^{bc}	04.09 ± 0.32^{c}	05.07 ± 0.34^{b}	05.97 ± 0.05^{a}	05.22 ± 0.04^{b}	04.99 ± 0.01^{b}
FCR	05.50 ± 0.96^{b}	02.95 ± 0.19^{c}	05.61 ± 0.49^{b}	08.09 ± 1.42^{a}	05.50 ± 0.96^{a}	03.55 ± 0.10^{b}	05.13 ± 0.11^{a}	05.04 ± 0.02^{a}
PER	01.24 ± 0.06	01.03 ± 0.02	00.97 ± 0.19	01.16 ± 0.01	01.24 ± 0.06	01.25 ± 0.13	01.37 ± 0.31	01.26 ± 0.30
S (%)	14.81 ± 3.20	20.37 ± 3.20	14.81 ± 3.20	16.66 ± 5.55	14.81 ± 3.20^{b}	25.92 ± 3.20^{a}	14.81 ± 3.20^{b}	20.37 ± 3.20^{ab}
HSI	02.93 ± 1.72	04.49 ± 0.00	02.74 ± 0. 27	03.34 ± 1.69	02.93 ± 1.72	05.58 ± 1.17	04.62 ± 0.17	03.38 ± 0.48
VSI	15.55 ± 2.42^{ab}	13.03 ± 0.29^{b}	17.36 ± 1.36^{a}	07.59 ± 0.18^{c}	15.55 ± 2.42^{a}	10.47 ± 0.80^{b}	11.22 ± 0.91^{b}	10.08 ± 0.28^{b}
K	00.47 ± 0.05	00.38 ± 0.01	00.37 ± 0.05	00.47 ± 0.02	00.47 ± 0.05^{a}	00.34 ± 0.04^{b}	00.33 ± 0.02^{b}	00.35 ± 0.01^{b}

Table 5. Digestive enzymatic activities of *A. tropicus* juveniles fed diets supplemented with *C. tropicalis* and *D. hansenii* compared with the control diet. Values are mean ± standard deviation. Significant control diets ($P < 0.05$). FI: feed intake; AWG: absolute weight gain; SGR: specific growth rate; FCR: feed conversion ratio; PER: protein efficiency ratio; S: survival; HSI: hepatosomatic index; VSI: viscerosomatic index; K: condition factor.

Activities (U mg $protein^{-1}$)	Control diet	*Candida tropicalis* (CFU g^{-1})			Control diet	*Debaryomyces hansenii* (CFU g^{-1})		
		10^4	10^5	10^6		10^3	10^5	10^7
Trypsin	1.75 ± 0.08^{a}	1.06 ± 0.05^{b}	0.86 ± 0.01^{b}	0.88 ± 0.00^{b}	1.75 ± 0.08^{b}	1.01 ± 0.01^{d}	2.19 ± 0.02^{a}	1.33 ± 0.05^{c}
Chymotrypsin	40.72 ± 0.12^{a}	28.76 ± 0.47^{b}	14.67 ± 2.15^{c}	10.91 ± 0.82^{d}	40.72 ± 0.12^{b}	12.75 ± 0.37^{d}	48.15 ± 0.49^{a}	38.87 ± 0.41^{c}
Leucine aminopeptidase	254.36 ± 2.01^{c}	108.64 ± 5.72^{a}	80.39 ± 4.51^{c}	68.18 ± 2.69^{d}	254.36 ± 2.01^{b}	90.15 ± 6.89^{c}	444.69 ± 31.69^{a}	163.98 ± 6.82^{c}
Amylase	22.14 ± 0.35^{c}	56.37 ± 4.03^{b}	71.02 ± 4.34^{a}	24.27 ± 4.59^{c}	22.14 ± 0.35^{c}	20.25 ± 4.22^{c}	100.75 ± 0.79^{a}	71.23 ± 1.71^{b}
Lipase	3.67 ± 0.75^{b}	5.98 ± 0.92^{b}	5.61 ± 0.07^{b}	8.02 ± 0.61^{a}	3.67 ± 0.75^{c}	8.55 ± 1.13^{a}	9.28 ± 0.13^{a}	8.64 ± 1.15^{a}

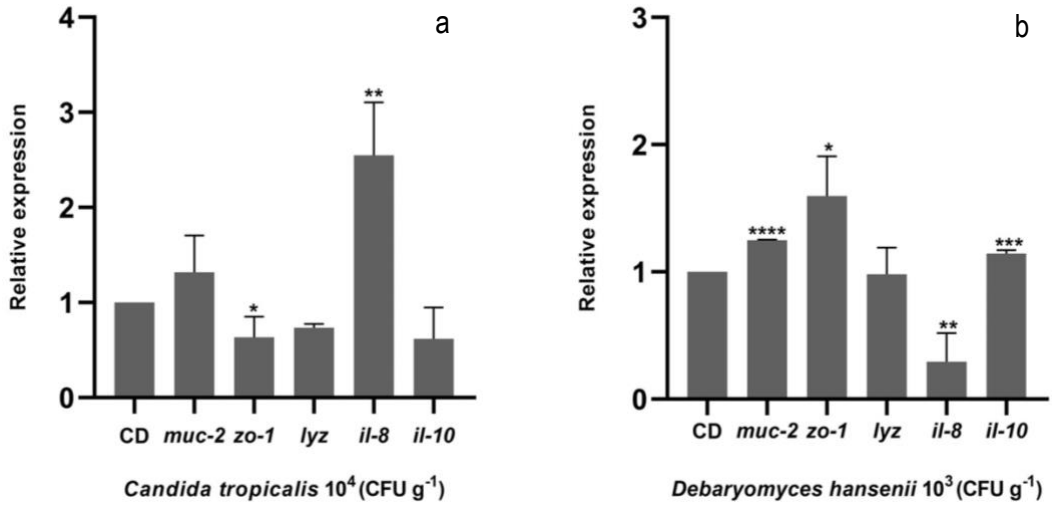

Figure 2. Relative expression levels of the intestinal barrier and immune system genes in *A. tropicus* juveniles fed with a) *C. tropicalis* (10^4 CFU g^{-1}) and b) *D. hansenii* (10^3 CFU g^{-1}), compared to the control diet (CD). Statistical analysis was conducted separately for *C. tropicalis* and *D. hansenii*. Data are presented as fold-change relative to control diet samples (set to 1). Values are mean ± standard deviation. Significant differences ($P < 0.05$) are indicated by asterisks, a) *$P < 0.0422$, **$P < 0.0084$; b) *$P < 0.0297$, **$P < 0.0056$, ***$P < 0.0009$, ****$P < 0.0001$.

Table 6. Alpha-diversity indices of bacterial communities present in the intestine of *A. tropicus* juveniles fed with *C. tropicalis* (10^4 CFU g^{-1}), *D. hansenii* (10^3 CFU g^{-1}), and control diet. Values are mean ± standard deviation. No significant differences were observed between treatments ($P > 0.05$).

Treatments	Richness		Diversity
	Chao 1	ACE	Shannon
Control diet	64.41 ± 7.14	64.53 ± 7.47	2. 83 ± 0. 07
Candida tropicalis (10^4 CFU g^{-1})	83.11 ± 7. 81	83.3 ± 8.05	2. 94 ± 0. 13
Debaryomyces hansenii (10^3 CFU g^{-1})	58.94 ± 21. 19	59.05 ± 21.36	2. 85 ± 0. 22

tropicalis at 75.33%; in contrast, in fish fed with the CD and *D. hansenii*, it was only found at 19.66 and 12.11%, respectively. The Moraxellaceae and Neisseriaceae families were present at percentages below 1% in fish from all treatments. As for the Enterobacteriaceae and Erysipelotrichaceae families, these were only found in fish fed with *D. hansenii*, although at less than 1% (Fig. 4).

Figure 5 shows the genus present in the intestinal microbiota of the evaluated fish. A total of six genera were identified, with *Mycoplasma* being the most abundant of the three treatments. In fish fed with the CD and *D. hansenii*, the genus *Mycoplasma* predominated with 82.03 and 77.42%, respectively, while in fish fed with *C. tropicalis*, it was found at a lower percentage of only 23.19%. Conversely, the genus *Desulfovibrio* was found at a greater percentage in fish fed with *C. tropicalis* (75.13%) than those fed with the CD and *D. hansenii* (12.12 and 19.67%, respectively). The genus *Acinetobacter* was observed in lower proportions, less than 5% in fish fed with the CD, while in fish fed with *C. tropicalis*, it was found at only 0.047%.

Community structure of the bacterial microbiota

The β-diversity index values showed significant differences ($P < 0.05$) in the intestinal microbiota of fish fed with *C. tropicalis* and *D. hansenii* compared to those fed with the CD. The distance matrix calculated using the Bray-Curtis index indicated a total variability of 99.74% ($P = 0.0038$). In contrast, the Jaccard index revealed an accumulated variance of 62.37% ($P = 0.004$) (Fig. 6b). The weighted UniFrac metric recorded a total variance of 99.87% ($P = 0.0048$) (Fig. 6c). In comparison, the unweighted UniFrac metric showed a total variance of 74.23 % ($P = 0.0042$) (Fig. 6d).

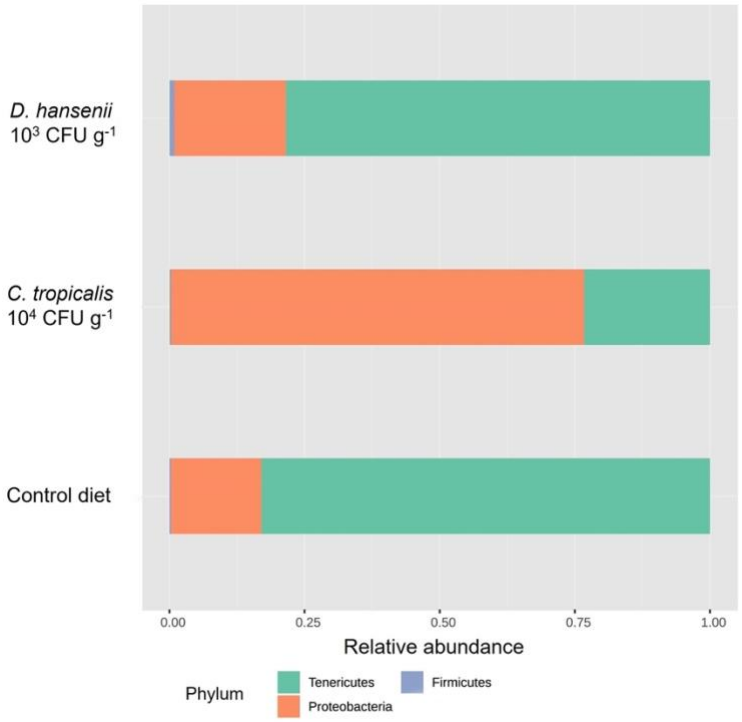

Figure 3. Composition and relative abundance of bacterial phyla present in the intestinal microbiota of *A. tropicus* juveniles fed with *C. tropicalis* (10^4 CFU g^{-1}), *D. hansenii* (10^3 CFU g^{-1}), and the control diet.

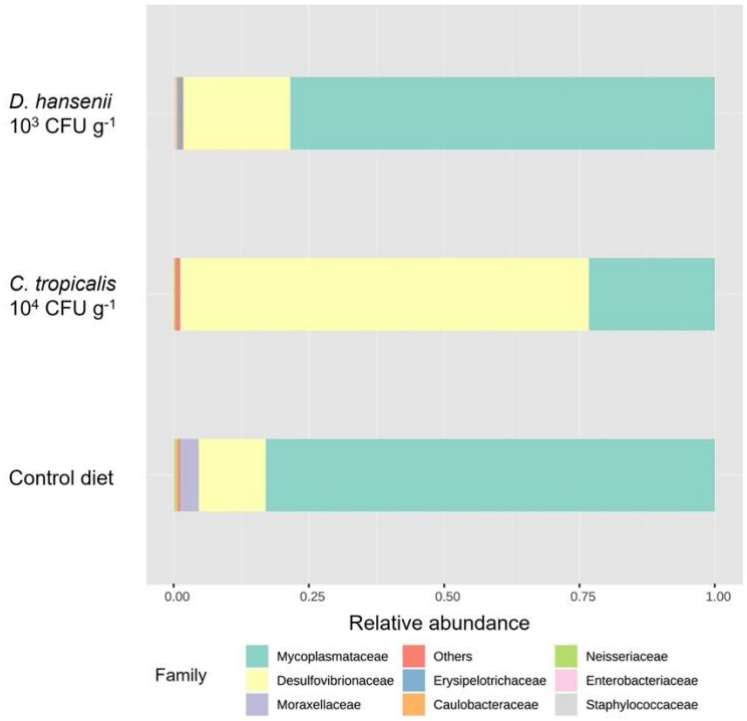

Figure 4. Composition and relative abundance of intestinal bacterial communities at family level in *A. tropicus* juveniles fed with *C. tropicalis* (10^4 CFU g^{-1}), *D. hansenii* (10^3 CFU g^{-1}), and the control diet.

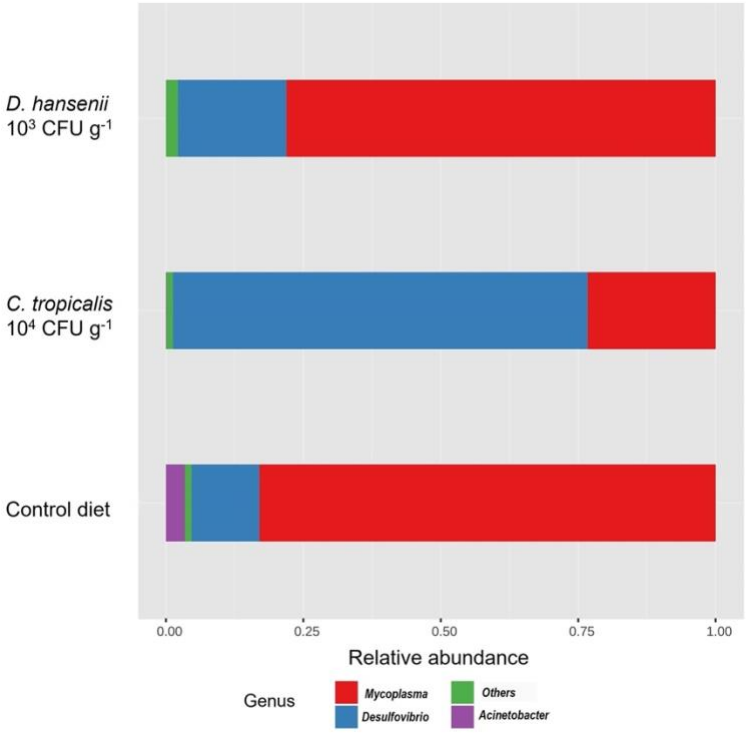

Figure 5. Composition and relative abundance of intestinal bacterial communities at genus level in *A. tropicus* juveniles fed with *C. tropicalis* (10^4 CFU g^{-1}), *D. hansenii* (10^3 CFU g^{-1}), and the control diet.

DISCUSSION

The effect of probiotic yeasts on host growth largely depends on the type and concentration administered. This study showed positive effects on *A. tropicus* juveniles fed with diets supplemented with the lowest doses of *D. hansenii* (10^3 CFU g^{-1}) and *C. tropicalis* (10^4 CFU g^{-1}). These fish exhibited higher weight, total length, AWG, SGR, and S rates than those fed the CD. However, higher levels of both yeasts resulted in similar or worse growth and feed utilization parameters than those observed with the CD. This finding aligns with the results of Hernández-López et al. (2021), who demonstrated that high concentrations of *D. hansenii* (10^{14}, 10^{15} and 10^{16} CFU g^{-1}) in the diet of *A. tropicus* juveniles have adverse effects on the growth and survival. The negative impact on growth and physiological variables is attributed to the high yeast concentrations, which produce elevated levels of polyamines that can be toxic, impair growth and development, and displace the resident microbiota (Tovar-Ramírez et al. 2004). FI was evaluated in this study to understand these effects further. The results showed that fish fed all three concentrations of *C. tropicalis* and the highest concentration (10^7 FCU g^{-1}) of *D. hansenii* exhibited lower FI values than the CD, which indicates that these yeast-supplemented diets might lead to reduced feed consumption.

Interestingly, despite the lower FI, significant growth improvement and better FCR were observed only in fish fed with the lowest concentrations of both yeasts, suggesting that while higher concentrations of yeasts may reduce FI, the optimal growth-promoting effects are achieved at lower concentrations, possibly due to better nutrient utilization or enhanced metabolic efficiency at these levels. Regarding K, our results showed that fish fed with *C. tropicalis* (10^4 and 10^5 CFU g^{-1}) obtained a lower K value than those fed with CD. Moreover, in the fish fed with the three concentrations of *D. hansenii*, K was significantly lower than CD. Although the K values are below the CD value, they agree with the values reported in juveniles of *A. tropicus* by Nájera-Arzola et al. (2018), Sepúlveda-Quiroz et al. (2020) and Hernández-López et al. (2021), which could represent a standard value or range for K in juveniles of this species. Positive effects on the growth and survival of fish fed with *D. hansenii* (5.7%) have also been reported in European seabass (*D. labrax*) larvae (Tovar-Ramírez et al. 2004), greater amberjack (*Seriola rivoliana*) (Telles et al. 2022), and gilthead seabream (*S. aurata*) (Sanahuja et al. 2023).

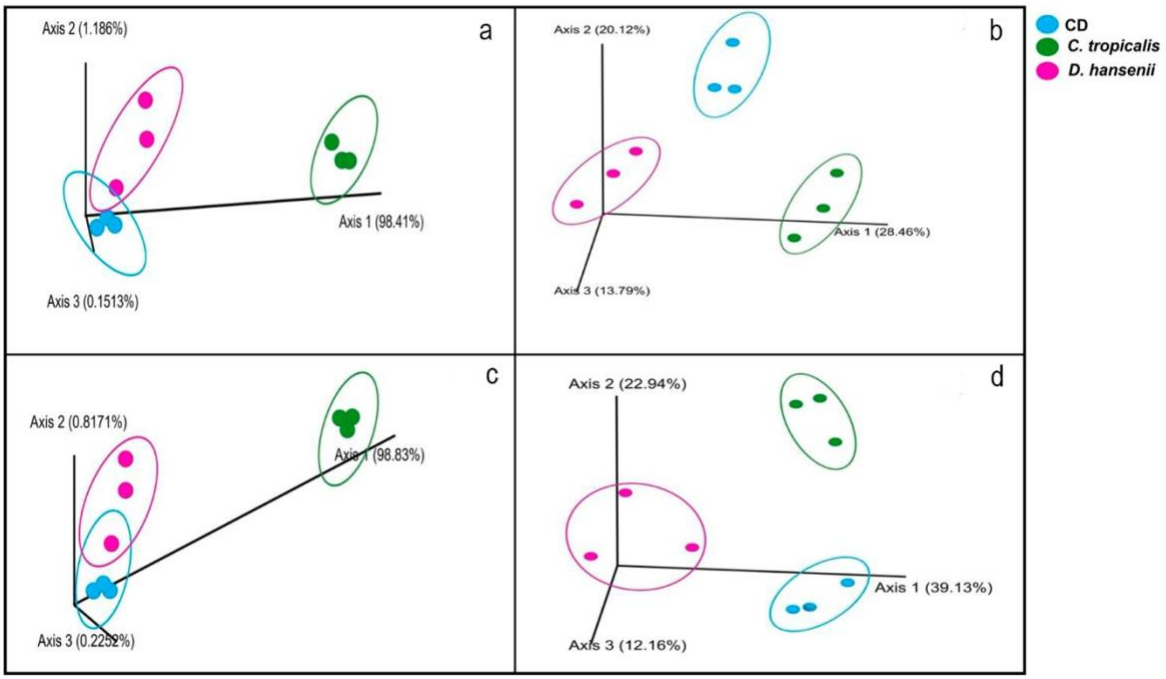

Figure 6. Beta diversity indices through Principal Coordinates Analysis based on distances of a) Bray-Curtis, b) Jaccard, c) Weighted Unifrac, and d) Unweighted Unifrac among the bacterial communities identified in the intestines of *A. tropicus* juvenile after the administration of *C. tropicalis* (10^4 CFU g^{-1}) and *D. hansenii* (10^3 CFU g^{-1}) compared to the control diet (CD).

Regarding aquaculture production, higher HSI and VSI values could indicate compromised muscle growth and higher fat storage. In this study, diets supplemented with 10^4 and 10^6 FCU g^{-1} of *C. tropicalis* and all three concentrations of *D. hansenii* showed significantly lower VSI values than the CD. Both yeasts might contribute to reduced visceral fat accumulation, which improves muscle growth quality. However, no significant differences were found in HSI values between fish fed with *C. tropicalis* and *D. hansenii*, compared to the CD, indicating that the yeasts do not significantly affect hepatic fat storage.

In addition to the beneficial effect on growth, this study observed that fish fed with all three concentrations of *C. tropicalis* and the two highest concentrations of *D. hansenii* (10^5 and 10^7 CFU g^{-1}) showed an increase in amylase enzymatic activity. Additionally, lipase activity was increased in fish fed with both yeasts compared to the CD. Fish fed with all three concentrations of *C. tropicalis* and *D. hansenii* at 10^3 and 10^7 CFU g^{-1} showed lower values of trypsin, chymotrypsin, and leucine aminopeptidase activity than those fed the CD. The lower enzymatic activity observed could be due to these yeasts altering the gut environment or microbiota composition, leading to changes in enzyme expression. However, the yeasts might compensate for the reduced enzymatic activity by providing essential nutrients or bioactive compounds that support growth through alternative metabolic pathways, ensuring that overall growth performance remains unaffected.

Since the best results in terms of growth, survival, and digestive enzymatic activity were observed in fish fed with the lowest concentrations of *C. tropicalis* (10^4 CFU g^{-1}) and *D. hansenni* (10^3 CFU g^{-1}) inclusion, these specimens were selected for the evaluation of their effect on the expression of genes related to intestinal barrier function (both physical and immunological) and to determine the composition of the intestinal microbiota. Gene expression results showed that the yeast *C. tropicalis* did not favor intestinal barrier function. However, it did not promote changes in the expression of the *muc-2* gene (main mucin of the mucus layer), it did lead to a reduction in the expression of the *zo-1* gene (responsible for maintaining tight junctions between intestinal epithelial cells), which likely resulted in increased intestinal permeability, triggering an adverse immune response by stimulating the overexpression of the proinflammatory cytokine *il-8*. In contrast, a favorable response

was observed in the fish fed with the diet supplemented with the probiotic yeast *D. hansenii*. The results demonstrated an increase in the expression of *muc-2*, *zo-1*, and the anti-inflammatory cytokine *il-10* (an important marker for the host's health status).

Additionally, it contributed to the reduction of *il-8* compared to the CD. This expression pattern could indicate improved mucus layer synthesis and reinforcement of junctions between epithelial cells, enhancing the intestinal barrier function and improving the immune response. The exact mechanism of immune system regulation promoted by yeasts is not known with certainty; however, it has been highlighted that components of their cell wall, such as β-glucans and mannan oligosaccharides, modulate it (Torrecillas et al. 2014). Particularly in *A. tropicus*, it has been reported that dietary administration of 1.0 and 1.5% β-glucan to juvenile *A. tropicus* promotes positive *il-10* expression (Nieves-Rodríguez et al. 2018), while in the larval stage, the inclusion of 0.4% promotes *lys* expression (Cigarroa-Ruiz et al. 2023).

In this study, we observed that the intestinal microbiota of *A. tropicus* juveniles obtained from induced spawning and reared under controlled conditions was primarily composed of Tenericutes and Proteobacteria. Although a low diversity was found, this is common in cultured fish compared to wild fish because the evaluated fish were in an early developmental stage (juveniles from 38 to 83 DPH). Wild fish typically have a more diverse microbiota than pond-cultured fish because their diet is more varied due to the abundance of natural food resources (Sun et al. 2021). The most dominant phyla in fish microbiota include Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Fusobacteria (Arumugam et al. 2011, Arboleya et al. 2012). In freshwater fish, the dominant phyla are typically Proteobacteria, Firmicutes, and Actinobacteria (Nayak, 2010, Talwar et al. 2018, Wang et al. 2018). In the case of *A. tropicus* juveniles isolated from the natural environment, it has been described that their microbiota mainly consists of the phyla Firmicutes, Proteobacteria, and Bacteroidetes.

In contrast, the phylum Actinobacteria is also found in cultured juveniles (Méndez-Pérez et al. 2020). Deng et al. (2019) showed that the most abundant phyla in the intestine of cultured *Acanthopagrus schlegelii* are Firmicutes and Proteobacteria, which aligns with our results. In *A. tropicus* juveniles fed with the CD, Tenericutes predominated at 82.43% compared to 17.14% for Proteobacteria. A similar abundance of Tenericutes was also observed in fish fed with *D. hansenii* (78.80%), with 20.86% for Proteobacteria. However, there was an evident change in the microbiota composition in fish fed with *C. tropicalis*, where the Tenericutes:Proteobacteria ratio was reversed (30% Tenericutes and 70% Proteobacteria). This opposite composition in the microbiota could be related to the negative gene expression response observed in fish fed with the yeast *C. tropicalis*, suggesting that this yeast leads to a decrease in beneficial microorganisms and an increase in microorganisms associated with the development of pathologies, a condition known as dysbiosis (Villamil et al. 2020, Yukgehnaish et al. 2020).

Conversely, the predominance of Tenericutes in fish fed with probiotic yeasts has also been reported in other fish species, such as rainbow trout (*O. mykiss*), where the inclusion of *S. cerevisiae* induced a predominance of Tenericutes (83%) compared to Proteobacteria (25%) (Huyben et al. 2018). Bacteria from the phylum Tenericutes are primarily considered mutualistic symbionts in the host's intestine (Wang et al. 2020). Mycoplasmataceae was the most abundant family in the microbiota composition of fish fed with the CD and *D. hansenii*, in contrast to fish fed with *C. tropicalis*. Members of the Mycoplasmataceae family inhabit symbiotically and commensally in rainbow trout, contributing to better digestion by producing lactic and acetic acid as energy sources for intestinal bacteria (Stadtländer et al. 1995). Particularly, *Mycoplasma* was the most abundant genus in fish fed with the CD and with *D. hansenii*, in contrast to fish fed with *C. tropicalis*. *Mycoplasma* is considered a symbiont in the host's intestine, not a pathogen (Huyben et al. 2021). It also predominates in other species, such as Atlantic salmon (*S. salar*) (Huyben et al. 2021, Rasmussen et al. 2021, Zhou et al. 2022) and rainbow trout (Lyons et al. 2017).

Additionally, we observed a significant change in the proportion of the genus *Desulfovibrio* in fish fed with the CD (19%) and *D. hansenii* (12%), compared to those fed with *C. tropicalis* (75%). Desulfovibrionaceae (sulfate-reducing bacteria) have been described as modifying the host's metabolic balance and potentially causing dysfunction in the intestinal barrier, promoting an inflammatory process (Carbonero et al. 2012), which aligns with the gene expression response pattern observed in fish fed with *C. tropicalis*. The positive effect observed in *A. tropicus* juveniles administered *C. tropicalis* (10^4 CFU g^{-1}) in terms of growth and production parameters is likely a result of the duration of the administration period (45 days). However, negative effects were observed on the expression of genes related to intestinal barrier function

(tight junctions and the immune system) and the composition of the intestinal microbiota (dysbiosis). It is unknown whether a more extended administration period could affect fish growth and generate an inflammatory response. Therefore, extending the administration period of *C. tropicalis* to other stages of fish development is recommended to assess the effects that may occur over time.

Given that dysbiosis and compromised intestinal barrier function can lead to increased susceptibility to infections, evaluating the antagonistic activity of the yeasts against fish pathogens is crucial. Some authors report that yeasts from the genera *Saccharomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia*, *Torulopsis*, and *Zygosaccharomyces* release antimicrobials and killer toxins that inhibit the growth and proliferation of certain pathogenic microorganisms (Liu et al. 2013, Wang et al. 2018). In this study, we evaluated the *in vitro* antagonistic capability of *C. tropicalis* and *D. hansenii* against seven pathogenic bacteria, which have been reported as significant pathogens in fish (*Aeromonas hydrophila*, *A. dhakensis*, *A. ichthiosmia*, *Staphylococcus arlettae* (two strains), *Vibrio harveyi*, *V. campbelli*, and *Photobacterium damselae*). Our results confirmed that both yeasts exhibit specific antagonistic activity against pathogens. *C. tropicalis* showed a broad-spectrum antagonistic effect, inhibiting both Gram-negative bacteria (*A. ichthiosmia* and *V. harveyi*) and Gram-positive bacteria (*S. arlettae*), while *D. hansenii* showed highly selective activity (only against *P. damselae*). The broader antagonistic effect observed in *C. tropicalis* could be attributed to its potential higher production of antimicrobial compounds (Siangpro et al. 2023), its ability to compete more effectively for nutrients and space, or the presence of specific killer toxins that might target a wider range of bacterial pathogens.

CONCLUSIONS

The present study demonstrates that incorporating low doses of the yeasts *C. tropicalis* (10^4 CFU g^{-1}) and *D. hansenii* (10^3 CFU g^{-1}) into the diet improves the growth, survival, and lipase activity in juveniles of *A. tropicus*. The inclusion of *C. tropicalis* in diets, even at low concentrations, induces a shift in the structure of the intestinal microbiota, promoting an increase in the Desulfovibrionaceae family, which suggests a relationship with the downregulation of *zo-1* expression and an increase in *il-8* expression. In contrast, yeast *D. hansenii* positively affects the expression of intestinal barrier genes, increasing *muc-2*, *zo-1*, and *il-10* expression while decreasing the *il-8* expression. However, it is not accompanied by changes in the composition of the core microbiota, where *Mycoplasma* predominates. Both yeasts showed specific *in vitro* antagonistic activity against pathogens, with *C. tropicalis* exhibiting a broad-spectrum antagonistic effect. Therefore, the dietary inclusion of *C. tropicalis* did not produce favorable outcomes for the intestinal health of the fish. On the other hand, including 10^3 CFU g^{-1} of *D. hansenii* in diets is recommended to promote better development, health, and disease resistance in *A. tropicus* juveniles.

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Ethics approval

The animals used in this study were handled following the technical specifications for the care and use of laboratory animals, as stated in the Official Mexican Standard NOM-062-ZOO-1999 from the Ministry of Agriculture, Livestock, Rural Development, Fisheries, and Food.

Author contribution

G.M. Pérez Jiménez: methodology, writing original draft, writing, review and editing; S. De la Rosa García: conceptualization, resources, formal analysis, writing original draft, writing, review and editing; M. Martínez Porcha: formal analysis, writing, review and editing; C.A. Sepúlveda-Quiroz: methodology, formal analysis, writing, review and editing; E. Garibay Valdez: methodology, formal analysis, writing, review and editing; D. Tovar Ramirez: formal analysis, review and editing; L.D. Jiménez Martínez: methodology, writing, review and editing; C.S. Alvarez Villagomez: conceptualization, formal analysis, resources, original draft, writing, review and editing; C.A. Alvarez Gonzalez: formal analysis, writing, review and editing.

Conflict of interest

All authors declared they have no conflict of interest.

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