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Research Article

Yeast *Rhodoturula glutinis* as a modulator of innate immune and oxidative stress-related genes in *Oreochromis niloticus* cultured in a Biofloc system

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ABSTRACT. The effect of live yeast *Rhodoturula glutinis* was evaluated on juvenile *Oreochromis niloticus* cultivated in a Biofloc system. Growth performance and the expression of innate response and relevant oxidative stress genes were evaluated after a 12-week feeding trial. Three experimental treatments were evaluated in a conventional tilapia culture (control), Biofloc culture (BFT), and Biofloc with the addition of the live yeast *R. glutinis* 1×10^6 CFU g^{-1} (BFT+Rg), with four replicates per treatment. In all cases, commercial food was supplied to the organisms (32% protein and 5% lipids). *O. niloticus* juveniles (7.02 ± 0.04 g) were randomly distributed in 12 tanks, each with 15 animals. BFT+Rg treatment showed a significant increase in weight gain compared with the other treatments. Significant improvements were found in Fulton's condition factor, feed conversion rate, and hepatosomatic index under Biofloc conditions, BFT, and BFT+Rg treatments. No significant differences were observed in survival. *R. glutinis* directly influenced gene expression in the liver and intestine. The expression of *tnfa*, *tgfb*, *hsp70*, and *gpx*, genes in the liver significantly increased in the BFT+Rg treatment compared with the other treatments. Similarly, a significant increase was found in intestinal *il1b*, *tnfa*, *tgfb*, *trf*, *hsp70 gpx*, and *cat* expression patterns in the BFT+Rg treatment. Based on the performance and immune response, the present study suggests the use of *R. glutinis* as a strategy to increase the productivity of tilapia in Biofloc culture.

Keywords: *Oreochromis niloticus*; innate immunity; yeast; probiotics; BFT; oxidative stress; gene expression

INTRODUCTION

In recent decades Biofloc technology (BFT) has attracted great attention. Its benefits compared to traditional aquaculture techniques provide a more sustainable approach with minimal water exchange and a reduced feed intake, making it a low-cost technology for aquaculture development (Khanjani & Sharifinia 2020, Kumar et al. 2021, Ogello et al. 2021). BFT reduces problems associated with the continuous increase of aquaculture activity, the environmental impact, and its strong dependence on fishmeal in the diet (Bossier & Ekasari 2017, Kumar et al. 2021). The system relies on adding an external carbon source under

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a high level of aeration to produce microbial bacterial floc, generally formed by the aggregation of heterotrophic microorganisms, heterotrophic bacteria are the predominant group of the BFT community (Ahmad et al. 2017, Kumar et al. 2021), yeast, algae (dinoflagellates and diatoms), flagellates, rotifers, ciliates, copepod, nematode, and detritus also constitute the components of the flocs (Khanjani & Sharifinia 2020). These microorganisms can assimilate inorganic pollutants from the water column and provide fish with microbial protein during the culture period (Emerenciano et al. 2017).

Some researchers mention that the microorganisms and their cellular components or metabolic products, have *in situ* probiotic and immunostimulant potential to enhance innate immunity and antioxidant capacity (Tepaamorndech et al. 2020, Van Doan et al. 2020). Microbes living in Bioflocs could stimulate species' immune systems in culture (Menaga et al. 2019, Tepaamorndech et al. 2020). Also, bioactive compounds (carotenoids, chlorophylls, free amino acids, essential fatty acids, vitamin C, trace minerals) found in BFT have a positive effect on cultured animals, such as growth, enhancement of the antioxidant status, and immune response (Ahmad et al. 2017, Bossier & Ekasari 2017).

Tilapia (*Oreochromis* spp.) is recognized as one of the most important aquaculture species due to its rapid growth, easy adaptation to intensive farming, and tolerance to moderate oxygen levels (Bosisio et al. 2017, Yilmaz 2019, El-Sayed 2020). As well as having ideal characteristics for its production in BFT, and a great capacity to feed on various substrates as suspended particles in the water column, microbial masses, and microorganisms present in the BFT (Lumsangkul et al. 2021, Ogello et al. 2021). Notwithstanding, the intensification of its production has led to the appearance of infectious diseases, giving rise to sizable death rates and significant economic losses (Lumsangkul et al. 2021).

Probiotic supplementation for BFT is a very recent area, and the information generated on the benefits of this procedure is still scarce (Daniel & Nageswari 2017, Bañuelos-Vargas et al. 2021). Traditionally, lactic acid bacteria have been widely used in aquatic organisms (Pandiyan et al. 2013). However, in aquaculture, a diverse genus of microorganisms have been considered as potential probiotics, mostly bacteria (Jahangiri & Esteban 2018, El-Saadony et al. 2021), as well as some yeasts, mainly brewer's and baker's yeast (Rima et al. 2012, Navarrete & Tovar-Ramírez 2014, Newaj-Fyzul et al. 2014).

The probiotic properties of yeasts in aquaculture have been scarcely studied. However, antioxidant and immunostimulant properties provide nutrients such as E and B vitamins, amino acids, proteins, mannan-oligosaccharides (MOS), and lipids (Navarrete & Tovar-Ramírez 2014, Schafberg et al. 2020, Ge et al. 2021).

Besides, some species provide a high content of carotenoids (Schafberg et al. 2020, Ge et al. 2021). Another advantage of using yeasts is that they are not affected by antibacterial compounds, and some strains have antagonistic activities against pathogenic bacteria (Rima et al. 2012, Navarrete & Tovar-Ramírez 2014, Caruffo et al. 2015). Yeasts are widely distributed in the water column, sediments, plants, and aquatic animals (Ferreira et al. 2022). Furthermore, play various roles in the aquatic environment; they participate in the decomposition of substrates, recycling of nutrients, biodegradation of oils, and recalcitrant compounds (Navarrete & Tovar-Ramírez 2014, Ferreira et al. 2022).

Rhodotorula is common in various environments and has been isolated from soil, grass, fresh and saltwater, food (milk, fruit juices), human skin, and feces. It has been reported as part of the normal microbiota of wild and farmed fish cultures (Gatesoupe 2007, Kot et al. 2016, Gerelmaa et al. 2018, Chen et al. 2019). Strains belonging to this genus have been used in aquaculture to promote immune response, antioxidant capacity, disease resistance, and increase pigmentation in species such as salmon, sea cucumber, and tilapia (Ueno et al. 2011, Wang et al. 2015, 2019).

Besides, *Rhodotorula* has been used to increase the nutritional value of live food in aquaculture, such as cladocerans (Khudyi et al. 2018), also are capable of using many compounds such as carbon sources, glucose, galactose, sucrose, maltose, ethanol, glycerol, and hexadecane (Navarrete & Tovar-Ramírez 2014, Kot et al. 2016, Gerelmaa et al. 2018). It is a good source of lipids, enzymes, vitamins, MOS, and carotenoids (β-carotene, torulene, and torularhodin) (Bhosale 2004, Kot et al. 2016, Gerelmaa et al. 2018). It is highly valued in pharmaceutical (Bhosale et al. 2002), cosmetic (Anunciato & Da Rocha 2012), food, and feed industries as well in medicine (Saenge et al. 2011, Hernández-Almanza et al. 2014), not only as precursors of vitamin A, but also for its coloration, antioxidant, possible tumor inhibitory activity, and potentiation of the immune response leading to protection against bacteria and fungi infections (Bhosale 2004, Hernández-Almanza et al. 2014, Kushniryk et al. 2015).

Adding probiotics and tilapia culture in Biofloc are independent practices commonly used in aquaculture. However, there is very little information about these technologies applied these technologies applied together. Due to the relevance of expanding the knowledge on the use of these two biotechnologies together, this study aimed to evaluate the effects of yeast *Rhodotorula glutinis* on the performance, expression of the immune response, and antioxidant genes Nile tilapia (*Oreochromis niloticus*) reared under BFT conditions.

MATERIALS AND METHODS

Biological material

Nile tilapias (*Oreochromis niloticus*) juveniles with an average weight of 3.5 ± 0.80 g collected from the Zacatepec-Morelos Aquaculture Center (México) were transported to the facilities of the Laboratory of Chemical Analysis of Live Food of the Autonomous Metropolitan University Xochimilco unit. Fish were acclimatized for four weeks in two 400 L polyethylene circular tanks (working volume of 300 L) on clear water and fed with commercial diets (32% of crude protein) until the beginning of the experiment.

Experimental design

The cultured organisms were randomly distributed in 12 plastic ponds at 75% capacity (100 L), at a density of 214 fish m^{-3}, with four replicates per treatment (15 fish per pond, with an average weight of 7.02 ± 0.04 g). Three experimental treatments were analyzed, Biofloc culture and the addition of the yeast *Rhodotorula glutinis* (BFT+Rg), Biofloc culture without yeast (BFT), and conventional culture without the addition of carbon source and yeast (control).

In all treatments, 15 mm air stones connected to an air pump were used to keep solids in suspension, freshwater was added regularly to compensate for the evaporation loss, and heaters (100 W) were used to maintain the temperature at 29°C. Fish were fed at a rate of 5% biomass with commercial food in three daily rations (09:00, 12:00, and 15:00 h) with a commercial food with a content of 32% protein, 5% fat, 5% fiber, and 5% ash, with a particle size of 1.5-2.0 mm. (Alimentos del Pedregal®, Toluca, State of Mexico, Mexico). It was adjusted every 16 days, according to biomass.

Culture conditions

In Biofloc treatments, at the beginning of the experiment, the ponds were filled with 30% water from a conventional tilapia culture and 70% freshwater to promote a faster maturation of the system. From day one until the end of the trial, molasses was added daily as an external carbon source in a 20:1 C/N ratio to guarantee the proper formation of flocs (Avnimelech 2015), adjusted every 16 days based on the feed ratio supplied. No water exchange was carried out. Only those requested by evaporation were compensated. In clear-water treatment (control), water exchange was carried-out every third day at a renewal rate of 40%.

For the BFT+Rg treatment, an aqueous suspension of *R. glutinis* (32765™ American Type Culture Collection), a concentration of 1×10^6 CFU g^{-1} yeast, was used as an additive. The inoculum was obtained by growing a pure culture of the yeast for two days in YM broth at 27°C. Spectrophotometric measurements of the optical density of the suspension using a bioSan, densitometer (DEN-1 McFarland) were performed to adjust the concentration of the microorganisms to 1×10^6 CFU g^{-1} (Kushniryk et al. 2015, Wang et al. 2019), the inoculum was added twice a day, by spraying on the food and pouring into growing water.

Water quality monitoring

The pH, temperature, and dissolved oxygen (DO) values were monitored daily at 09:00 h, using a Hanna model HI 9829 multiparametric meter. The total ammonia nitrogen (TAN), nitrite, and nitrate levels were measured every 10 days using a Hanna auto-analyzer Aquaculture Photometer (model HI83203) following the manufacturer's procedures. Settleable solids were measured once a week by pouring 1 L of water from the tank for 30 min into an Imhoff cone, as mentioned by Kishawy et al. 2020.

Sampling

After a 12 weeks trial, the fish of each replicate were weighed individually at the beginning of the experiment to obtain initial body weight. Then, fish were weighed at two-week intervals, and a daily mortality record was kept.

Weight gain (WG), survival rate (SR), Fulton's condition factor (CF), feed conversion rate (FCR), and hepatosomatic index (HSI) were calculated as follows:

WG (g) = final weight - initial weight

SR (%) = total number of fish harvested / total number of fish stocked ×100

$CF = (W / L3) \times 100$

where W: total weight of fish (g), L: length of fish (cm) measured from the tip of the snout to the end of the middle caudal fin.

FCR = feed intake / weight gain

HSI (%) = (liver weight / whole fish weight) × 100

At the end of the experiment, eight fish per treatment were randomly captured and sacrificed according to the AVMA recommendations (Underwood et al. 2013) with an overdose of benzocaine. The liver and distal intestine were extracted in cold under aseptic conditions, 50 mg of each tissue stored in RNAlater (Sigma Aldrich, Saint Louis, MI, USA), kept at 4°C overnight, and subsequently stored at -80°C until further processing and analysis (Tröβe et al. 2010).

RNA extraction and quantitative PCR

According to the manufacturer's manual, total RNA was extracted from the liver and intestine using PureLink™ RNA Mini Kit (Invitrogen, USA). The quantity and quality of RNA were measured using gel electrophoresis and spectrophotometrically (Eppendorf BioPhotometer, Hamburg, Germany). Only RNA samples with OD260-OD280 nm ratios between 1.90 and 2.10 were used for expression quantification.

Total RNA (500 ng) was reverse-transcribed in a 20 µL reaction using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Carlsbad CA, USA) in a thermal cycler MyCycler™ (Bio-Rad, USA). The reverse transcription program consisted of 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and finally kept at 4°C. qPCR reactions were performed with one ng of cDNA, sense, and antisense primers (200 nM each, indicated in Table 1) and SYBR® Select Master Mix (Applied Biosystems).

Relative gene quantification was calculated by the $\Delta\Delta C_T$ method (Livak & Schmittgen 2001), using an automated threshold and walking baseline for determining the C_T values. PCR conditions were: an initial denaturation and polymerase activation step for 10 min at 95°C; 40 cycles of denaturing for 15 s at 95°C, annealing and extension for 45 s at 60°C; and a final melting curve from 60 to 95°C for 20 min to check for primer-dimer artifacts (Rotor-Gene 6000, Corbett Life Sciences). The qPCR optimization conditions were made on primer annealing temperature (60°C), primer concentration (200 nM), and template concentration (five 1:10 dilution series in triplicate from 10 ng to 1 pg of input RNA). β-actin (*actb*) was used as the internal reference gene (GenBank acc. no. KJ126772.1). GenBank accession numbers for the studied genes are: GQ853451.1 for *gpx*, JF801727.1 for *sod*, JF801726.1 for *cat*, FJ213839.1 for *hsp70*, XM_003459454.2 for *tgfb*, KJ192194.1 for *trf*, AY428948.1 for *tnfa* and XM_003443911.5 for *il-1b*. The primer sequences and product sizes are shown (Table 1). Primers were designed through Primer3 Software version 0.4.0. reaction efficiencies (E). E and coefficients of determination (R^2) were calculated using the Rotor-Gene 6000 (Corbett Life Sciences) thermal cycler software.

Data analysis

Statistical analysis was performed using the software Statistica 8.0™ (StatSoft, Inc. USA). Water quality, growth, SR, CF, HSI, and gene expression of the culture animals were analyzed using one-way ANOVA followed by a Tukey's posthoc test. For all cases, statistical significance was set at $P < 0.05$.

RESULTS

Water quality

Water quality parameters at the end of the experiment are shown (Table 2). Control tanks have significantly higher DO values (5.71 ± 0.05 mg L^{-1}) than BFT treatments. Water pH tended to be more acidic in BFT+Rg (6.97 ± 0.04) and BFT (7.04 ± 0.05) treatments than in the control group (7.55 ± 0.07). Settleable solids were significantly increased in BFT+Rg (18.18 ± 0.51 mL L^{-1}), compared to BFT (16.36 ± 0.33) and control (3.16 ± 0.08) treatments. No significant differences ($P = 0.18$) were found in nitrite. Meanwhile, TAN and nitrate showed significant differences ($P > 0.05$) between all experimental groups, with higher values in BFT and control groups.

Performance and biological index

The WG, CF, SR, FCR, and HSI are shown in Table 3. The dietary and water culture incorporation of live yeast *Rhodotorula glutinis*, a concentration of 1×10^6 CFU g^{-1} (BFT+Rg), significantly increased FW (46.30 ± 1.75 g), WG (39.31 ± 1.71 g) and showed the lowest FCR (1.59 ± 0.10) in contrast to fish in culture without yeast (BFT), and control treatment. However, survival rates of Nile tilapia were not influenced among treatments, but a tendency to increase was observed in BFT and BFT+Rg treatments. The HSI (2.39 ± 0.10) revealed a significantly higher value ($P > 0.05$) on fish reared in BFT. Concerning CF, a significant increase was observed in the control treatment (1.99 ± 0.25).

Gene expression

In the liver, the expression patterns of innate immune and oxidative stress genes are affected by *R. glutinis* (Figs. 1-2). Regarding natural immune expression, *tnfa*, *hsp70*, and *tgfb* genes significantly increase their expression in BFT+Rg. Where as *trf*, and *il1b*, showed

Table 1. Primer pairs used for qPCR. Primer sequences, amplicon sizes in base pairs (bp), reaction efficiencies (E), and coefficients of determination (R^2) are indicated.

Gene (symbol)	Fwd sequence (5′-3′)	Rev sequence (5′-3′)	Size (bp)	E	R^2
gpx	GGAACGACAACCAGGGACTA	TCCCTGGACGGACATACTTC	160	0.92	0.98
sod	GACGTGACAACACAGGTTGC	TACAGCCACCGTAACAGCAG	386	0.95	0.99
cat	GGCCGGGTTTCTAAAAGAAG	GCTGTAAACGTGCAAAGTGG	154	0.91	0.99
hsp70	CAAGATCACCATCACCAACG	TCTTGTCCTCCTCGCTGATT	206	0.94	0.98
tgfb	CGAGCAGCTGTCCAATATGA	AGGTCCATGGCTTAATGTGC	211	1.05	0.94
tfr	GAGCATCGTCCATTCCCTTA	CTCTGGCATTCAATGGAGGT	159	0.95	0.99
tnfa	TCTGGAGTGGAGGAATGGTC	TCTGAGTAGCGCCAGATCCT	204	0.95	0.99
actb	GAGCGTGGCTACTCCTTCAC	GCAGGATTCCATACCAAGGA	234	0.98	0.99
il-1b	TTT TGG ATC CTC AGG ACA GG	GTA GCA GAA CAT TGG CAG CA	231	0.96	0.98

Table 2. Water quality parameters of experimental groups in the 12 weeks culture trial. Data are presented as means ± standard error. Values in the same row with different superscripts mean significant difference ($P < 0.05$). DO: dissolved oxygen, TAN: total ammonia nitrogen.

Parameter	Treatment			
	Control	BFT	BFT+Rg	*P*
T (°C)	29.45 ± 0.16	29.09 ± 0.15	29.21 ± 0.14	0.22
DO (mg L^{-1})	5.71 ± 0.05^{a}	4.75 ± 0.04^{b}	4.73 ± 0.04^{b}	>0.01
pH	7.55 ± 0.07^{a}	7.04 ± 0.05^{b}	6.97 ± 0.04^{b}	>0.01
Settleable solids (mL L^{-1})	3.16 ± 0.08^{a}	16.36 ± 0.33^{c}	18.18 ± 0.51^{b}	>0.01
TAN (mg L^{-1})	0.52 ± 0.04^{b}	0.71 ± 0.04^{a}	0.60 ± 0.03^{ab}	>0.01
Nitrite (mg L^{-1})	0.89 ± 0.04	0.84 ± 0.01	0.83 ± 0.01	0.18
Nitrate (mg L^{-1})	21.5 ± 0.29^{a}	13.37 ± 0.21^{c}	14.71 ± 0.37^{b}	>0.01

a similar expression in all treatments (Fig. 1). Regarding the expression of oxidative stress genes in the liver (Fig. 2), *sod* and *cat* genes showed significant differences in BTF in comparison with the other treatments. In the case of the hepatic level *gpx* gene, no significant differences were revealed among all treatments (Fig. 2).

The relative expressions of intestinal immune and oxidative stress-related genes are shown in Figures 3-4. The addition of *R. glutinis* significantly affects the expression level in all studied genes (*tnfa*, *hsp70*, *tgfb*, *trf*, and *il1b*) compared with BFT and control treatments (Fig. 3). Similarly, a significant increase was observed in the *cat*, *sod*, and *gpx* expression ($P > 0.05$), in the BFT+Rg treatment in comparison with the other treatments (Fig. 4).

DISCUSSION

It is well known that water quality is very important to maintain the good condition of the species under culture conditions. Poor water quality can limit performance parameters, immunity, and antioxidant indicators (Khanjani & Sharifinia 2020). In the present study, the control treatment has significantly higher DO values than the other treatments due to the regular exchange of water or the absence of bacterial biomass (Lima et al. 2018). On the contrary, in treatments with BFT, the decrease in DO can be attributed to the development of heterotrophic microbial communities, which use it for their metabolic activities (Piñeiros-Roldan et al. 2020, Gálvez-Cantero et al. 2022). Likewise, Faizullah et al. (2015) indicate that these communities' oxygen consumption is very high, reaching more than 70% of total oxygen consumption. The pH of the water tended to be more acidic in the BFT groups compared to the control group. At the same time, the BFT+Rg treatment showed the lowest pH value and the one with the highest volume of flocs, which can translate into a greater number of microorganisms present in the culture tank (Khanjani & Sharifinia 2020, Kumar et al. 2021). Ebeling et al. (2006) mentioned that the respiration processes of the microorganisms in the BFT community influence the pH of the water; in the same way, the acidification of the water can be due to the processes of photosynthesis and nitrification (Boyd et al. 2011, Hlordzi et al. 2020).

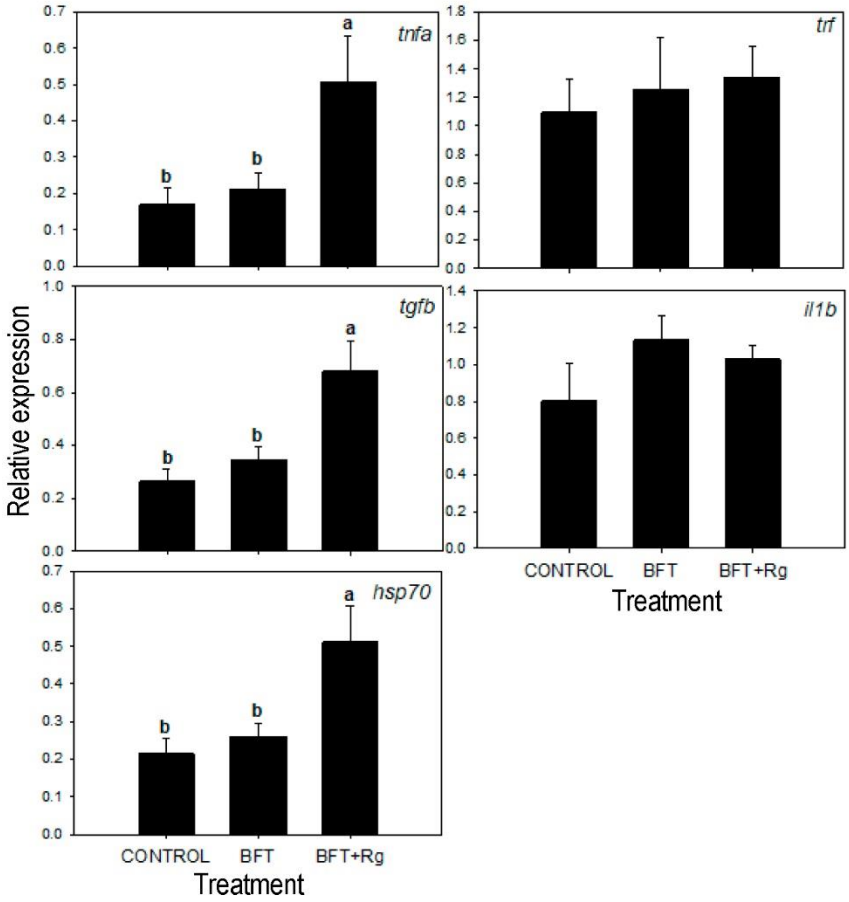

Figure 1. Relative expression of genes related to innate immune response (*tnfa*, *trf*, *tgfb*, *il1b*, and *hsp70*) in the liver of *Oreochromis niloticus* under different rearing conditions after 12 weeks. Data are presented as means ± standard error. Different letters represent significantly different values ($P < 0.05$) between treatments (n = 6).

The assimilation process by heterotrophic bacteria increases the concentration of CO_2 in the water and reduces both the alkalinity and the pH of the water (Boyd et al. 2011, Emerenciano et al. 2017, Kishawy et al. 2020).

The contribution of mannan oligosaccharide (MOS) in BFT by the genus *Rhodotorula* could cause the diversification of probiotic bacteria, which increases the proliferation of lactic acid bacteria that assimilate MOS and secrete lactic acid in the water, which contributes to the acidic pH of the water (Sak-Ubol et al. 2016, Kishawy et al. 2020). Furthermore, most probiotic bacteria that are heterotrophic efficiently use the toxic nitrogenous materials available in the water for their growth (Bossier & Ekasari 2017, Hlordzi et al. 2020). In our study, nitrogenous compounds (TAN, NO_2, NO_3) were kept at appropriate levels for tilapia cultivation in Biofloc (Emerenciano et al. 2017). Water changes (30%) were carried out every three days to maintain good water quality in the control treatment. In contrast, in the BFT and BFT+Rg treatments, the good quality of the water can be attributed to the fact that the microorganisms present in the flocs can assimilate inorganic contaminants from the water column and transform them into microbial protein (Kuhn et al. 2010, Avnimelech 2015, Bossier & Ekasari 2017, Jin et al. 2018, Kishawy et al. 2020). The lowest concentration of TAN was recorded in the BFT+Rg treatment; this reduction can be attributed to the addition of the live yeast *Rhodotorula glutinis* to the water and food. Yeasts are used frequently in the bioremediation of water quality to reduce nitrogen from metabolism excretion and uneaten food (Jin et al. 2018, Ceseña et al. 2021). Authors like Saenge et al. (2011) and Zhang et al. (2016) mentioned the potential of *R. glutinis* and *Rhodotorula* spp. to remove ammoniacal nitrogen in culture water through ammonium assimilation, which is an important way of utilizing an inorganic nitrogen source.

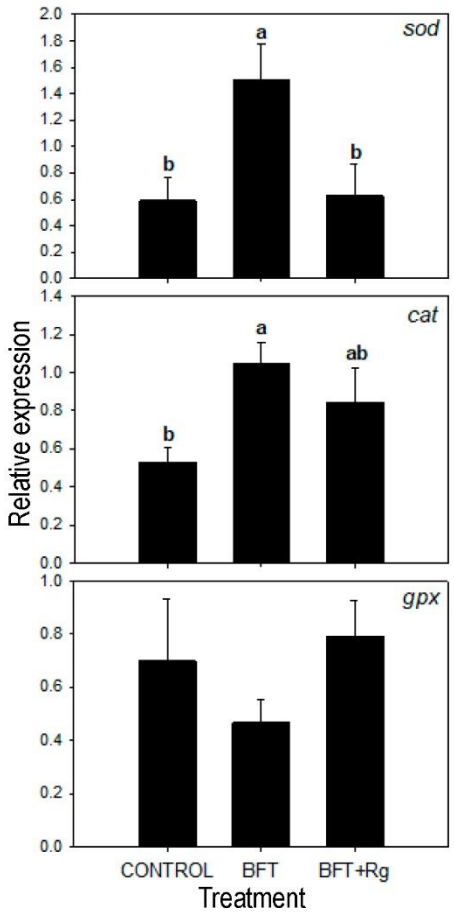

Figure 2. Relative expression of genes related to oxidative stress response (*sod*, *cat*, and *gpx*) *i*n the liver of *Oreochromis niloticus* under different rearing conditions after 12 weeks. Data are presented as means ± standard error. Different letters represent significantly different values ($P < 0.05$) between treatments (n = 6).

Growth performance is an important index commonly used to evaluate the effects of probiotic compounds on BFT trials. Yeasts, in addition, are considered part of the normal microbiota of both wild and farmed fish (Caruffo et al. 2015, Ceseña et al. 2021). In addition, its ability for artificial colonization of the intestinal host has been demonstrated (Ueno et al. 2011, Navarrete & Tovar-Ramírez 2014). Therefore, numerous studies have evaluated the effects of live yeast or its subcomponents and derivatives alone or in combination on the growth of aquatic animals (Navarrete & Tovar-Ramírez 2014, Wang et al. 2015, Chen et al. 2019, Ge et al. 2021). Cells of *Rhodotorula* are rich in several nutrients like E and B vitamins, amino acids, proteins, lipids, carotenoids, and MOS (Chen et al. 2019, Schafberg et al. 2020). In the present study, WG and FCR in fish reared on BFT+Rg were significantly better than those in control and BFT without yeast after 12 weeks of feeding. In addition to the nutritional contribution of the yeast, the growth results observed may be related to the modulation of MOS on gut microbiota. MOS participates in the increased villus integrity and promotes the efficiency of digestion and absorption as well as the energy and building protein produced from β-glucan degenerated by glucanases in the digestive gland (Yuji-Sado et al. 2015, Gelibolu et al. 2018, Chen et al. 2019, Yun et al. 2021). Our results agree with other studies, where strains of the *Rhodotorula* genus, such as *R. benthica* and *R. mucilaginosa*, improved the growth of cultivated organisms (Wang et al. 2015, Chen et al. 2019, Ge et al. 2021). Regarding FCR, the lowest value was obtained in the BFT+Rg treatment; this agrees with Chen et al. (2019), who, when supplementing the diet of *Oreochromis niloticus* with 1% hydrolyzed *R. mucilaginosa* reported a value of 1.12. Similarly, Kishawy et al. (2020) reported the lowest value of FCR when using MOS as a carbon source. The values obtained in BFT and BFT+Rg, fall within the indications of Craig et al. (2017), who mention acceptable FCR values between 1.5 to 2. The increase in growth and decrease in FCR can also be attributed to BFT, as it provides significant amounts of nutrition to farm animals. Previous authors indicate that increased animal growth is due to the intake of microbial flocs that contain the proper nutrients that support growth (Ahmad et al. 2017, Daniel & Nageswari 2017, Khanjani & Sharifinia 2020).

According to Chen et al. (2019), the body condition indices are useful indicators for assessing fish's nutritional and physiological status. The current study exhibited a higher HSI in BFT+Rg compared to the fish in the control and BFT groups. Rocha et al. (2017) mentioned the effect of diet on liver size; the increase in HSI is proportional to the increase of protein in the diet since the higher content of absorbed protein recharges the work of the liver, which responds with an increase in size.

Innate immunity is a very important defense mechanism in fishes because the organism requires mechanisms to protect itself against a large variety of microorganisms immersed in its surrounding environment (Lieschke & Trede 2009, Biller-Takahashi & Urbinati 2013, Lumsangkul et al. 2021). Consequently, this immunity is influenced by diverse internal and external factors (Biller-Takahashi & Urbinati 2013). Regarding internal factors (depending on the organism *per se*), like age, nutritional status, stress, hormonal levels, and sexual maturation cycles

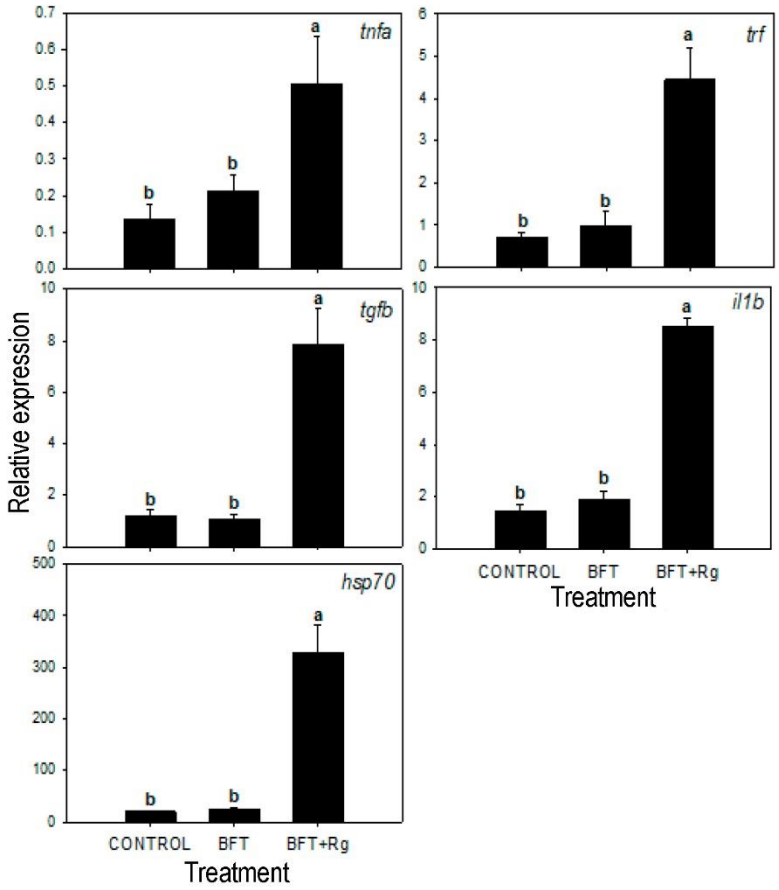

Figure 3. Relative expression of genes related to innate immune response (*tnfa*, *trf*, *tgfb*, *il1b*, and *hsp70*) in the intestine of *Oreochromis niloticus* under different rearing conditions after 12 weeks. Data are presented as means ± standard error. Different letters represent significantly different values ($P < 0.05$) between treatments (n = 6).

(Lumsangkul et al. 2021). Regarding the external ones (attributable to the environment), like temperature, salinity, pH, oxygen level, the organic load of pollutants in the water, and handling of the organisms (Lumsangkul et al. 2021).

In the present study, the expression of different immune-related genes (*tgfb*, *trf*, *tnf*-α, *hsp70*, and *il-1b*) was analyzed in the liver and distal intestine of the experimental fish. The liver is one of the central immune organs in teleost fishes. Likewise, the intestine, especially the posterior segment, is immunologically active and shielded with various immune cells (Wu et al. 2016). It was noticed that there was a significant increase in the expression of target genes in the BFT+Rg fish compared to BFT and control, in both organs, except for *trf* and *il1b*, which only presented upregulation in the intestine, indicating the probiotic influence of live yeast *R. glutinis* in immune response activation. This stimulation is attributable to components present in the yeast, such as β-glucans, nucleic acids, carotenoids, and MOS (Caruffo et al. 2015, Chen et al. 2019, Kishawy et al. 2020). β-glucans are structural components of the cell walls of plants, bacteria, fungi, and yeasts. In yeasts, they have shown interesting immune (modulatory properties) found within the so-called pathogen-associated molecular patterns. They are recognized by different cell receptors of macrophages, dendritic cells, and neutrophils. The interaction with said receptors induces the production of various cytokines (Navarrete & Tovar 2014, Ching et al. 2021, Ge et al. 2021).

Different authors mentioned that various species of yeasts, including *Rhodoturula*, have great benefits as immune promoters (Chen et al. 2019, Nakano & Wiegertjes 2020, Yun et al. 2021). According to Martin & Król (2017), the transcription of immune and stress-related genes is normally applied when investigating the impact of functional feed additives on aquatic animals to understand the mode of action on a gene basis. Cytokines (primarily generated by white blood

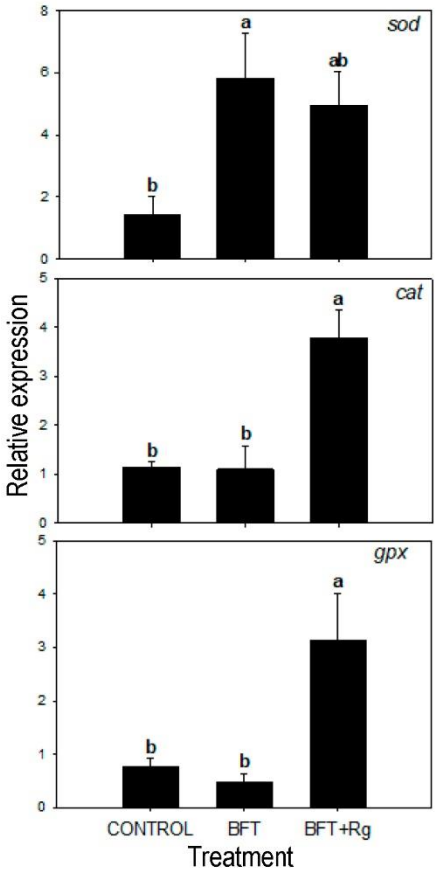

Figure 4. Relative expression of genes related to oxidative stress response (*sod*, *cat*, and *gpx*) in the intestine of *Oreochromis niloticus* under different rearing conditions after 12 weeks. Data are presented as means ± standard error. Different letters represent significantly different values ($P < 0.05$) between treatments (n = 6).

cells) are particularly important and one of the most studied mediators of innate immunity response. Among them, we can mention interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), transforming growth-β (TGF-β), and heat shock protein 70 (HSP-70) (Chen et al. 2014, Wu et al. 2018). TNF-α and IL-1β play multiple functions, like the regulation of cell proliferation, are immune regulators and activate lymphocytes, macrophages, and natural killer cells, stimulating the release of other cytokines during the microbial invasion or when there is a tissue injury (Menaga et al. 2019, Lumsangkul et al. 2021). However, more research needs to be done in this regard. In fish, it has been shown that Hsp70 plays an essential role in immune defense against bacterial or viral infection, can stimulate the cytolytic activity of natural killer cells, and the production of NO and chemokines by macrophages and dendritic cells (Chen et al. 2014, Wu et al. 2018). The TGF-β superfamily plays active roles in immune cell functional regulation, cell proliferation, differentiation, and migration (Lin et al. 2014, Zhan et al. 2015). Transferrin is considered an activator of macrophages and an inhibitor of bacterial growth by sequestering the iron in the blood, limiting its availability for pathogens (Yin et al. 2018). This glycoprotein presents polymorphism, possibly as an adaptation to the capacity that they have developed pathogenic bacteria to obtain iron. Evidence indicates that some proteolytic fragments of transferrin can produce nitric oxide, which is also a powerful bactericidal agent (Stafford & Belosevic 2003). Our results agree with Wang et al. (2015) and Yang et al. (2015). They have mentioned that *Rhodotorula* sp. and *R. benthica* effectively colonized the gut of sea cucumbers and improved resistance against disease. *R. mucilaginosa* has been used as part of the diet with a mixture of microorganisms or alone and has been shown to promote innate immunity in species such as Atlantic salmon (*Salmo salar*), juvenile Nile tilapia, and rainbow trout (*Oncorhynchus mykiss*) (Chen et al. 2019, Wang et al. 2019, Schafberg et al. 2020).

When fish are under stressful conditions such as temperature, hypoxia, handling, crowding, and nutrition, the level of reactive oxygen species (ROS) increases and can result in DNA hydroxylation, protein denaturation, lipid peroxidation, apoptosis, and ultimately cell damage (Yilmaz 2019, Hoseinifar et al. 2020, Schafberg et al. 2020). The production of ROS is elevated by several biochemical reactions in the organism, such as immune defense, oxidative stress, or degenerative diseases. Our results also show that supplementation of live *R. glutinis* improves the antioxidant defense response in tilapia, as adding yeast to BFT produced a significant increase in the expression of genes *cat* and *gpx* in the distal intestine. A tendency to increase *gpx* expression in the liver was observed in the BFT+Rg group, which could be due to the participation of the carotenoids absorbed in the intestine in the mechanisms of activation of the antioxidant system that protect the organism from the oxidation of free radicals of biomolecules caused by stress, as mentioned by Hoseinifar et al. (2020).

Fish have an antioxidant defense system to reduce the negative effects of ROS. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes represent a cellular defense mechanism against the toxicity of ROS (Hoseinifar et al. 2020). CAT, catalyze hydrogen peroxide (H_2O_2) to create water and O_2 control free radicals. SOD converts superoxide radicals into H_2O_2, thus protecting against

damage caused by free radicals (Yilmaz 2019, Lumsangkul et al. 2021). GPx catalyzes the oxidation of glutathione, using H_2O_2 or lipid hydroperoxides as substrates, producing water and alcohols, respectively (Ighodaro & Akinloye 2018, Galeana-López et al. 2021). This enzyme plays an important role in inhibiting lipid peroxidation processes and, consequently, protecting cells from oxidative damage (Hoseinifar et al. 2020).

Exogenous antioxidants have a significant physiological impact as radical or scavenging promoters. Rathod et al. (2021) found that vitamins (i.e. ascorbic acid or tocopherol), phenolic compounds (i.e. flavonoids), and pigments (carotenoids or chlorophylls) are additives that should be included in the daily diet. Some of these can be provided by *R. glutinis* (Navarrete & Tovar-Ramírez 2014, Ceseña et al. 2021). Finally, yeasts are well known for their role in alleviating oxidative stress, growth promoter, and immunostimulant in fish and crustaceans, and *R. glutinis* have the potential to be considered as a probiotic (Navarrete-Ramírez & Tovar 2014, Wang et al. 2015, Zhang et al. 2016, Hoseinifar et al. 2020, Ge et al. 2021). Therefore, organisms under the influence of *R. glutinis* should be in a better condition to respond to stress due to environmental factors or pathogens. More studies testing different yeast species (alive or processed) are necessary to broaden the understanding of their action mechanisms in aquaculture species.

In conclusion, the results showed that supplementing live yeast *R. glutinis* (1×10^6 CFU g^{-1}) in the culture of Nile tilapia reared in BFT promotes growth, enhances immunity, and reduces antioxidative stress. Moreover, based on the results obtained, the application of this yeast species in important aquaculture species deserves to be evaluated.

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