Research, Society and Development, v. 10, n. 16, e183101623385, 2021
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v10i16.23385

Effects of different stocking densities and supplementation with Saccharomyces cerevisiae in juvenile Nile tilapia cultivated in a recirculating water system (RAS)

Efeitos de diferentes densidades de estocagem e da suplementação com Saccharomyces cerevisiae em juvenis de tilápia-do-Nilo cultivados em um sistema de recirculação de água (RAS)

Efectos de diferentes densidades de población y suplementación con Saccharomyces cerevisiae en juveniles de tilapia del Nilo cultivadas en un sistema de recirculación de agua (RAS)

Received: 11/16/2021 | Reviewed: 11/28/2021 | Accept: 11/29/2021| Published: 12/11/2021

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Abstract
The objective of this study was to evaluate the productive performance, organosomatic indices and liver-intestinal histomorphometric parameters of Nile tilapia (*Oreochromis niloticus*) juveniles, fed diets containing probiotics (yeast - *Saccharomyces cerevisiae*) and reared at different densities of storage. In this experiment, 450 juveniles of tilapia (2.9 ± 0.23 g) were distributed in 18 experimental units arranged in RAS and arranged in a randomized block design in a 2x3 factorial scheme. These animals were fed two diets, the first without yeast and the second containing 0.1% yeast inclusion and distributed in three stocking densities of 15, 25 and 35 fish / m^3 (500, 833 and 1166 fish / m^3). There was an improvement in the oxygenation of the medium with the use of yeast, as well as in the lowest stocking densities evaluated (15 and 25 fish / m^3). However, lower specific growth rates and higher viscerosomatic index were observed in animals supplemented with 0.1% yeast. For the average area of hepatocytes, there was an interaction between the evaluated factors (use of probiotic x storage density), where the best values were found in the lowest storage densities (15 and 25 fish / m^3), regardless of use or not of the probiotic. It is recommended to use a stocking density of 15 to 25 fish / m^3 and a supplementation of 0.1% with yeast as a probiotic, in order to improve liver health and oxygenation of the water used to grow Nile tilapia juveniles cultivated in RAS.
Keywords: Density stocking; Probiotic; Saccharomyces cerevisiae; Water quality.

Resumo
O objetivo deste estudo foi avaliar o desempenho produtivo, os índices organossomáticos e os parâmetros histomorfométricos hepático-intestinais de juvenis de tilápia-do-Nilo (*Oreochromis niloticus*), alimentados com dietas contendo probióticos (levedura - *Saccharomyces cerevisiae*) e criados em diferentes densidades de estocagem. Neste experimento, 450 juvenis de tilápia (2,9 ± 0,23 g) foram distribuídos em 18 unidades experimentais dispostas em RAS e dispostas em delineamento de blocos ao acaso em esquema fatorial 2x3. Esses animais foram alimentados com duas dietas, sendo a primeira sem levedura e uma segunda contendo 0,1% de inclusão de levedura e distribuídas em três

densidades de estocagem de 15, 25 e 35 peixes / m^3 (500, 833 e 1166 peixes / m^3). Observou-se melhora na oxigenação do meio com o uso de levedura, bem como, nas menores densidades de estocagem avaliadas (15 e 25 peixes / m^3). No entanto, menores taxas de crescimento específico e maior índice viscerossomático, foram observados em animais suplementados com 0,1% da levedura. Para a área média dos hepatócitos, observou-se interação entre os fatores avaliados (uso de probiótico x densidade de estocagem), onde os melhores valores foram encontrados nas menores densidades de estocagem (15 e 25 peixes / m^3), independentemente do uso ou não do probiótico. Recomenda-se o uso de uma densidade de estocagem de 15 a 25 peixes / m^3 e uma suplementação de 0,1% com levedura como probiótico, com o intuito de melhorar a saúde hepática e a oxigenação da água de cultivo de juvenis de tilápia-do-Nilo cultivados em *RAS*.
Palavras-chave: Densidade de estocagem; Probiótico; *Saccharomyces cerevisiae;* Qualidade da água.

Resumen
El objetivo de este estudio fue evaluar el comportamiento productivo, índices organosomáticos y parámetros histomorfométricos hígado-intestinales de juveniles de tilapia del Nilo (*Oreochromis niloticus*), alimentados con dietas que contienen probióticos (levadura - *Saccharomyces cerevisiae*) y criados en diferentes densidades de almacenamiento. En este experimento, se distribuyeron 450 juveniles de tilapia (2.9 ± 0.23 g) en 18 unidades experimentales dispuestas en RAS y ordenadas en un diseño de bloques al azar en un esquema factorial 2x3. Estos animales fueron alimentados con dos dietas, la primera sin levadura y la segunda con 0,1% de inclusión de levadura y distribuidas en tres densidades de población de 15, 25 y 35 peces / m^3 (500, 833 y 1166 peces / m^3). Hubo una mejora en la oxigenación del medio con el uso de levadura, así como en las densidades de siembra más bajas evaluadas (15 y 25 peces / m^3). Sin embargo, se observaron tasas de crecimiento específicas más bajas y un índice viscerosomático más alto en animales suplementados con levadura al 0,1%. Para el área promedio de hepatocitos, hubo interacción entre los factores evaluados (uso de probiótico x densidad de almacenamiento), donde los mejores valores se encontraron en las densidades de almacenamiento más bajas (15 y 25 peces / m^3), independientemente del uso o no del probiótico. Se recomienda utilizar una densidad de población de 15 a 25 peces / m^3 y una suplementación del 0,1% con levadura como probiótico, con el fin de mejorar la salud del hígado y la oxigenación del agua utilizada para el cultivo de juveniles de tilapia del Nilo cultivada en RAS.
Palabras clave: Densidad de población; Probiótico; Saccharomyces cerevisiae; Calidad del agua.

1. Introduction

Aquaculture is the fastest growing activity in comparison to other agricultural food production sectors (FAO, 2018). From 2001 to 2018, world aquaculture production grew by more than 5% per year, according to data from the State of The World Fisheries and Aquaculture - SOFIA, reaching over 82.1 million tons of fish in the last year. Brazil is currently the 13th largest fish producing country in the world (FAO, 2020), and the incessant search for alternatives aimed at improvements in the aquaculture sector, such as in fish nutrition, is a constant challenge for this activity and for fish farmers (MPA, 2015). Feed costs are one of the most expensive inputs in aquaculture production making alternative ingredients that maintain or even improve the animals performance worth serious consideration (Boscolo *et al.*, 2001; Meurer *et al.*, 2002).

Tilapia is the second most produced species in the world but the most cultivated species in Brazil (FAO, 2020; PeixeBR, 2020) due to its favorable traits, such as good acceptance by consumers, rusticity, rapid growth and meat quality (Meurer *et al.*, 2003; 2008). Nevertheless, throughout the fingerling development stage, high stocking densities are usually adopted (Marengoni, 2006) with occurrences of high mortality rates, since the immune system is still underdeveloped and the natural resistance to diseases is limited (Kirkan *et al.*, 2003; Yin *et al.*, 2008). According to Filipetto *et al.* (2015), proper nutrition during this developmental stage, when fish grow at accelerated rates (Hayashi *et al.*, 2002), can ensure an effective nutritional support for its growth and health maintenance (Kim *et al.*, 2003). Therefore, the provision of a balanced diets containing food additives might be an efficient strategy to mitigate losses in this stage and provide additional benefits in subsequent phases (Hayashi *et al.*, 2002).

The rational for inclusion of additives in diets for tilapia, also known as functional feeds (Ribeiro *et al.*, 2012), are benefits to both water quality and the animal's immune system and growth performance during fingerlings development and fattening stages (Schwarz, 2009; Iwashita *et al.*, 2014). Among several known additives, probiotics are one of the most commonly used in aquaculture. Probiotics are living microorganisms that, when supplied in adequate quantities, can benefit the

host and may reduce the occurrence of diseases (Reid *et al.*, 2003). Probiotics exert their effects in several ways and have been linked to producing enzymes in the intestinal tract, improving its morphology and functionality (Heidarieh *et al.*, 2012). In this sense, when probiotics are ingested by the fish, in addition to modifying its intestinal microbial composition, it positively affects the immune system and, as a consequence, the animals health status (Borquez *et al.*, 2010) and its growth performance (Dharmaraj& Dhevendaran, 2010).

The use of probiotics for aquatic organisms has been gaining attention over the last few years mainly due to the need to improve sustainable management of aquaculture (Gatesoupe, 1999). Among the most used probiotics in fish nutrition, yeasts and their processing derivatives are characterized by their biosafety, easy incorporation to diets and positive effects on growth performance (Hisano *et al.*, 2006; 2008). According to Butolo (2001), yeasts are fundamental ingredients that must be used in diet formulations, as they act like immunostimulants and pro-nutrients - compounds that promote intrinsic nutritional values, which are demanded in lower quantities in animal diets.

The yeast of S. cerevisiae comes from the bakery industry and contains several immunostimulants compounds, such as beta-glucan, nucleic acids, mannan-oligossacharides and chitin (Gopalakannan & Arul, 2010). This yeast has high levels of proteins, amino acids, vitamins of the B complex, fatty acids, minerals and enzymes (Pardo-Gamboa *et al.*, 2011). Studies reported improvements in growth performance (Schwarz *et al.*, 2010) and intestinal health status of animals supplemented with this strain of yeast (Carvalho *et al.*, 2011; Schwarz *et al.*, 2011; Heidarieh *et al.*, 2012); however, no studies that encompasses the hepatic health of animals were performed. Therefore, the aim of this study was to evaluate the inclusion of a probiotic composed by S. cerevisiae yeast and different stocking densities in the growth development, somatic indexes and hepatic-intestinal histomorphometry of Nile tilapia juveniles, in a water recirculation system during the pre-fattening stage.

2. Methodology

2.1 Experimental design

This study was conducted in the Laboratory of Aquaculture and Animal Pathology, of the Barriga Verde University Center (UNIBAVE), located in the municipality of Orleans, Santa Catarina, Brazil and lasted 35 days, between the months of August and September 2019.The experiment was approved by the Ethics Committee on Animal Use (CEUA-UNIBAVE), under protocol number 003/2018. A total of 450 Nile tilapia juveniles (*Oreochomis niloticus*) were acquired from a commercial fish farm (lineage GIFT - EPAGRI SC02), with a mean initial weight of 2.9 ± 0.23g.

The animals were distributed in 18 experimental units in a randomized blocks design, a 2x3 factorial scheme, with the inclusion of probiotics and stocking density as factors. Fish were submitted to two diets, one without the addition of yeast (SP) and one with a 0.1% inclusion level of yeast (CP) containing 100% living cells of S. cerevisiae (strain NCYC Sc 47 - ACTISAF PWD, Phileo, France), and distributed in three stocking densities of 500, 833 and 1166 fish / m-3 (15, 25 and 35 fish per tank, respectively). Each tank was considered as an experimental unit.

The inclusion of the probiotic in the diets was performed every three days. The lyophilized yeast powder was diluted in 100 mL of water from the experimental system's supply (SAMAE – Orleans/SC, Brazil, low salinity water), and sprayed on a micro-extruded commercial diet (40% crude protein CP and 1.77 mm of thickness – BIOBASE®), with the aid of a sprayer (ULTRAJET VERDE – GUARANY®), before being provided to fish. The characterization of the commercial diet is presented in Table 1.

Table 1. Nutritional information of the commercial diet (according to the manufacturer) provided to Nile tilapia juveniles (*O.s niloticus*), supplemented with a probiotic composed of living cells of *S. cerevisiae* in distinct stocking densities.

Nutrient	**Concentration (min.)**
Crude protein (min)	400.0 g kg^{-1}
Ether extract (min)	85.00 g kg^{-1}
Fiber matter (max)	40.00 g kg^{-1}
Mineral matter (max)	70.00 g kg^{-1}
Calcium (min)	10.0 g kg^{-1}
Calcium (max)	25.0 g kg^{-1}
Phosphorus (min)	10.0 g kg^{-1}
Moisture (max)	100.00 g kg^{-1}
Sodium (min)	2.000.00 mg kg^{-1}
Vitamin A (min)	11.200.00 Ul kg^{-1}
Vitamin D3 (min)	2.240.00 Ul kg^{-1}
Vitamin E (min)	128.00 Ul kg^{-1}
Vitamin k3 (min)	10.00 mg kg^{-1}
Vitamin B1 (min)	16.0 mg kg^{-1}
Vitamin B2 (min)	16.0 mg kg^{-1}
Vitamin B6 (min)	16.0 mg kg^{-1}
Vitamin B12 (min)	16.0 mg kg^{-1}
Biotin (min)	0.06 mg kg^{-1}
Nicotinic acid (min)	80.0 mg kg^{-1}
Pantothenic acid (min)	40.00 mg kg^{-1}
Folic acid (min)	5.00 mg kg^{-1}
Choline (min)	2.000.00 mg kg^{-1}
Vitamin C (min)	1.100.00 mg kg^{-1}
Iodine (min)	1.50 mg kg^{-1}
Selenium (min)	0.30 mg kg^{-1}
Iron (min)	85.0 mg kg^{-1}
Copper (min)	11.50 mg kg^{-1}
Zinc (min)	35.0 mg kg^{-1}
Manganese (min)	25.50 mg kg^{-1}
Cobalt (min)	0.50 mg kg^{-1}

Source: Biobase Alimentação Animal LTDA, Av. Anita Boaro 734, Águas Frias, Downtown. ZIP Code 89843-000, Santa Catarina, Brazil.

Each days feed ration was weighed on a precision scale (Electronic Analytical Scale BG 100, Gehaka, Brazil), kept in identifiable plastic containers and stored in an aerated site. Feeding was provided three times a day at 08h00min, 13h00min and 19h00min, at a feeding rate of 15% of the animals live weight in the first week, 8% in the second week and 5% in the following weeks until the end of the experiment. The reduction of feeding rates was defined by means of weekly biometrics, with the aim of establishing a nutritional restrictive challenge.

The animals were allocated in experimental units (polypropylene tanks with 30 L of useful volume) that were oxygenated with porous stones (2 cm) coupled through individual air compressors (SUNSUN® Hp-200, China). These tanks were interlinked by a water recirculation system composed of a circular matrix tank (500 L useful volume) that served as a reservoir/sump. The filtration system was constituted by a mechanical (60-µm perlon mesh) and a biological filter (particulate material like gravel with an approximate volume of 0.1 m3), in order to provide sufficient adhesion surface to bacteria, and assist in the nitrification process. The system was equipped with a heater (2,500 W) and a submerged pump with a capacity of 4,000 liters / hora (ATMAN® model PH4000) that pumped the water to the experimental units, which returned by gravity.

Tanks were cleaned every two days with the recharge water coming from the municipal water and sewage company (SAMAE). Before using this water, it was stored in a 500-L recipient with strong aeration for 2 days, in order to remove the residual chloride. Water quality parameters were measured periodically, with temperature and dissolved oxygen being measured on a daily basis with an oximeter ALFAKIT (model AT-155, Alfakit, Florianópolis/SC, Brazil), and the pH assessed weekly, with the aid of a pH-meter SENSOGLASS (model SP1800, São Paulo/SP, Brazil). Weekly water samplings were conducted with samples kept in a freezer (-20 °C) until analysis. Nitrogenous parameters (total ammonia, nitrite and nitrate) were measured, as well as alkalinity and orthophosphate, with the aid of a photo colorimeter ALFAKIT (model AT-100P, Florianópolis/SC, Brazil). These analyses were performed in a Laboratory of Aquaculture of the Santa Catarina State University (LAQ - UDESC).

At the end of the experimental period, all the fish were captured with nets and gloves, and were manually immobilized (using moistened towels and gloves) for the final biometrics. Growth performance was evaluated according to the following variables: mean initial weight (MIW) (g), mean final weight (MFW) (g), survival rate (S) (%), mean weight gain (MWG) (g), apparent feed conversion (AFC), and specific growth rate (SGR) (% day-1), by means of the equation [(final weight – initial weight) / time in days] x 100.

2.2 Somatic indexes and hepatic-intestinal histomorphometry

Twenty percent of the animals from each experimental unit were randomly selected to measure the somatic indexes and the hepatic-intestinal morphometry, by means of collecting samples from the gastrointestinal tract and the liver. For this purpose, fish were kept in water containing ice for 10 min until loss of balance and reduction of operculum movements (Matushima & Mariano, 1996) with a subsequent medullar transection being performed on those animals (Pedrazzani *et al.*, 2007; CONCEA, 2013). The sampled organs were weighted to calculate the hepatosomatic (HSI) and viscerosomatic (VSI) indexes, according to equations, below:

Subsequently, two hepatic fractions and two portions of the intestines were sent to the Laboratory of Veterinary Pathology of the Barriga Verde University Center (UNIBAVE) for histological procedures, and later for histomorphometric analyses. The histological processing of samples was performed according to the routine methods proposed by Nunes and Cinsa (2016). Fragments of the intestines and liver were placed in sterile plastic containers that were pre-identified and filled with a formaldehyde solution (10%) (this solution was renewed and the tissues were maintained in this solution until histological slides were prepared). Tissue samples were cut in 0.3 cm portions, diaphanized and included in histological paraffin, and then cut in 3 µm sections with the aid of a semi-automatic microtome, according to the methodology of Mello *et al.* (2013), with adaptations. Subsequently, the slides were prepared and stained with hematoxylin-eosin (HE) (Prophet *et al.*, 1992), and then the intestinal villi were measured, as well as the area of hepatocytes (MAH). Samples were analyzed in a light microscope (Bioval®, L-2000) coupled with a camera. Pictures were registered with the aid of the software TCapture®. The other fish remaining in the experimental units were weighted and measured for external evaluation parameters and then these were sent to the Experimental Farm of UNIBAVE.

2.3 Analysis of intestinal and hepatic histomorphometry

The slides prepared for histological evaluations were analyzed with a 10x lens and selected by means of a criterion of integrity, considering four villi per animal, totaling 360 villi. Measurements (Figure 1) were adapted from the methodology described by Picoli *et al.* (2019), being: i) villi's height (VH) as the distance from the apex of the villi until the beginning of the muscle layer; ii) total height of villi (THV) as the height from the villi's apex to the end of the serosa; iii) width of villi (VW); and iv) thickness of the villus epithelium (TV). All measurements were performed with the software ImageJ®. Random

images were taken from slides containing the hepatic tissue stained with HE, from five image fields (40x) for each animal. Then, the area of 20 hepatocytes in each image captured was measured (Figure 2), totaling 100 hepatocytes per animal, according to Picoli *et al.* (2019). The MAH was estimated for each treatment, as recommended by Tessaro *et al.* (2012).

2.4 Statistical Analysis

The data obtained was analyzed for normality of residues (Shapiro-Wilk test) and homogeneity of variances (Levene test). The parameters relating to water quality, growth performance, as well as somatic and histomorphometric indexes, were evaluated by means of a factorial analysis (Myers, 1990; Quinn & Keough, 2002). In case no interaction between variables was found, a one-way variance analysis (ANOVA) was carried out separately for each factor (stocking density and use of yeast), and when significant effects were visualized, the Tukey test of multiple comparison of means was applied at a 5% probability level (Sampaio, 2010).

3. Results

3.1 Water Quality

The water quality parameters evaluated in this study did not show any interaction with stocking density or with the addition of the probiotic ($p > 0.05$); however, significant differences were found when these were individually evaluated (Table 2). Improvement in the oxygenation (DO) of the culture water with the use of probiotic was observed in the lowest evaluated stocking densities (15 and 25 fish / m³). The other parameters (temperature, ammonium, nitrate and pH) were statistically similar ($p > 0.05$) when evaluated individually (Table 2).

Table 2. Water quality parameters in an experimental set-up for rearing juvenile Nile tilapia (*Oreochromis niloticus*) supplemented with a probiotic made of *Saccharomyces cerevisiae*, at different stocking densities.

Treatments		T (ºC)	DO (mg / l)	pH	TAN (mg / l)	N-NO_2 (mg / l)
With probiotic	**15**	25.11 ± 0.18	6.67 ± 0.35	7.23 ± 0.04	2.44 ± 0.21	0.022 ± 0.003
	25	25.17 ± 0.10	6.07 ± 0.46	7.24 ± 0.07	2.29 ± 0.28	0.022 ± 0.003
	35	25.21 ± 0.06	5.48 ± 0.45	7.17 ± 0.07	2.54 ± 0.03	0.023 ± 0.001
Without probiotic	**15**	25.22 ± 0.08	5.85 ± 0.74	7.22 ± 0.11	2.51 ± 0.27	0.023 ± 0.003
	25	25.11 ± 0.10	5.91 ± 0.35	7.19 ± 0,06	2.44 ± 0.19	0.022 ± 0.003
	35	25.27 ± 0.01	5.06 ± 0.17	7.12 ± 0.09	2.48 ± 0.13	0.023 ± 0.002
15		25.16 ± 0.14	6.26 ± 0.69 a	7.22 ± 0.07	2.47 ± 0.22	0.022 ± 0.003
25		25.14 ± 0.10	5.99 ± 0.37 a	7.22 ± 0.06	2.36 ± 0.23	0.022 ± 0.002
35		25.24 ± 0.05	5.27 ± 0.38 b	7.15 ± 0.08	2.51 ± 0.09	0.023 ± 0.001
With probiotic		25.16 ± 0.12	6.07 ± 0.63 a	7.22 ± 0.04	2.42 ± 0.21	0.023 ± 0.002
Without probiotic		25.20 ± 0.09	5.61 ± 0.59 b	7.18 ± 0.09	2.48 ± 0.18	0.023 ± 0.002
p value	Probiotic	NS (0.49)	0.04*	NS (0.27)	NS (0.53)	NS (0.19)
	Density	NS (0.26)	0.00*	NS (0.14)	NS (0.42)	NS (0.52)
	Interaction	NS (0.37)	NS (0.46)	NS (0.92)	NS (0.66)	NS (0.79)

T: Temperature; DO: dissolved oxygen; pH: hydrogen potential; TAN: total ammonium nitrogen; N-NO_2: nitrite; NS: not significant; *: $p < 0.05$. Source: Authors.

3.2 Growth performance and somatic indexes

No significant interaction between stocking densities and/or the use of the probiotic supplement was observed in relation to the evaluated growth performance parameters or somatic indexes ($p > 0.05$); however, the animals supplemented with the probiotic presented a lower SGR (2.65 ± 1.11% day $^{-1}$) and VSI (7.88 ± 2.53%) in comparison to the animals that did not receive the yeast, which had a SGR of 3.27 ± 1.12% day $^{-1}$ and a VSI of 9.87 ± 2.68% ($p < 0.05$) (Table 3). The other parameters (MIW, MFW, MWG, S, AFC and HSI) did not present any statistical difference relating to supplementation ($p > 0.05$). Similarly, no differences were observed regarding the effects of stocking densities ($p > 0.05$) on the growth parameters and somatic indexes evaluated (Table 3).

Table 3. Growth performance and somatic indexes of Nile tilapia (*O. niloticus*) juveniles, supplemented with a probiotic (*S. cerevisiae*) at different stocking densities for 35 days.

Treatments		**MIW (g)**	**MFW (g)**	**S (%)**	**MWG (g)**	**FC**	**SGR (% day $^{-1}$)**	**VSI (%)**	**HSI (%)**
With probiotic	15	2.96 ± 0.45	12.15 ± 1.20	60.00 ± 17.64	9.19 ± 1.16	1.48 ± 0.30	2.71 ± 0.82	7.50 ± 2.16	2.34 ± 0.70
	25	2.90 ± 0.15	10.10 ± 0.23	78.67 ± 16.17	7.20 ± 0.21	1.90 ± 0.15	2.47 ± 1.26	7.69 ± 3.01	2.29 ± 1.14
	35	3.00 ± 0.39	11.24 ± 0.84	70.48 ± 14.38	8.19 ± 0.96	1.75 ± 0.29	2.76 ± 1.13	8.15 ± 2.39	2.40 ± 0.67
Without probiotic	15	3.00 ± 0.06	11.76 ± 1.22	68.89 ± 3.85	8.76 ± 1.21	1.70 ± 0.18	3.92 ± 0.49	10.94 ± 2.31	2.84 ± 0.65
	25	2.96 ± 0.24	12.17 ± 1.57	77.33 ± 11.55	9.21 ± 1.36	1.65 ± 0.18	3.43 ± 1.26	10.26 ± 3.31	2.14 ± 0.77
	35	3.05 ± 0.09	10.84 ± 0.26	74.29 ± 9.90	7.79 ± 0.26	1.84 ± 0.03	2.92 ± 1.11	9.21 ± 2.28	2.73 ± 0.93
15		2.98 ± 0.29	11.96 ± 1.10	64.44 ± 12.41	8.98 ± 1.09	1.59 ± 0.25	3.37 ± 0.89	9.38 ± 2.78	2.61 ± 0.69
25		2.93 ± 0.18	11.14 ± 1.51	78.00 ± 12.59	8.20 ± 1.40	1.77 ± 0.20	2.55 ± 1.33	8.98 ± 3.35	2.21 ± 0.95
35		3.03 ± 0.26	11.04 ± 0.60	72.38 ± 11.24	7.99 ± 0.67	1.80 ± 0.19	2.84 ± 1.10	8.70 ± 2.35	2.57 ± 0.82
With probiotic		2.95 ± 0.31	11.16 ± 1.16	69.71 ± 16.14	8.19 ± 1.15	1.71 ± 0.29	2.65 ± 1.11 b	7.88 ± 2.53 b	2.35 ± 0.84
Without probiotic		3.01 ± 0.14	11.59 ± 1.16	73.50 ± 8.67	8.58 ± 1.11	1.73 ± 0.15	3.27 ± 1.12 a	9.87 ± 2.68 a	2.55 ± 0.86
p value	Probiotic	NS (0.66)	NS (0.44)	NS (0.52)	NS (0.46)	NS (0.85)	0.03*	0.00*	NS (0.36)
	Density	NS (0.81)	NS (0.34)	NS (0.19)	NS (0.30)	NS (0.24)	NS (0.43)	NS (0.78)	NS (0.26)
	Interaction	NS (0.99)	NS (0.09)	NS (0.79)	NS (0.08)	NS (0.19)	NS (0.27)	NS (0.36)	NS (0.49)

MIW: mean initial weight; MFW: mean final weight; S: survival rate; MWG: mean weight gain; FC: feed conversion; SGR: specific growth rate; VSI: viscerosomatic index; HSI: hepatosomatic index; NS: not significant; *: $p < 0.05$. Fonte: Autores.

3.3 Histomorphometric analysis

A lack of significant differences were observed in the evaluations of intestinal variables (THV, VH, VW and TV) ($P > 0.05$) among treatments and stocking densities, as well as the interaction between them (Table 4). Contrarily, for the average area of hepatocytes, an interaction was observed between the determination factors (use of probiotic x stocking density), where the best values were found in the lowest stocking densities (15 and 25 fish / m^3), regardless of using or not the probiotic (Table 4).

Table 4. Hepatic-intestinal histomorphometry of Nile tilapia juveniles (*O .niloticus*) supplemented with a probiotic made of *S. cerevisiae*, at different stocking densities.

Treatments		THV (*μm*)	VH (*μm*)	VW (*μm*)	TV (*μm*)	MAH (*μm*^{2})
With probiotic	15	395.33 ± 6.50	289.66 ± 43.13	95.14 ± 11.33	69.18 ± 36.72	249.09 ± 13.35 a
	25	401.19 ± 58.19	297.86 ± 49.44	113.91 ± 33.87	53.71 ± 10.68	166.91 ±40.53 ab
	35	390.34 ± 40.81	297.57 ±31.02	99.50 ± 1.84	49.50 ±1.06	157.74 ± 12.40 b
Without probiotic	15	362.62 ± 23.43	285.30 ± 21.06	107.54 ± 22.99	50.53 ± 10.75	182.93 ± 8.82 ab
	25	414.13 ± 28.86	316.45 ± 23.54	103.63 ± 18.17	47.84 ± 7.72	147.44 ± 11.85 b
	35	416.54 ± 89.77	316.39 ± 71.40	106.09 ± 11.54	50.69 ± 3.30	157.75 ± 61.65 b
15		378.98 ± 26.61	287.48 ± 30.45	101.34 ± 17.57	59.85 ± 26.26	216.01 ± 37.62 a
25		407.66 ± 41.69	307.15 ± 36.10	108.77 ± 24.95	50.77 ± 8.94	107.17 ± 70.68 b
35		403.44 ± 60.11	306.98 ± 50.30	102.80 ± 8.22	50.09 ± 2.29	157.75 ± 39.77 ab
With probiotic		395.62 ± 36.01	295.03 ± 36.51	102.85 ± 19.80	57.46 ± 21.13	191.25 ± 48.90 a
Without probiotic		397.76 ± 52.48	306.05 ± 42.02	105.75 ± 15.84	49.69 ± 6.96	129.37 ± 70.00 b
p value	Probiotic	NS (0.92)	NS (0.74)	NS (0.32)	NS (0.57)	0.00*
	Density	NS (0.52)	NS (0.63)	NS (0.77)	NS (0.51)	0.00*
	Interaction	NS (0.54)	NS (0.87)	NS (0.59)	NS (0.59)	0.02*

THV = total height of villi; VH = villi's height; VW = villi width; TV = thickness of the villi's epithelium (EV); MAH = mean area of hepatocytes; NS: not significant; *: $p < 0.05$. Source: Authors.

4. Discussion

Over the years, probiotics have been used to improve water quality in aquacultural enterprises due to their beneficial effects in reducing the concentration of nitrogen compounds and competing with pathogenic microorganisms in the rearing Environment (Porubcan, 1991a; 1991b; Wang *et al.*, 2000; 2008; Wang & Xu, 2004; Zhou *et al.*, 2009; Martínez Cruz *et al.*, 2012). In this study, the water quality parameters evaluated remained within the adequate limits for the cultivated species (El Sayed, 2020). The improvement in the levels of DO with the probiotic inclusion (6.07 ± 0.63 mg / l) in relation to the treatment without the yeast (5.61 ± 0.59 mg / l) ($p < 0.05$) corroborates with the findings of Abdulrahman and Muhammad (2012), who evaluated *S. cerevisiae* inclusion levels for common carp (*Cyprinus carpio*). Those authors reported higher concentrations of DO (5.31 mg/l in the water cultivated with 7% of dry yeast in comparison to the control treatment (3.09 mg / l). This substantially higher value of DO might be related to the abundance of yeasts in the water, which increased the photosynthetic activity of the environment and led to the production of oxygen (Abdulrahman & Muhammad, 2012).

Although the levels of DO are within the adequate range for tilapia (El Sayed, 2020), the treatments with lower stocking densities (15 and 25 fish per experiment unit) suggest a higher availability of oxygen to be used by the fish, seen that at a lower density in a same volume of water, the use of oxygen in comparison to a denser environment (35 fish per unit) is

lower. This result corroborate the study of Tucker *et al.* (1979), who observed a lower weight gain, survival and feed conversion of catfish (*Ictalurus punctatus*), when the mean levels of DO in the morning were reduced from 4.5 to 2.1 mg / l, due to an increased stocking density in the ponds.

In a study conducted by Ferreira *et al.* (2018), the authors supplied *S. cerevisiae* for tilapia reared in treated sludge and observed a mean value of 1.98 ± 0.51 mg / l of DO, which is considerably lower to the values observed in the present study. Taoka *et al.* (2006a) reported improvements in the survival of fish and in water quality parameters such as pH, $N\text{-}NH_4$, $N\text{-}NO_2$ and $P\text{-}PO_4$ when rearing Japanese sole (*Paralichthys olivaceus*) supplemented with several probiotics in their diet (*Bacillus subtilis* at 1.6 × 107 CFU g-1; *Lactobacillus acidophilus* at 1.2 × 108 CFU g-1; *Clostridium butyricum* at 2.0 × 107 CFU g-1; and *S. cerevisiae* at 1.6 × 107 CFU g-1). In another study, Taoka *et al.* (2006b) demonstrated the beneficial effects of *S. cerevisiae* supplementation in immunological parameters of Nile tilapia, such as lysozyme activity, neutrophil migration and plasma bactericidal activity, resulting in improved resistance to infection by *Edwardsiella tarda*.

The results obtained for the animals' SGR corroborated with Lara-Flores *et al.* (2003), who included *S. cerevisiae* in diets for Nile tilapia juveniles and verified a positive effect on SGR; however, regarding the other growth performance parameters (MFW, AFC and survival), the results of that study are in discordance with the ones presented here. Conversely, Oba *et al.* (2011) did not observe significant differences in the SGR of tambaqui (*Colossoma macropomum*) supplemented with *S. cerevisiae*, while Asadi Rad *et al.* (2012) reported values (8.13± 0.14%) that were similar to the present study (8.98 ± 2.65%), when supplementing tilapia with 1% of the yeast.

When rearing tilapia in conditions similar to this study (0.1% *S. cerevisiae* and a sanitary challenge), Meurer *et al.* (2007) did not observe any influence on FMW, survival and HSI of the animals; however, the probiotic's inclusion efficiently reduced the number of total coliforms in the water and in the animals' intestines (Meurer *et al.*, 2004). It was suggested that the probiotic inclusion may have colonized the gastrointestinal tract of the fish and modified the local bacterial community. In addition, Meurer *et al.* (2006) tested the yeast during the sex reversal stage and did not find improvements in the fish's performance.

When using 0.2% of *S. cerevisiae* yeast in diets for Nile tilapia juveniles, Schwarz *et al.* (2016) observed a higher deposition of crude protein in the carcass, as well as a higher AFC and weight at 60 days, in comparison to the fish fed a diet with an inclusion level of 0.4%, and the control. Assumpção (2011) provided diets supplemented with the same yeast (1%) to tilapia reared in brackish water ponds during the sexual reversal stage and reported improved AFC and higher weight gain up to 4 months of age, corroborating with Baccarin and Pezzato (2001), and Castro and Cervon (2004). Finally, in the study developed by Sutthi and Thaimuangphol (2020), the authors fed Nile tilapia with a 0.5% inclusion level of *S. cerevisiae* and observed a higher growth performance, body composition and better blood chemistry parameters in treatments where the fish were reared in low salinity (0 and 5 ppt).

Meurer *et al.* (2009) also used *S. cerevisiae* at a concentration of 0.1% as a probiotic in diets for Nile tilapia; however, those authors reported low values of HSI in animals supplemented with the yeast. This index indicates if the fish is using its glycogen and lipids reserves (Young & Cech, 1994; Yogata & Oku, 2000), making a correlation between the animal's nutritional status and its growth rate (Busacker *et al.*, 1990). Under adequate handling conditions, the animals' performance is scarcely influenced by probiotic supplementation (Lima *et al.*, 2003), which is based on the lower probability of contact between the animals and pathogenic microorganisms in these conditions (Loddi *et al.*, 2000; Zuanon *et al.*, 1998), as also mentioned by Meurer *et al.* (2004). It is noteable that, although the animals in this study were maintained in high densities and with a restrictive decreasing feeding rate, they were submitted to adequate conditions of feed management and hygiene. This might justify the lack of clear improvements of the growth performance parameters, seen that the effect of the probiotic tends

to be boosted when the animals are exposed to stress or extreme sanitary conditions (Lima *et al.*, 2003; Macari & Furlan, 2005). Even though no significant effects ($p > 0.05$) were observed for the evaluated parameters (MFW, AFC, HSI and survival), regardless of stocking density and presence of the probiotic, it was evident that no harms was caused to the animals by the addition of the yeast.

Regarding the intestinal villi of animals, no significant differences ($p > 0.05$) were observed for the evaluated parameters. In addition, no damages were observed in the intestinal mucosa, regardless of probiotic inclusion or stocking density. According to Hisano *et al.* (2006), when supplementing Nile tilapia with intact yeasts of *S. cerevisiae* (2.0%), beneficial intestinal morphological alterations are expected, such as an increased number of villi. These benefits occur due to the presence of nucleotides and polysaccharides in the yeast's cell wall, which have an important function in the differentiation and growth of intestinal cells (Hisano *et al.*, 2006). It is noteworthy that larger intestinal villi provide a higher absorptive area in the mucosa, and as a consequence, benefits are observed, such as a higher rate of nutrients absorption (Cechim, 2013; Hisano *et al.*, 2006; Picoli *et al.*, 2019).

When adopting an inclusion rate of 0.4% of yeast throughout an experimental period of 30 days (similar to the present study), Cechim (2013) observed beneficial effects in the animals' intestinal villi. Similar results were reported by Lima (2014), who supplemented diets for yellowtail *Astyanax altiparanae* with *S. cerevisiae*, which has the capacity to adhere in the bonding sites of enterocytes, and by means of competitive exclusion with pathogenic bacteria, it reduces their colonization in the intestines (Noga, 1995; Macari & Furlan, 2005; Mello *et al.*, 2013).

Despite all the benefits that yeasts can provide to the health of intestines, a 0.1% inclusion level of *S. cerevisiae* during 35 days of experiment was not sufficient to positively alter the histomorphometry of intestines of supplemented fish, even though it did not harm the animals. Different food additives have beneficial results in terms of fish intestinal morphology (Schwarz *et al.*, 2011; Lima, 2014; Honorato *et al.*, 2014), with similar results in comparison to this study. For instance, studies with Bacillus subtilis have also demonstrated increases in villi's height and thickness of the intestinal epithelium (Mello *et al.*, 2013; Carvalho *et al.*, 2011; Nakandakare *et al.*, 2013). Mello *et al.* (2013) attributed the results to an inhibitory action of probiotic bacteria on the multiplication of undesirable bacteria in the intestines, which leads to a higher integrity, digestibility and absorption of nutrients.

The liver plays several functions in the organism, such as accumulating substances as energy reserves, especially in the form of glycogen and lipids (Roberts, 2012; Wolf *et al.*, 2015). Hepatocytes constitute the greatest share of the hepatic parenchyma (Cavichiolo, 2009). In conditions where there is a lack of nutrients, these reserves are used and the volume of these cells tend to reduce (Roberts, 2012; Wolf *et al.*, 2015). In this study, an increased area of hepatocytes was observed ($p < 0.05$) at different stocking densities and probiotic inclusion levels, and an interaction was found between them (Table 4). This suggests an excellent effect of the yeast on the hepatic function of supplemented animals, seen that the higher areas of hepatocytes observed in the supplemented group at the lowest stocking density (15 animals per experimental unit), represented higher energy stocks to be used. In addition, it was estimated that the animals in these conditions did not have any scarcity of nutrients, which may justify the non-treated group at the densities of 25 and 35 fish per experimental unit. It was speculated that stocking density might be related to a stressful factor for the fish, seen that this limitation could interfere in the amount and quality of available nutrients on an individual level. This fact could justify the lower area of hepatocytes observed in treatments where fish were cultivated in higher densities, which also showed the beneficial effect of S. cerevisiae in relation to the control, where those effects were not observed.

Hassaan *et al.* (2018) observed an improved structure of hepatocytes when supplementing diets for Nile tilapia with 5 g kg-1 (0.05), 10 g kg-1 (0.1%) and 15 g kg-1 (1.5) of *S. cerevisiae*, in addition to reporting lower levels of alanine

aminotransferase (ALT) and aspartate aminotransferase (AST). Jarmolowicz *et al.* (2012) also reported lower activities of AST and ALT, which are hepatic enzymes liberated in the blood when hepatic cells are damaged, and its reduction might indicate an improvement of the hepatic function (Metwally, 2009). Bombardelli et al (2010) observed an increased area of hepatocytes with lipid deposition caused by a more energy-rich diet, which is common in diets containing high energy levels, since the liver is responsible for storing excess energy (Wolf *et al.*, 2015). It is suggested that an increased area of hepatocytes, and consequently the hepatic energy reserve, as observed in this study, can be related to an increment of the nutrients use obtained with the addition of *S. cerevisiae*, considering that this yeast has a high protein content and good digestibility for nutrients and amino acids.

In addition, improvements in the digestibility of dry matter, crude protein, crude energy and most essential and non-essential amino acids were also observed (Fuller, 1989; Lara-Flores *et al.*, 2003; Hisano *et al.*, 2008; Cornélio, 2009). In accordance with this, Picoli *et al.* (2019) observed an increased area of hepatocytes and villi's height when using bee pollen as a feed additive for Nile tilapia. The authors justified those improvements due to the higher supply of nutrients to the fish, especially regarding amino acids coming from the pollen, which supplemented the hepatic glycogen reserves. Campos *et al.* (2008) suggested that such an increase can come from essential amino acids that are present in the composition of the pollen, which after meeting the animal's basic needs, were deposited in the liver as an energy reserve (Wolf *et al.*, 2015).

5. Final Considerations

It is recommended to use a stocking density of 15 to 25 fish / m³ and the supplementation of 0.1% *of S. cerevisiae* as a probiotic to improve hepatic health and water oxygenation in a water recirculation system of Nile tilapia (*O. niloticus*) juveniles cultived in *RAS.* More studies are needed to elucidate the use of *S. cerevisiae* as a health promoter in Nile tilapia diets.

Ethics approval

The experiment was approved by the Ethics Committee on Animal Use (CEUA-UNIBAVE), under protocol number 003/2018.

Conflicts of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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