

Article

Study of the Effect of Dietary Agavin Supplementation in Blood Parameters and Antioxidant Enzymes of Juvenile Nile Tilapia (*Oreochromis niloticus*) under Stress Conditions

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Abstract: High-density aquaculture produces oxidative stress that affects fish welfare and leads to poor growth. Agavin is a fructan that regulates the antioxidant response and inhibits stress-related alterations in blood metabolites (cortisol and blood lipids), preventing oxidative damage in fish. This study evaluated the effect of dietary agavin on the hepatic antioxidant response and blood metabolites in Nile tilapia under high rearing density. A negative control, a positive control, and experimental diets supplemented with 20 and 40 g/kg agavin were formulated. Nile tilapia (1.04 g ± 0.01 g initial body weight) were fed for 80 days to evaluate the antioxidant response and blood metabolites. After 90 days, fish were exposed to high density (63 kg/m³) for twenty days, except for the negative control (low-density). Under high-density stress, cortisol, cholesterol, and triglyceride levels decreased in fish fed diets with agavin compared with the positive control (high density). CAT and SOD activity significantly increased in fish fed agavin diets; malondialdehyde levels decreased under high density compared with both controls. These results suggest that dietary agavin supplementation promotes the antioxidant response and prevents stress-related alterations in blood parameters in Nile tilapia under high rearing density through its antioxidant properties and, probably, has a prebiotic effect.

Keywords: aquaculture; agavin; lipid peroxidation; stress hormone; serum lipids



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1. Introduction

Nile tilapia (*O. niloticus*) is among the most extensively produced aquaculture species worldwide, mainly for its importance as a protein source in human food and its favorable characteristics in terms of adaptation capacity, reproduction, and high tolerance to adverse aquaculture conditions [1,2]. However, this activity faces several challenges due to stressful conditions and diseases on farms, which may lead to significant economic loss [2].

Among the main stress factors is intensive aquaculture, whereby fish are reared at high densities that cause several metabolic alterations affecting cultured organisms. Fish subjected to stressful conditions develop an adaptive physiological response consisting of a cascade of metabolic signals at three levels [3]. The first involves endocrine changes in the production of catecholamines and corticosteroids, such as increased cortisol. The second includes changes in blood parameters related to energy mobilization (glucose, cholesterol, and triglycerides) in response to an alert phase [4]. Metabolic pathways involving the disruption of cellular redox homeostasis can also be affected, leading to an increase in reactive oxygen and nitrogen species (ROS and RNS, respectively) and oxidative stress, which causes oxidative damage (lipid peroxidation) to tissues, mainly the liver

and important biomolecules. The third level causes poor growth and disease resistance, affecting behavior and survival [3,5,6].

To offset oxidative stress, fish have antioxidant defense mechanisms that include four levels. The primary level is the most important; it is formed by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase, which stabilize ROS production and reduce oxidative stress [7–9]. Therefore, alternative strategies to prevent and reduce the negative effects of high-density stress in Nile tilapia by regulating the antioxidant response of fish are urgently needed to ensure healthy production. The use of feed additives with bioactive properties such as antioxidant and prebiotic effects has gained popularity in recent decades. Several sources can be used for bioactive compound extraction, particularly agro-industrial wastes such as mango peel and corn husk [10,11], as well as agave waste, which can be used as antioxidant sources for aquatic species. Agave wastes are a source of important bioactive compounds with antioxidant and prebiotic properties [12–14]. Agave species contain agavin, a fructan formed by fructose and glucose polymers with a non-linear structure, a 2–30 degree of polymerization (DP), and β -2,1 y β -2,6 linkages; it can be obtained from agave waste [12,14]. The tequila industry produces approximately 300,000 tons of agave waste annually [15,16]. Studies addressing the effect of dietary agavin on aquatic organisms are scarce. The addition of dietary fructans has improved growth performance, with beneficial effects on the biological index, stress resistance, and blood metabolites, promoting fish welfare of different species [13,17,18].

Dietary agavin supplementation has been reported to play several biological roles, such as obesity prevention and immunostimulating and antioxidant effects [13,19,20]. Previous studies in mammals have demonstrated that fructans can promote growth performance and reduce blood glucose, triglycerides, and cholesterol levels, mainly by its resistance to hydrolysis by digestive enzymes, leading to the generation of microbial metabolites such as short-chain fatty acids (SCFAs) from fructan fermentation [13,21,22], which in turn can stimulate enzymatic antioxidant response (ROS scavengers) by triggering the expression of antioxidant enzymes (SOD, CAT and glutathione peroxidase) improving the redox status of the organism and therefore resistance to stress [9,23,24]. However, to our knowledge, no study has reported the effects of dietary agavin supplementation in diets for juvenile tilapia under intensive aquaculture systems. The present study hypothesizes that dietary agavin can protect Nile tilapia from high-density stress that induces side effects. This study aimed to evaluate the effect of dietary agavin on the hepatic antioxidant response and biochemical blood metabolites in Nile tilapia under high rearing density.

2. Materials and Methods

The agavin used in this study was donated by the IMAG company and was extracted from the cones of *Agave tequilana* Weber Var. Blue, with a degree of purity of 95%.

2.1. Agavin Analyses by MALDI-TOF-MS

MALDI-TOF-MS analyses were performed as described by Campos-Valdez [25] using a Microflex LT (Bruker Daltonics, Billerica, MA, USA) in the positive-ion mode. The instrument was operated at 10 kV. The agavin sample was dissolved in distilled water (10 mg/mL). The ionization matrix was 2,5-dihydroxybenzoic acid in ethanol: water (50% v/v); 1 μ L of water sample mixtures were applied to the reading plate and allowed to dry at room temperature. The equipment was calibrated from 13 to 14 m/z. A commercial mixture of agave fructooligosaccharides was used as the calibration standard (3 mg/mL).

2.2. Experimental Diets

Fish were fed either a control diet (D0) or two experimental diets with agavin supplemented at 20 g/kg (D20) or 40 g/kg (D40) (Table 1) [26]. The agavin inclusion dose of 20 g/kg was selected based on the best growth, and intestinal health results in *Totoaba macdonaldi* fed diets containing 10, 20, and 30 g agavin/kg diet (Fuentes-Quesada) [18], while that the dose of 40 g/kg was selected taking as a reference the eating habits of Nile tilapia

since unlike totoaba (carnivorous) tilapia is omnivorous and tolerates a greater amount of non-digestible carbohydrates FAO [2]. The experimental diets were isolipidic (7%) and isoprotein (40%). All diets had the same levels of fish meal, soybean meal, polished rice meal, wheat meal, sorghum meal, meat and bone meal, krill meal, hydrolyzed fish, fish and vegetable oil, soybean lecithin, dicalcium phosphate; the vitamin and mineral mix was kept constant in all diets. The macro ingredients were ground in a hammer mill to a particle size of 250 μm and mixed in a Hobart mixer, (model AT-200, Troy, OH, USA), followed by the addition of micro-ingredients. Fish oil and soybean lecithin were added until a homogeneous mixture was obtained, which was pelletized in a meat grinder (Tor-Rey 22, model M-22 R2, Monterrey, Nuevo León, Mexico). Pellets were dried with forced air at 40 °C for 6 h, manually ground to approximately 1300 μm , sieved to remove fine particles, and stored in sealed containers at 4 °C until use.

Table 1. Formulation and chemical composition of the experimental diets.

Ingredient (g/100 g)	D0	D20 *	D40 *
Fish meal ^a	32.60	32.60	32.60
Soybean meal ^b	33.00	33.00	33.00
Polished rice meal ^b	4.00	4.00	4.00
Sorghum meal ^b	3.00	3.00	3.00
Meat and bone meal ^b	7.00	7.00	7.00
Soybean lecithin ^b	0.65	0.65	0.65
Corn meal ^b	8.40	6.20	4.00
Krill meal ^b	2.00	2.00	2.00
Wheat meal ^c	5.00	5.00	5.00
Calcium phosphate dibasic ^d	0.20	0.20	0.20
Hydrolyzed fish ^e	3.00	3.00	3.00
Vitamin and mineral mix ^f	1.00	1.00	1.00
Fish and vegetable oil ^g	0.20	0.20	0.20
Agavin (fructan) ^h	0.0	2	4
	Proximal composition		
Dry matter	93.41	93.07	93.79
Crude protein	35.90	35.81	35.63
Crude lipid	6.98	7.01	6.90
Ash	18.96	18.79	18.49
Nitrogen-free extract (NFE)	31.57	31.46	32.77

* D20: 20 g/kg agavin diet; D40: 40 g/kg agavin diet. Mean \pm SE ($n = 3$). ^a "Premium"-grade fish meal, Selecta de Guaymas, S.A. de C.V. Guaymas, Sonora, Mexico. ^b Proteínas Marinas y Agropecuarias, S.A. de C.V., Guadalajara, Jalisco, México. ^c PROAQUA, S.A. de C.V. Mazatlán, Sinaloa, México. ^d Sigma-Aldrich Chemical, S.A. de C.V. Toluca, México, México. ^e Trouw Nutrition México S.A. de C.V. * Vitamin mix: Vitamin A, 10,000,000 IU o mg/g; Vitamin D3, 2,000,000 IU; Vitamin E, 100,000 g; Vitamin K3, 4.00 g; Thiamine B1, 8.00 g; Riboflavin B2, 8.70 g; Pyridoxine B6, 7.30; Vitamin B12, 20.00 mg; Niacin, 50.00 g; Pantothenic acid, 22.20 g; Inositol, 153.80 g; Nicotinic acid, 160.00 g; Folic acid, 4.00 g; 80 mg; Biotin, 500 mg; Vitamin C, 100.00 g; Choline 300.00 g, Excipient q.s. 2000.00 g. Mineral mix: Manganese, 100 g; Magnesium, 45.00 g; Zinc, 160 g; Iron, 200 g; Copper, 20 g; Iodine, 5 g; Selenium, 400.00 mg; Cobalt 600.00 mg. Excipient q.s. 1500.00 g. ^f DSM Nutritional Products México S.A. de C.V., El Salto, Jalisco, México. ^g Enzymatic hydrolysate of fish subproducts. ^h IMAG S.A. de C.V., Guadalajara. NFE (nitrogen-free extract) = 100 - (% moisture + % crude protein + % crude lipids + % ash).

2.3. Fish and Experimental Conditions

Masculinized Nile tilapia (*O. niloticus*) were obtained from Aquamol Company S.C. of R. L. After 14 days of acclimatization, a total of 200 Nile tilapia (1.04 ± 0.01 g) were stocked into 120 L fiberglass tanks assigned to one of four treatments a negative control without high-density stress (C-), a positive control under high-density stress (C+), and two experimental diets with dietary agavin supplementation at 20 g/kg (D20) and 40 g/kg (D40). Each treatment included five replicates and an initial fish density of 1.73 kg/m³ [1]. Fish were fed to 10% of body weight thrice daily (9:00, 12:00, and 16:00). The feeding rate and water volume were adjusted every fifteen days based on fish biometry. The tanks were aerated and cleaned daily; 50% of the water was replaced by dechlorinated water to maintain optimum water quality. Water temperature (28 ± 2 °C), dissolved

oxygen 5.5 ± 0.5 mg/dL (Pro20, Professional series, YSI oximeter, USA), pH (7.01), and total ammonia <0.01 mg/L, nitrites, and nitrates <2.0 mg/L in water were monitored daily during the experiment.

The experiment was conducted in two parts. First, we evaluated the effect on growth performance and biological indexes under low rearing density on day 80 of feeding. Then, on day 90, fish were subjected to high-density stress (63 kg/m³) by reducing water level (120 L), except the negative control (C−) [27]. Fish were maintained under this condition for 20 days, and samples were obtained before and after high-density stress. During the high-density stress challenge, the water quality parameters remained at optimal values (temperature 28 ± 2 °C; dissolved oxygen 5.5 ± 0.5 mg/dL, pH (7.01), total ammonia <0.01 mg/L, nitrites, and nitrates <2.0 mg/L) for tilapia, by performing daily 50% water changes (9:00 and 16:00 h) and fecal siphoning (1 h after the second feeding), to prevent the accumulation of waste excretion. The effects on growth performance, biological index, and biochemical blood metabolites were analyzed and compared in fish fed agavin-supplemented diets subjected to high-density stress.

2.4. Growth Performance and Body Composition

Biometrics were determined every fifteen days. To this end, fish were carefully anesthetized with clove oil (0.2 mL/L) via immersion. The selection of clove oil for anesthesia (0.2 mL/L) was based on the AVMA Guidelines for the Euthanasia of Animals: 2020 [28], which accepts the use of this compound as an anesthetic and for euthanasia. The dose of the anesthetic was based on published studies with similar species [5,29]. Fish were weighted to calculate the mean body weight. Weight gain (WG), specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), and survival (S) were determined as follows:

$$\text{WG (g)} = \text{final body weight} - \text{initial body weight}$$

$$\text{SGR (\%/day)} = \left(\frac{\ln \text{ final weight} - \ln \text{ initial weight}}{\text{number of days}} \right) \times 100$$

$$\text{FI (\%BW/day)} = \sum i \left(\frac{\text{total feed consumption (g)}}{\text{number of days (days)}(\text{final weight})} \right) \times 100$$

$$\text{FCR} = \frac{\text{FI}}{\text{final weight} - \text{initial weight}}$$

$$\text{S (\%)} = \left(\frac{\text{final number of fish}}{\text{initial number of fish}} \right) \times 100$$

The condition (CI), hepatosomatic (HSI), and viscerosomatic (VSI) indexes were calculated using the following formulas:

$$\text{CI} = \left(\frac{\text{Total fish weight}}{\text{Lenght}^3} \right) \times 100$$

$$\text{HSI} = \left(\frac{\text{Liver weight}}{\text{Total fish weight}} \right) \times 100$$

$$\text{VSI} = \left(\frac{\text{Viscera weight}}{\text{Total fish weight}} \right) \times 100$$

2.5. Sample Collection

The effect of dietary agavin on Nile tilapia subjected to high-density stress was evaluated using two sampling points. The first sampling point was performed on day 80 of feeding and before the stress challenge, while the second sampling was conducted on day 110, after twenty days of high rearing density [27]. At both sampling points, one fish per tank was anesthetized with oil clove (0.2 mL/L) to collect blood to be tested for blood metabolites (5 fish per treatment); then, the same fish was euthanized (0.5 mL oil clove) to

determine body composition. For the hepatic antioxidant response, one fish per tank (5 fish per treatment) was euthanized (0.5 mL oil clove), and livers were excised on day 80 and day 110 of feeding.

2.6. Body Composition

One fish per tank was captured before (day 80) and after (day 110) high-density stress and stored at $-20\text{ }^{\circ}\text{C}$ for analysis. The whole-body composition was determined in triplicate using standard methods of the Association of Official Analytical Chemists [30]. Dry matter content was determined by gravimetry, drying samples in an oven at $105\text{ }^{\circ}\text{C}$ for 12 h (Heraeus, D-63450, Hanau, Germany). Ash was determined by incineration of samples in a muffle furnace (Felisa[®], model FE-363) at $550\text{ }^{\circ}\text{C}$ for 5 h. Crude protein was measured by micro-Kjeldahl ($\text{N} \times 6.25$; AOAC method 954.0). Lipid content was determined in a microFoss Soxtec Avanti 2050 Automatic System (Fos Soxtec, Hoganäs, Sweden), using petroleum ether as the extraction solvent.

2.7. Blood Metabolites

On days 80 and 110, one fish was randomly sampled from each tank and immediately anesthetized with 0.2 mL/L clove oil. Blood samples were collected from the caudal vein using heparinized syringes (5 mg heparin per mL of blood), poured into sterile vials, and centrifuged at $1200 \times g$ for 10 min. Serum and plasma were poured into new tubes and stored at $-80\text{ }^{\circ}\text{C}$ until analysis [31]. Serum levels of glucose, total protein, cholesterol, and triglycerides were measured spectrophotometrically using commercial kits (Mexlab Co., Ltd., Guadalajara, Jalisco, Mexico). Plasma cortisol level was estimated using the ELISA method and a commercial kit (NEOGEN, Gesellschaft für Immunchemie und Immunbiologie, Hamburg, Germany). All analyses were performed in triplicate.

2.8. Antioxidant Analyses

One fish per tank was euthanized with clove oil (0.5 mL/L) on days 80 and 110; the liver was excised to be tested for antioxidant enzymes and lipid peroxidation. Livers were homogenized (Ultra-Turrax D25 basic, IKA-Werke GmbH & Co., Staufen, Germany) with PBS buffer (pH 7.4), with the buffer volume (mL) corresponding to one-third of the liver weight (g). Homogenized samples were centrifuged (Beckman Allegra X-30R) at 12,000 rpm for 15 min at $4\text{ }^{\circ}\text{C}$; then, supernatants were recovered and tested for antioxidant enzymes and lipid peroxidation [10].

2.8.1. Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined using the SOD-WST kit (19160 Sigma Aldrich, St. Louis, MA, USA) following the kit instructions. Volumes of 20 μL of the supernatant and 200 μL of the WST solution were transferred to a 96-well plate. Briefly, 20 μL of the enzyme working solution was added and mixed. The plate was incubated at $37\text{ }^{\circ}\text{C}$ for 20 min. Absorbance was read at 450 nm in an EPOCH 2NS microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA). The results were expressed as units of SOD activity per milligram of protein (U/mg protein), where one unit of SOD activity was defined as the quantity of enzyme that inhibits formazan formation by 50%.

2.8.2. Catalase Activity

Catalase (CAT) activity was determined according to Aebi [32] with slight modifications. Briefly, 10 μL of the supernatant was mixed with 200 μL of 15 mM H_2O_2 dissolved in 100 mM phosphate buffer (pH 6.5) and the reaction kinetics were immediately run for two minutes, with readings every 10 s at 240 nm in an EPOCH 2NS microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA). CAT activity was expressed as U/mg protein. One unit of catalase activity was defined as the quantity of enzyme needed to catalyze 1 mmol of H_2O_2 per minute at $25\text{ }^{\circ}\text{C}$.

2.8.3. Lipid Peroxidation: Malondialdehyde Content Determination

Malondialdehyde (MDA) was determined as described by Lizárraga-Velazquez [10]. Briefly, 100 μL of the supernatant was mixed with 650 μL of 1-methyl-2-phenylindole (10.3 mM) in methanol:acetonitrile (1:3; v/v), 100 μL of water, and 150 μL of 37% HCl. The mixture was incubated at 45 $^{\circ}\text{C}$ for 40 min, cooled on ice for 10 min, and centrifuged at 12,000 rpm for 15 min at 4 $^{\circ}\text{C}$. Absorbance was read at 586 nm in an EPOCH 2NS microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA). MDA levels were expressed as mmol MDA/g of tissue using a standard solution of 1,1,3,3-tetra methoxy propane (10 mM).

2.8.4. Protein Concentration

Protein concentration in the supernatant was determined according to Bradford [33], expressing enzymatic activity as U/mg protein. Briefly, 5 μL of supernatant was mixed with 250 μL of Bradford reagent (B6916, Sigma Aldrich) and incubated for 15 min at room temperature (25 $^{\circ}\text{C}$). Absorbance was read at 595 nm in an EPOCH 2NS microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT, USA). The results were calculated from a bovine serum albumin standard curve (0.1–1.4 mg/mL). Measurements were read in triplicate ($n = 3$).

2.9. Statistical Analyses

Data were tested for normality and homoscedasticity using the Shapiro–Wilk and Levene’s tests, respectively. Percent data (SGR, S, HIS, VSI and IFR) were arcsine transformed prior to analysis. One-way analyses of variance (ANOVA) were used to test for differences among the experimental groups. When significant effects of treatments were detected, means were compared using Tukey’s multiple comparison tests [34]. All statistical tests were carried out using the software STATISTICA (TIBCO Software Inc., Palo Alto, CA, USA). A significance level of $p > 0.05$ was used for all statistical tests.

3. Results

3.1. Agavin Analyses by MALDI-TOF-MS

The mass spectrometry of the agavin sample used in the experiments was determined by MALDI-TOF-MS (Figure 1). The mean molecular weight ranges from 527 kDa to 2311 kDa, corresponding to a degree of polymerization (DP) of 3–16. Additionally, a mean molecular weight of 1210 kDa and a polydispersity index (PDI) of 1.2 were recorded. The mass spectrum of agavin matches with the calibrators.

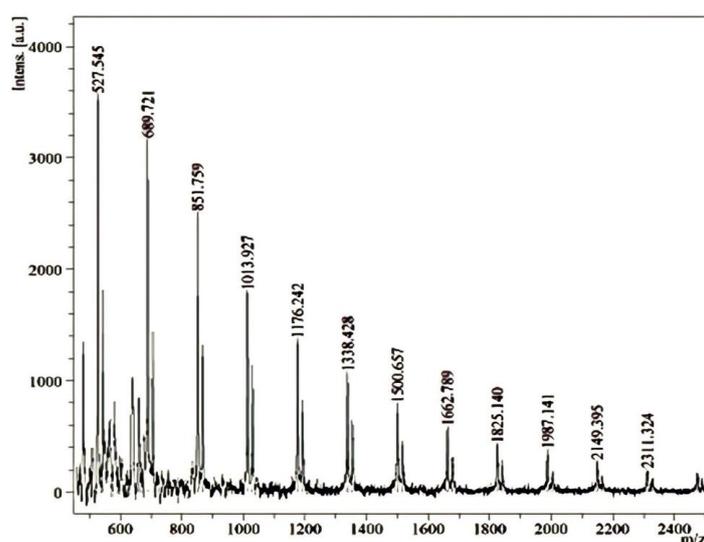


Figure 1. MALDI-TOF-MS mass spectrum of agavin from *A. tequilana* Weber, blue variety, with 2,5-dihydroxybenzoic acid as the matrix. All m/z values correspond to $[\text{M}_p\text{Na}]_p$ and $[\text{M}_p\text{K}]_p$ adducts.

3.2. Growth Performance and Body Composition

On day 80 of feeding, dietary agavin supplementation had no significant effects ($p > 0.05$) on WG, FI, FCR, and body composition of Nile tilapia under low-density culture (Table 2). The survival rate was not affected by agavin supplementation to diets.

Table 2. Growth performance and body composition of Nile tilapia (*O. niloticus*) fed an agavin-supplemented experimental diet after 80 days under low-density conditions.

	C−	C+	D20	D40
Initial body weight (g)	1.04 ± 0.01	1.04 ± 0.01	1.03 ± 0.01	1.03 ± 0.01
Final body weight (g)	39.0 ± 9.4	40.7 ± 7.3	39.9 ± 7.6	38.3 ± 9.6
Weight gain (g)	37.9 ± 4.1	39.5 ± 2.9	38.9 ± 1.6	37.5 ± 2.5
SGR (%/day)	4.49 ± 0.1	4.48 ± 0.1	4.51 ± 0.05	4.50 ± 0.1
IFC	21.2 ± 2.7	22.8 ± 2.1	23.3 ± 1.1	23.0 ± 2.3
FCR	1.17 ± 0.07	1.18 ± 0.06	1.14 ± 0.05	1.18 ± 0.04
Survival (%)	100 ± 0.0	98 ± 0.0	100 ± 0.0	100 ± 0.0
Body composition				
Moisture (%)	76.4 ± 0.1	76.5 ± 0.1	77.1 ± 0.5	75.1 ± 0.4
Crude protein (%)	12.7 ± 0.8	13.1 ± 0.2	12.5 ± 0.9	13.8 ± 0.3
Crude lipids (%)	5.1 ± 0.2	5.1 ± 0.1	4.9 ± 0.1	5.1 ± 0.2
Ash (%)	1.4 ± 0.6	1.4 ± 0.1	1.4 ± 0.1	1.7 ± 0.1

IFC, individual feed consumption; FCR, feed conversion ratio. Data obtained from five biological replicates are shown as the mean ± SE values ($n = 20$). C−, control without high-density stress; C+, control under high-density stress; D20, experimental diet supplemented with 20 g/kg agavin under high-density stress; D40, experimental diet supplemented with 40 g/kg agavin under high-density stress.

In this study, the diet supplemented with 20 g/kg agavin (D20) reduced the negative effect only of FBW under high-density rearing, with similar results to those observed in the negative control (Table 3). WG, SGR, S, FI, FCR, VSI, and HIS were not significantly affected by dietary agavin supplementation to the diet of Nile tilapia under high rearing density. Body composition showed no significant differences ($p > 0.05$) (Table 3).

Table 3. Growth performance and body composition of Nile tilapia (*O. niloticus*) under high rearing density (63 kg/m³) for 20 days.

	C−	C+	D20	D40
Initial body weight (g)	39.0 ± 9.4	40.7 ± 7.3	39.9 ± 7.6	38.3 ± 9.6
Final body weight (g)	235.6 ± 22.8 ^a	159.6 ± 27.5 ^c	219.8 ± 24.5 ^{ab}	193.2 ± 16.6 ^{bc}
Weight gain (g)	196.0 ± 13.7 ^a	118.9 ± 14.2 ^c	179.9 ± 14.4 ^{ab}	154.9 ± 24.4 ^b
SGR (%/day)	7.4 ± 0.3 ^a	6.1 ± 0.6 ^b	6.9 ± 0.5 ^{ab}	6.5 ± 0.7 ^{ab}
IFC	147.8 ± 19.3 ^a	117.8 ± 15.4 ^b	137.0 ± 10.2 ^{ab}	129.8 ± 16.7 ^{ab}
FCR	0.94 ± 0.1	1.0 ± 0.06	1.0 ± 0.09	1.0 ± 0.1
K (%)	1.9 ± 0.1	1.8 ± 0.1	1.8 ± 0.05	1.8 ± 0.1
HSI (%)	1.5 ± 0.3	2.2 ± 0.7	2.7 ± 0.7	1.9 ± 0.8
VSI (%)	5.1 ± 0.9	5.8 ± 0.9	6.2 ± 1.6	6.1 ± 1.9
Survival (%)	97.0 ± 0.0	97 ± 0.0	94 ± 0.0	100 ± 0.0
Body composition				
Moisture (%)	74.2 ± 0.4	73.8 ± 0.3	73.5 ± 0.5	74.6 ± 0.4
Crude protein (%)	14.1 ± 0.6	14.4 ± 0.6	14.8 ± 0.2	14.3 ± 0.8
Crude lipids (%)	6.2 ± 0.1	5.7 ± 0.1	5.8 ± 0.1	5.3 ± 0.1
Ash (%)	1.9 ± 0.2	2.1 ± 0.1	2.0 ± 0.1	2.2 ± 0.2

IFC, individual feed consumption; FCR, feed conversion ratio; K, condition factor; HSI, hepatosomatic index; VSI, viscerosomatic index. Negative control (C−): low-density control; positive control (C+): high-density control; D20 and D40 correspond to 20 and 40 g/kg of agavin supplementation, respectively, under high rearing density. Data obtained from all biological replicates are shown as the mean ± SE values ($n = 20$). Different letters correspond to significant differences ($p < 0.05$) according to Tukey's HSD test.

3.3. Blood Metabolites

No significant differences in plasma cortisol, serum glucose, and total protein were observed between fish in both control groups and fish fed agavin diets for 80 days at a low density ($p > 0.05$). Total cholesterol levels were lower in fish fed the 20 g/kg agavin diet, with no significant differences between treatments, while both control diets yielded triglyceride levels higher than the values recorded in fish fed agavin diets for 80 days and reared at a low density (1.73 kg/m^3) (Table 4).

Table 4. Blood metabolites of Nile tilapia (*O. niloticus*) fed for 80 days under low-density conditions.

	C−	C+	D20	D40
Cortisol (ng/mL)	45.5 ± 4.8	44.9 ± 2.3	45.9 ± 1.6	44.0 ± 1.7
Glucose (mg/dL)	63.0 ± 2.2	68.1 ± 2.5	64.9 ± 2.9	69.6 ± 2.1
Cholesterol (mg/dL)	143.6 ± 4.7	154.5 ± 3.1	134.0 ± 7.3	156.5 ± 8.4
Triglycerides (mg/dL)	330.2 ± 8.8	283.0 ± 12.5	251.0 ± 8.1	260.0 ± 14.8
Total protein (g/dL)	2.8 ± 0.1	2.7 ± 0.1	2.5 ± 0.1	2.7 ± 0.2

Data from five biological replicates are shown as the mean ± SE values ($n = 5$). Different letters correspond to significant differences ($p < 0.05$) according to Tukey's HSD test. C−, control without high-density stress; C+, control under high-density stress; D20, experimental diet supplemented with 20 g/kg agavin under high-density stress; D40, experimental diet supplemented with 40 g/kg agavin under high-density stress.

Plasma cortisol, serum glucose, cholesterol, and triglyceride levels were lower in fish fed agavin diets (D20 and D40) under the high rearing density (63 kg/m^3) for 20 days, compared with the positive control group ($p < 0.05$), whereas total protein showed no significant differences under these conditions (Figure 2). Cortisol levels were lower in tilapia fed agavin-supplemented diets (D20 and D40) and subjected to high-density stress (63 kg/m^3) for 20 days, compared with both controls (Figure 2a). On the other hand, glucose levels increased in fish fed the D40 diet during the high-density challenge compared to the other treatments (Figure 2b). Regarding cholesterol levels, the addition of 40 g/kg agavin (D40) in diet maintained this parameter at similar levels as those in the negative control, while the D20 diet produced a slight increase in cholesterol, with a pattern resembling the one in the positive control (Figure 2c). Both supplementation levels maintained triglycerides during the high-density stress at similar concentrations as those in the negative control, whereas the positive control showed the highest increase in this parameter (Figure 2d). Finally, no significant differences in total protein levels were observed in any treatment ($p < 0.05$).

The highest plasma cortisol level was recorded in the positive control (fish reared under high density) (Figure 2a). Stock density significantly increased glucose levels in fish fed D40 than in the other treatments (Figure 2b). Total cholesterol under high density decreased in fish fed the D40 diet but was significantly higher in fish fed D20 and in the positive control (C+) (Figure 2c). Triglyceride levels differed significantly ($p < 0.05$) across treatment groups, with the highest values observed in fish in the positive control (C+, high rearing density) for 20 days (Figure 2d).

3.4. Hepatic Antioxidant Response

There were no significant differences in CAT activity between fish fed the control diet and those fed the experimental diets after 80 days under low-density conditions ($p > 0.05$). However, fish that consumed any of the agavin diets showed lower MDA levels than fish in both control groups (Table 4). Furthermore, SOD activity was higher in fish fed the D20 diet ($11.2 \pm 2.1 \text{ U SOD/mg protein}$) and lower in fish that consumed the D40 diet ($3.7 \pm 0.1 \text{ U SOD/mg protein}$) (Table 5).

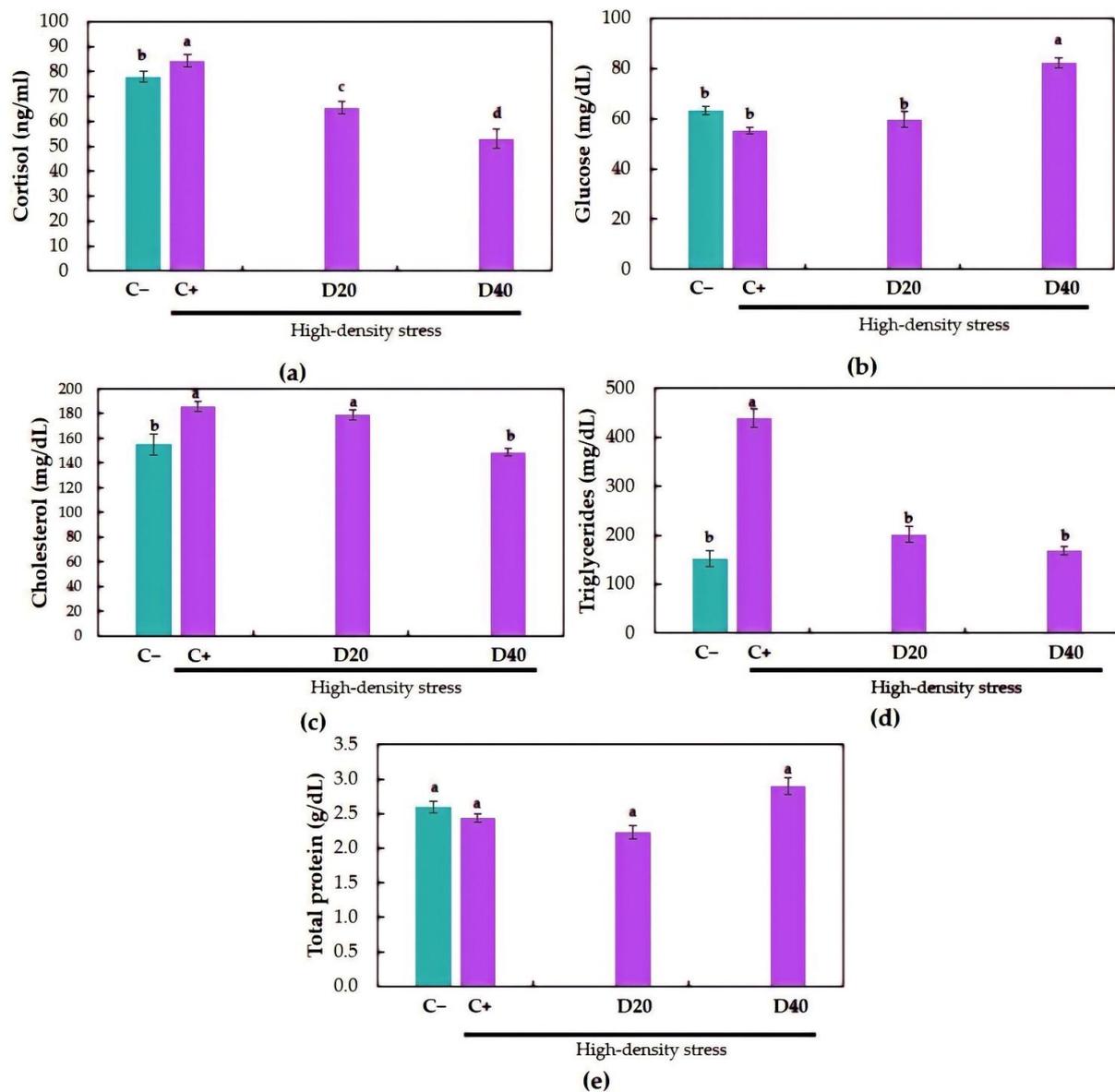


Figure 2. Effect of dietary agavin on blood metabolites of Nile tilapia (*O. niloticus*) after 20 days under high rearing density (63 kg/m³). (a) Cortisol; (b) glucose; (c) cholesterol; (d) triglycerides; and (e) total blood protein. C−, control without high-density stress; C+, control under high-density stress; D20, experimental diet supplemented with 20 g/kg agavin under high-density stress; D40, experimental diet supplemented with 40 g/kg agavin under high-density stress. Data from five biological replicates are shown as the mean ± SE values. Different letters correspond to significant differences ($p < 0.05$) according to Tukey's HSD test ($n = 5$).

Table 5. Hepatic antioxidant response to Nile tilapia fed for 80 days under low-density conditions.

	C−	C+	D20	D40
CAT	31.8 ± 5.3	27.3 ± 7.1	33.9 ± 2.3	24.7 ± 1.3
SOD	7.1 ± 1.4 ^b	3.8 ± 0.7 ^c	11.2 ± 0.6 ^a	3.7 ± 0.1 ^c
MDA	0.14 ± 0.06	0.15 ± 0.12	0.08 ± 0.04	0.04 ± 0.02

CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde. Data obtained from five biological replicates are shown as the mean ± SE values ($n = 5$). Different letters correspond to significant differences ($p < 0.05$) according to Tukey's HSD test. C−, control without high-density stress; C+, control under high-density stress; D20, experimental diet supplemented with 20 g/kg agavin under high-density stress; D40, experimental diet supplemented with 40 g/kg agavin under high-density stress.

Dietary agavin supplementation was associated with increased SOD and CAT activities and decreased MDA levels in fish fed the D20 and D40 diets after 20 days under high rearing density, while the positive control showed the opposite effect ($p < 0.05$) (Figure 3). Fish fed the D20 diet showed a higher SOD activity than fish fed with the D40 diet and those in the positive control but showed a similar behavior as fish in the negative control (Figure 3a). CAT activity was higher in fish fed D20 and D40 diets than in fish fed control diet under both densities (Figure 3b). The MDA level was higher in the positive control compared with the rest of the groups. Dietary agavin supplementation significantly reduced the increases in MDA levels under high-density stress ($p < 0.05$) (Figure 3c).

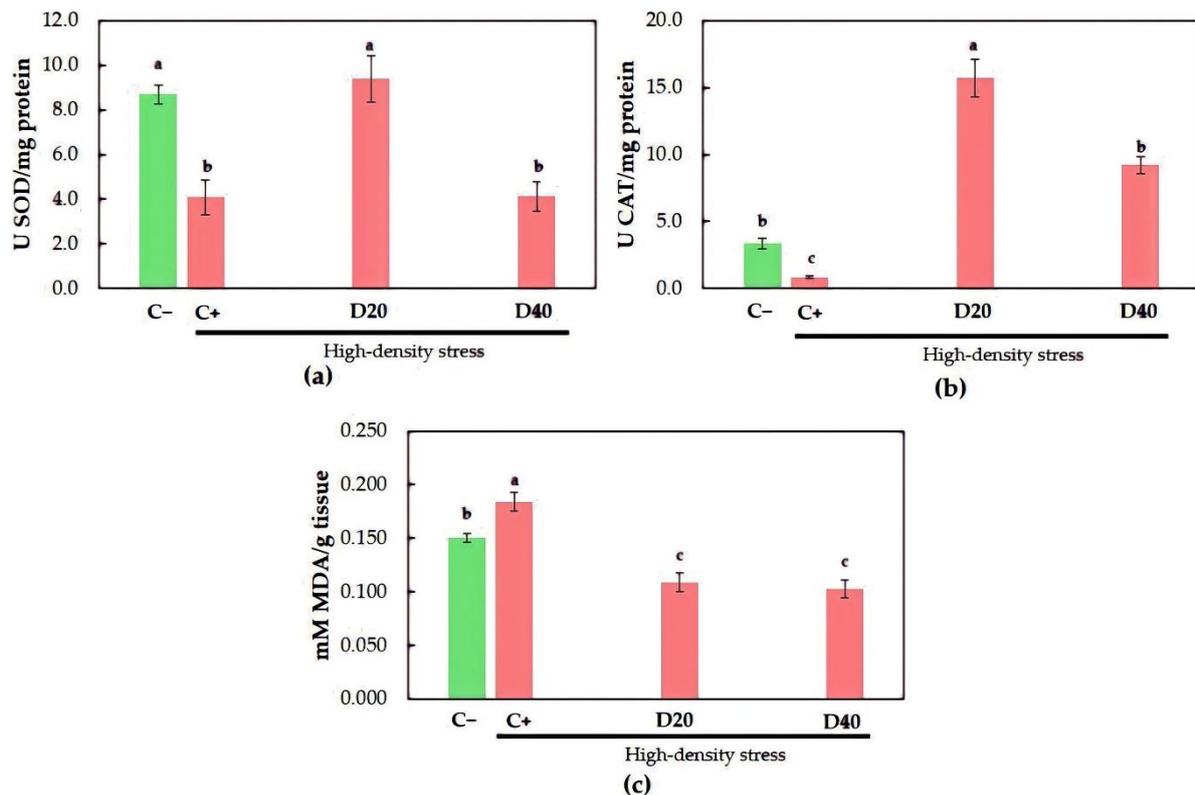


Figure 3. The effect of dietary agavin on (a) SOD activity, (b) CAT activity, and (c) MDA levels in Nile tilapia (*O. niloticus*) after 20 days under high rearing density (63 kg/m^3). C−, control without high-density stress; C+, control under high-density stress; D20, experimental diet supplemented with 20 g/kg agavin under high-density stress; D40, experimental diet supplemented with 40 g/kg agavin under high-density stress. Data obtained from five biological replicates are shown as the mean \pm SE values. Different letters correspond to significant differences ($p < 0.05$) according to Tukey's HSD test.

4. Discussion

High rearing density produces oxidative stress affecting the health status and survival of aquatic organisms [5,6,35,36]. The present study demonstrated that dietary agavin supplementation prevent the hepatic oxidative damage and alterations related with the energetic metabolism in Nile tilapia under high rearing density.

Agave plants store high fructan concentrations as reserve carbohydrates in the stem. In this study, agavin MALDI-TOF analyses showed a lower degree of polymerization (DP of 3–16 units) than values reported in other studies (3–30 units) [12,37,38]. Differences in DP and molecular weight may be attributed to several factors such as fructan source, plant age, agave species, extraction process, and others [12,38]. The authors showed that the beneficial effect of agave fructan could be attributed to DP, molecular weight, molecular complexity, and linkage types, highlighting that a low DP, a low molecular weight, and

β -linkages confer antioxidant and prebiotic properties to feed, leading to improved fish health [17,19,39].

Diets with 20 or 40 g/kg of agavin had no significant effects on Nile tilapia growth performance at 90 days under low rearing density. The results on fish growth observed in the present study are consistent with those observed with inulin or FOS supplementation at 5 and 10 g/kg in rainbow trout; 2.5–10 g/kg inulin in Nile tilapia; and 5–20 g/kg in Asian seabass (*Lates calcarifer*) [17,40,41]. To date, studies evaluating dietary agavin supplementation in aquaculture organisms are scarce. Fuentes-Quesada [18] reported that 20 g/kg agavin in the diet promotes growth performance in totoaba (*Totoaba macdonaldi*) through its prebiotic effect. However, in the present study, dietary agavin supplementation at the doses used did not improve growth performance in Nile tilapia, although it did not adversely affect fish growth either. Therefore, agavin with a DP of 3 to 16 units from *Agave tequilana* at the doses used in this study is a safe functional additive in tilapia feed.

The blood parameters evaluated (cortisol, glucose, cholesterol, triglycerides, and total protein) are important biomarkers of stress and the health status of fish [42–45]. Cortisol is the main corticosteroid produced in high concentrations by the hypothalamic-pituitary-adrenal (HPA) axis in response to homeostasis disruption caused by a stressor that elicits an alert phase in organisms, so the evaluation of plasma cortisol is considered an excellent indicator of stress [4,6,45]. Increased cortisol levels stimulate the mobilization of energy reserves such as glucose (via gluconeogenesis and glycogenolysis), triglycerides (via lipogenesis), and cholesterol (cortisol precursor) [46–49], allowing fish to meet its energy requirements during an alert phase in the first few minutes or hours (acute stress), and even over days and weeks (chronic stress). The latter, however, may lead to the depletion of energy reserves, reducing growth and food efficiency, and ultimately leading to death [42,46].

Studies in different fish species exposed to acute or chronic high-density stress have reported increases in cortisol, glucose, triglycerides, or cholesterol levels. For instance, Naderi [50], reported increased cortisol and glucose levels in rainbow trout (*Oncorhynchus mykiss*) grown at a high density (200 kg/m³) for 45 min. In addition, Mirghaed [27] reported increases in cortisol, glucose, triglycerides, and cholesterol levels in the same species under a culture density of 60 kg/m³ for 14 days. On the other hand, Yaghoobi [51] reported increased cortisol and glucose levels in catfish (*Pangasianodon hypophthalmus*) grown at densities of 100 kg/m³ for 3 h. In the present study, the positive control group showed increases in cortisol, triglycerides, and cholesterol levels, but not in glucose levels. Previous studies have reported that exposure of fish to chronic stress depletes plasma glucose levels due to the rapid consumption of plasma glucose as an energy source during the alert phase [52,53]. However, some studies indicate that plasma glucose levels in fish may remain unchanged under stress [54]. Therefore, our results indicate that the culture density used in the present study elicited physiological changes (increased cortisol, triglycerides, and cholesterol levels) in Nile tilapia in response to stress.

The lower cortisol, triglycerides, and cholesterol levels in tilapia fed a diet supplemented with 40 g/kg agavin may be attributed to this fructan. Studies in mammals have reported that fructans can regulate cortisol levels by modulating the HPA axis through stimulation of the vagus nerve. This inhibits the expression of pituitary adenylate-cyclase-activating polypeptide (PACAP) receptors (such as G and PACAP-R1 proteins), components that regulate the HPA axis and corticotropin (CRH) production, which, in turn, suppresses the release of cortisol in plasma [6,55,56]. In turn, lower cortisol levels inhibit lipogenesis and, consequently, plasma triglyceride levels are also reduced. In addition, mammalian studies have also reported that fructans reduce triglyceride levels by suppressing the expression of G protein-coupled receptors (GRP43/GRP41), which participate in the regulation of lipid metabolism [56–59]. In addition, cholesterol reduction is linked to cortisol levels, as it is its main precursor; in turn, lower cortisol levels reduce cholesterol synthesis by inhibiting the HPA axis. Further, it has been observed that inulin-type fructans may reduce the activity of enzymes involved in lipogenesis (3-hydroxy-3-methylglutaraldehyde

CoA reductase, acetyl-CoA carboxylase, malic enzyme, ATP citrate lyase, and fatty-acid synthase), thereby reducing triglyceride synthesis attributed to increased bile acid secretion [46,57,60,61]. In fish, information on the mechanism through which agavin reduces the plasma cholesterol and triglyceride levels of stressed organisms has not yet been elucidated. However, since agavin is a fructan mainly composed of fructose, we hypothesize that agavin reduces cortisol levels by inhibiting the HPA axis. Further studies are needed to support this assumption.

The increased glucose level observed in organisms fed a diet supplemented with 40 g/kg agavin is directly attributed to this supplement rather than chronic high-density stress because the cortisol level was lower than in the positive control. That is, we believe that glucose was not regulated by cortisol but by other unknown mechanisms. Studies in animal models have reported that inulin fructans do not influence plasma glucose levels [19,54]. In fish, information on the use of agavin as a food supplement is scarce. Therefore, further studies are needed on the mechanisms through which agavin may influence glycolytic pathways in tilapia. To note, the glucose level reported herein lies within the range reported for tilapia grown under optimal conditions (40.1–80.5 mg/dL), i.e., the glucose level (74.2 ± 4.7 mg/dL) observed in fish fed the D40 diet lies within the range reported for healthy organisms [61,62].

On the other hand, the disruption of cellular homeostasis by a stressor leads to oxidative stress characterized by the suppression of the endogenous antioxidant system (CAT and SOD) and oxidative damage to lipids (lipid peroxidation) that make up the lipid bilayer of cell membranes. The latter can affect the health of organisms and lead to increased mortality [9,62,63]. Malondialdehyde (MDA) is a product of oxidative damage to lipids [59,64], so it is used as an excellent marker of oxidative stress. On the other hand, measuring CAT and SOD activities provides information on the antioxidant response, so the measurement of the enzymes MDA, and, CAT and SOD enzymes is a valuable tool to elucidate the aspects or pathways involved in the inhibition or increase in oxidative damage in organisms exposed to stressors [64,65].

In the present study, tilapia fed an agavin-supplemented diet and grown at high density showed an increase in SOD and CAT activity and lower MDA levels. Liu [65] reported that fructans from *Arctium lappa* L. (at 1.25 and 2.5 mg/mL) increased in vitro antioxidant activity measured by their ability to donate hydrogen atoms and chelate metals (such as Fe^{2+}). These same authors also reported that the dietary administration of fructans from *Articum lappa* L. (100 mg/kg) increases CAT and SOD activity and reduces MDA levels in the serum of stressed mice. On the other hand, Zou [66] observed that inulin fructans from *Radix Codonopsis* increased the expression of genes encoding the antioxidant enzymes CAT and SOD and decreased MDA levels in porcine jejunal epithelial cells (IPEC-J2).

Studies in fish have evaluated the antioxidant response of different types of fructans. For example, the dietary administration of commercial fructooligosaccharides (FOS) (at 10 and 20 g/kg) and inulin (4 g/kg) has been reported to increase the activity of antioxidant enzymes (SOD and CAT) and reduce the levels of malondialdehyde in carp (*Megalobrama amblycephala* Yih) and Nile tilapia (*O. niloticus*), respectively [22,67]. Previous studies have found that agavin from *Agave tequilana* and *A. vulgaris* and inulin from *Radix Codonopsis* elicit an antioxidant response by increasing CAT, SOD, and GPX activities and lowering MDA levels in intestinal epithelial cells, serum, and liver of mice [24,68,69]. The observed antioxidant response was mainly attributed to the prebiotic effect of fructans that produces fermented metabolites, such as short-chain fatty acids (SCFAs), which can trigger the expression of antioxidant enzymes (SOD, CAT) by activating transcription factors such as Nrf2 (Nuclear factor erythroid-derived 2-related factor 2) or inhibition NF- κ B (nuclear factor kappa B) [66,67,69]. The present study suggests that agavin may act as a direct (ROS neutralization) or indirect antioxidant by regulating antioxidant enzymes, attributed to the chemical structure of the compound or its prebiotic effect [69,70]. However, further studies are needed to determine the mechanism by which agavin exerts its antioxidant effect in tilapia exposed to chronic stress from high culture density.

5. Conclusions

In the present study, the high culture density used (63 kg/m³) caused oxidative stress in tilapia. Further, supplementation with 20 g/kg agavin reduced blood cortisol, cholesterol, and triglycerides, increased liver antioxidant activity (SOD and CAT), and exerted a strong inhibitory effect on peroxidation (decreasing MDA) in tilapia cultured at high density. Therefore, this study suggests that dietary supplementation with 20 g/kg agavin can be used as a safe additive to reduce oxidative stress in Nile tilapia reared at high densities. However, future studies are required to explore the mechanisms involved in regulating the antioxidant response and preventing alterations of blood metabolites in fish reared under stress, as well as the prebiotic effect and its relationship with the antioxidant response and blood metabolites.

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Data Availability Statement: The data presented in this study are available in this article.

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