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Research article

Formulation strategy to reach a balance among dietary essential amino acids for Nile tilapia juveniles

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ABSTRACT: Diets with high protein levels but unbalanced amino acid (AA) profiles can lead to poor AA utilization, increasing nitrogenous compound excretion and feed costs. Our study aimed to develop a formulation strategy to maintain a balanced dietary AA profile with a low protein level for Nile tilapia juveniles without compromising growth. Fish (6.75 ± 0.07 g) were fed on one of four isoenergetic diets with varied protein concentrations and AA profiles twice daily to apparent satiation for 41 days. The trial included four dietary treatments containing either 321 (32HighLys and 32LowLys) or 292 and 222 g protein kg^{-1} (29BAL and 22BAL, respectively) with five replicates per diet, making a total of 20 experimental units in a completed randomized design. The growth of fish fed the 29BAL diet was not compromised; weight gain (WG) and thermal growth coefficients were similar to those fed the 32HighLys and 32LowLys diets. However, the protein gain of those fish fed the 32HighLys diet was significantly higher than that of those fed the other diets. The 22BAL diet promoted the lowest growth, and its higher protein-energy ratio led to increased body lipid content. Therefore, the formulation strategy to supply balanced dietary AA to Nile tilapia reduced digestible protein from 32 to 29 %, without compromising fish growth and allowing a 12 % decrease in the excretion of nitrogenous waste.

Keywords: protein quality, environmentally friendly diets, nitrogen excretion

Introduction

Nile tilapia (*Oreochromis niloticus*, Linnaeus 1758) is the third most produced aquaculture species worldwide (FAO, 2022). For maximum growth and successful tilapia farming, diets must meet their nutritional requirements (Ghomi et al., 2012). Protein is the costliest nutrient in feed formulations (Ballester-Moltó et al., 2017), so excessive dietary protein should be avoided (Teles et al., 2020). Diets with excess or low digestibility protein and an unbalanced amino acid (AA) profile can promote the deamination of ingested AA, resulting in the excretion of nitrogenous metabolic waste into the environment (Bureau et al., 2002). This negatively impacts aquatic ecosystems through eutrophication (Peres and Teles, 2001). However, increased utilization of dietary protein and the development of feed formulation strategies to improve protein retention could make fish farming more sustainable and profitable (Cho and Bureau, 2001).

Crystalline AAs are often added to commercial diets to achieve the AA required by the fish, which contributes to a reduction in dietary protein levels (Gaylord and Barrows, 2009; Gan et al., 2012). The requirements for AA, such as lysine, methionine, threonine, and tryptophan, often limited in conventional ingredients, are quickly addressed from crystalline sources. In contrast, the remaining essential AA (EAA) usually ends up in excess in commercial diets. When increasing the dietary protein concentration of experimental diets, EAA that were not deficient in the ingredients exceeded the recommended levels. With increasing protein inclusion rates, this excess is exacerbated and compromises the balance of the AA profile. In addition, when in excess, some AAs can have antagonistic effects on others, resulting in compromised growth. A study conducted on rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792), reported that an increase in dietary arginine resulted in increased arginine digestibility but decreased the digestibility of lysine, suggesting that there is competition between these two AA for absorption in the intestine (Kaushik et al., 1988). In another study on the same species, excess leucine had an antagonistic effect on isoleucine and valine absorption, reducing the concentration of these AA in the blood plasma and tissue (Yamamoto et al., 2004).

The present study proposes a formulation strategy to maintain a balanced dietary AA profile while decreasing the protein level for Nile tilapia juveniles without compromising production performance. Such a strategy avoids imbalances in the dietary AA profile and reduces the excretion of nitrogenous waste into aquatic environments.

Materials and Methods

The feeding trial was carried out in Florianópolis, Santa Catarina, Brazil (27°43′45″ S, 48°30′31″ W, altitude 3 m). The Ethics Committee on the Use of Animals of the Universidade Federal de Santa Catarina (UFSC) approved all procedures described below through protocol # 9527201021.

Fish and experimental design

Nile tilapia juveniles of the Genetically Improved Farmed Tilapia (GIFT) strain that had been sexually inverted to male were obtained from the Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (EPAGRI, Camboriú, Santa Catarina, Brazil). Before the feeding trial, all fish were acclimated to laboratory conditions for two weeks in six tanks (1,000 L) connected to a water recirculation system (RAS) with biological and mechanical filtration, air supply, and a heating system. Water temperature and photoperiod were fixed at 28 °C and 12 h, respectively.

After the adaptation period, 400 fish weighing of 6.75 ± 0.07 g were used in the feeding trial. Groups of 20 fish were stocked into 115 L experimental units and randomly received one of four diets (32HighLys, 32LowLys, 29BAL, and 22BAL; Table 1) with five replicates per diet, totaling 20 experimental units. These units were connected to another RAS equipped with biological and mechanical filtration, an air supply, and a heating system. The inlet water flow rate was 50 mL s^{-1} per tank, and the total volume of the RAS was 25 m^3. During the feeding trial, the average water temperature was 27.45 ± 0.31 °C, dissolved oxygen 6.29 ± 0.24 mg L^{-1}, and the pH was 7.91 ± 0.26. The water quality variables were maintained within the optimum for this species (El-Sayed, 2019).

Table 1 – Formulation of experimental diets.

Ingredients, g kg^{-1}	32HighLys[5]	32LowLys[6]	29BAL[7]	22BAL[8]
Corn	349.6	354.3	384.0	453.6
Soybean meal	290.0	290.0	290.0	290.0
Wheat bran	155.0	155.0	160.0	160.0
Poultry by-product meal	135.0	129.5	95.0	0.0
Corn gluten	30.0	50.0	20.0	0.0
Biolys® (L-Lysine)[2]	9.0	0.0	11.8	9.3
Soybean oil	5.0	5.0	5.9	45.0
Mineral and vitamin premix[1]	5.0	5.0	5.0	5.0
Sodium chloride	5.0	5.0	5.0	5.0
MetAMINO® (DL-Methionine)[2]	5.0	1.4	4.1	3.4
ThreAMINO® (L-Threonine)[2]	2.9	0.6	5.2	4.1
Dicalcium phosphate	2.7	3.2	8.0	20.5
L-Valine[3]	1.5	0.0	0.0	0.0
L-Isoleucine[3]	1.3	0.0	1.8	1.2
L-Histidine[3]	1.2	0.0	0.4	0.0
L-TrypAMINO® (L-Tryptophan)[3]	0.7	0.0	0.7	0.4
L-Phenylalanine[3]	0.1	0.0	2.1	1.5
BHT[4]	1.0	1.0	1.0	1.0

[1]Mineral and vitamin premix (produced by Rovimix, DSM), composition per kg product: folic acid = 2,666.66 mg; pantothenic acid = 26,666.66 mg; biotin = 333.33 mg; niacin = 53.33 g; copper = 3,333.33 mg; iron = 20 g; iodate = 666.66 mg; manganese = 16.6 g; selenite = 166.66 mg; vitamin (vit) A = 5,333,333.00 IU; vit B1 = 6,666.66 mg; vit B12 = 13.33 µg; vit B2 = 10.00 g; vit B6 = 10.00 g; vit C = 100.00 g; vit D3 = 1,000,000.00 IU; vit E = 66,666.66 IU; vit K3 = 3,333.33 mg; zinc = 26.66 g. [2]Produced by Evonik Operations GmbH. [3]Produced by Ajinomoto. [4]Butylated hydroxytoluene. [5]Formulated to meet Evonik's minimum amino acid recommendation (AMINOTilapia®) with some amino acid levels exceeding the recommended ideal ratio. [6]Formulated to meet Santiago and Lovell's (1988) minimum amino acid recommendation, with some amino acids exceeding the recommended ideal ratio. [7]Santiago and Lovell's (1988) amino acid recommendation and 292.1 g kg^{-1} digestible protein balanced to meet the ideal amino acid ratios. [8]Santiago and Lovell's (1988) amino acid recommendation and 221.6 g kg^{-1} digestible protein balanced to meet the ideal amino acid requirement.

Experimental diets and feeding management

Diets were formulated to be isoenergetic and to meet the nutritional requirements of Nile tilapia (Furuya et al., 2010; NRC, 2011), except for AA requirements, which followed different criteria (Tables 1 and 2).

The 32HighLys diet was formulated following the EAA recommendation of AMINOTilapia®, proposed by Evonik Operations GmbH, whose lysine level was 20.9 g kg^{-1}, whereas the 32LowLys diet was formulated to meet Santiago and Lovell's (1988) AA recommendation with lysine level at 16.1 g kg^{-1}; however, both diets had the same digestible protein level of 320 g kg^{-1}. On the other hand, the 29BAL diet was formulated based on a basal diet (22BAL) but matched the same lysine level used in the 32HighLys diet for further comparison. The main objective of the last two diets was to keep the balance among essential amino acids.

The basal diet (22BAL) was used to formulate the 29BAL diet to ensure a balanced amino acid profile. The 22BAL diet was designed to meet the estimated essential amino acid requirements determined by Santiago and Lovell (1988). Only EAA levels were considered, and no attention was paid to the digestible protein content of the 22BAL diet. After achieving the desired EAA profile, the level of dietary digestible protein in the 22BAL diet was obtained. The result was a digestible protein content of 221.6 g kg^{-1} on a dry matter basis (195.0 g kg^{-1} on an as-fed basis), which was the outcome of the desired amino acid profile. The 22BAL diet met the EAA ratios determined by Santiago and Lovell (1988), except for leucine, which was higher than the recommended ratio to Lys (103 vs 66 %). The protein and EAA content of the 29BAL diet were calculated using the lysine level of the 32HighLys diet (20.9 g kg^{-1}) with the same EAA ratio as the 22BAL diet (Table 3). As a result, all essential amino acids increased proportionally based on the lysine level, reaching a digestible protein level of 292.1 g kg^{-1}. Experimental diets were formulated based on the digestibility coefficients of energy, protein, and AA reported in the Brazilian Tables for Nutrition of Tilapia (Furuya et al., 2010) and the database provided by Evonik Operations GmbH.

Before manufacturing diets, all ingredients were individually ground in a hammer mill (1.0 mm mesh) and manually sieved (0.6 mm). Samples of all raw materials were sent to Evonik (Guarulhos, SP, Brazil) for AA analysis. Diets were extruded using a single-thread extruder (model MX-40; Inbramaq). During the extrusion process, the temperature of the extruder barrel was maintained at 100 °C and the moisture of the mixtures at 21 %. The resulting floating pellets (2-3 mm) were dried in an oven with forced air circulation at

Table 2 – Nutrient composition of experimental diets.

Composition, g kg^{-1} dry matter	32HighLys[2]	32LowLys[3]	29BAL[4]	22BAL[5]
Dry matter	914.3	905.8	898.2	927.3
Crude protein	351.9	353.5	320.4	243.5
Digestible protein (DP)[1]	320.6	320.6	292.1	221.6
Crude ash	65.3	63.8	65.0	60.4
Ether extract	58.0	56.3	51.2	83.0
Crude fiber	35.0	35.3	36.1	34.5
Gross energy, kcal kg^{-1}	4,650	4,587	4,553	4,722
Digestible energy (DE)[1], kcal kg^{-1}	3,750	3,684	3,645	3,748
DE: DP, kcal g^{-1} protein	11.70	11.49	12.48	16.91
Essential amino acids				
Arginine	19.8	20.3	17.9	13.9
Phenylalanine	13.8	14.4	14.5	11.3
Tyrosine[6]	12.2	12.7	11.0	8.1
Histidine	8.2	7.6	7.0	5.4
Isoleucine	13.0	12.3	12.5	9.6
Leucine	23.8	25.8	21.2	16.4
Lysine	20.9	16.1	20.7	15.9
Methionine	9.8	6.6	8.3	6.4
Cystine[6]	4.0	4.3	3.9	3.1
Threonine	13.6	11.7	14.8	11.4
Tryptophan	4.3	3.5	4.0	3.0
Valine	14.5	13.5	11.8	9.1
Non-essential amino acids				
Alanine	18.5	19.7	16.2	11.2
Aspartic acid	30.5	31.5	27.7	22.3
Glutamic acid	55.1	58.2	50.1	40.4
Glycine	18.8	19.0	16.1	9.8
Proline	19.5	21.2	17.9	13.3
Serine	15.1	16.0	13.6	11.0

[1]Apparent digestibility coefficients: corn = protein 79 %; energy 77 %; lysine 77 %; methionine 86 %; threonine 71 %; arginine 74 %; isoleucine 81 %; leucine 90 %; valine 81 %; histidine 91 %; phenylalanine 75 %; tryptophan 86 %; soybean meal = protein 94 %; energy 85 %; lysine 94 %; methionine 93 %; threonine 92 %; arginine 95 %; isoleucine 87.5 %; leucine 92 %; valine 90 %; histidine 95 %; phenylalanine 93 %; tryptophan 97 %; wheat bran = protein 73 %; energy 55 %; lysine 73 %; methionine 60 %; threonine 69 %; arginine 83 %; isoleucine 76 %; leucine 78 %; valine 70 %; histidine 77 %; phenylalanine 72 %; tryptophan 83 %; poultry by-product meal = protein 90 %; energy 89 %; lysine 94 %; methionine 90 %; threonine 85 %; arginine 90 %; isoleucine 83 %; leucine 83 %; valine 77 %; histidine 92 %; phenylalanine 89 %; tryptophan 93 %; corn gluten = protein 91 %; energy 88 %; lysine 92 %; methionine 95 %; threonine 89 %; arginine 93 %; isoleucine 90 %; leucine 91 %; valine 89 %; histidine 93 %; phenylalanine 92 %; tryptophan 93 %; soybean oil = energy 90 %. [2]Formulated to meet the minimum Evonik amino acid recommendation (AMINOTilapia®) with some amino acid levels exceeding the recommended ideal ratios. [3]Formulated to meet the minimum Santiago and Lovell's (1988) minimum amino acid recommendation, with some amino acids exceeding the recommended ideal ratio. [4]Santiago and Lovell's (1988) amino acid recommendation and 292.1 g kg^{-1} digestible protein, balanced to meet the ideal amino acid ratios. [5]Santiago and Lovell's (1988) amino acid recommendation and 221.6 g kg^{-1} digestible protein, balanced to meet the ideal amino acid ratios. [6]Conditionally essential.

55 °C and then packaged and stored. During the 41 days of the feeding trial, fish were fed the experimental diets twice daily until apparent satiation, and the amount of feed provided to each tank was recorded daily.

Measured variables, sample collection, and laboratory analyses

At the beginning of the feeding trial, all fish were individually weighed and sorted, maintaining a coefficient of variation of 5 % within each experimental unit and across all treatments. At the end of the feeding trial, all fish were individually weighed. Average weight gain Eq. (1), feed intake Eq. (2), and number of fish were used to calculate feed conversion ratio Eq. (3), thermal growth coefficient (TGC) Eq. (4), and survival rate Eq. (5), as follows:

$$\text{Weight gain (g fish}^{-1}) = \text{final mean body weight} - \text{initial mean body weight} \quad (1)$$

$$\text{Feed intake (g fish}^{-1}\ \text{d}^{-1}) = \frac{\text{daily feed intake}}{\text{number of fish}} \quad (2)$$

$$\text{Feed conversion ratio (g g}^{-1}) = \frac{\text{feed intake}}{\text{weight gain}} \quad (3)$$

$$\text{Thermal growth coefficient (\%)} = \frac{\text{final live weight}^{(0.333)} - \text{initial live weight}^{(0.333)}}{\text{mean daily temperature} \times \text{days}} \times 100 \quad (4)$$

$$\text{Survival rate (\%)} = \frac{\text{number of final fish}}{\text{number of initial fish}} \times 100 \quad (5)$$

Shortly before the beginning of the feeding trial, 60 fish were fasted for 24 h and then euthanized with 200 mg L^{-1} Eugenol® to analyze the proximate composition of the whole body. Five fish per tank were randomly collected at the end of the trial and stored at – 20 °C for further analysis. Proximate analyses of diets and body composition were performed at the Laboratório de Nutrição de Espécies Aquícolas (LABNUTRI, UFSC) following procedures standardized by the Association of Official Analytical Chemists (AOAC, 2005): moisture (drying at 105 °C to a constant weight, method 950.01), ether extract (Soxhlet, method 920.39C), ash (incineration at 550 °C, method 942.05), crude protein (using an FP-528 Leco) and the Dumas method (AOAC 990.03) as previously described (Etheridge et al., 1998), and energy (using a calorimeter (Model 6200, Parr Instrument Company) according to the manufacturer's instructions. Amino acids were analyzed using high-performance liquid chromatography (HPLC) by Evonik Operations GmbH – Nutrition & Care.

Diet and body composition variables were used to calculate nutrient deposition as protein gain Eq. (6), nitrogen loss Eq. (7), and protein retention Eq. (8) using the following equations:

$$\text{Protein gain (g fish}^{-1}) = (\text{final body protein content} \times \text{final body weight}) - (\text{initial body protein content} \times \text{initial live weight}) \quad (6)$$

Nitrogen loss (g N kg^{-1}) = (crude nitrogen intake to produce one kilogram of fish – nitrogen gain to produce one kilogram of fish) (7)

$$\text{Protein retention (\%)} = \frac{\text{body gain in protein}}{\text{digestible protein intake}} \times 100 \quad (8)$$

Statistical analyses

Data were analyzed by one-way ANOVA using the SAS GLM procedure (SAS Inst. Inc.). Differences among dietary treatments were compared using Tukey's multiple comparison test. The significance level adopted was 5 %.

Results

The mortality rate was less than 1 % throughout the experiment and was unrelated to the experimental diets. The performance variables are presented in Table 4. Fish responded well to the experimental diets and reached approximately 12 times their initial weight by the end of the trial. Weight gain (WG) and TGC differed marginally between fish fed the 32HighLys and 29BAL diets ($p \leq 0.05$). However, the 32HighLys and 32LowLys diets, despite having the same protein content, promoted distinct growth responses; WG and TGC were higher in fish fed the 32HighLys diet than in those fed the 32LowLys diet ($p \leq 0.05$). Fish fed the 22BAL diet had the lowest WG and TGC of all diets.

Fish fed the 32HighLys diet had the highest protein gain of all diets, and those fed 22BAL had the lowest ($p \leq 0.05$). Fish fed 32LowLys and 29BAL diets showed a similar response regarding protein gain ($p > 0.05$). Protein retention was highest in fish fed 29BAL and 22BAL diets ($p \leq 0.05$) (42 and 54 %, respectively).

The FCR was lower in fish fed the 32HighLys diet than in those fed the 22BAL diet ($p \leq 0.05$); all other diets promoted intermediate responses. Feed intake was highest in fish fed the 32HighLys diet and lowest in fish fed the 22BAL diet ($p \leq 0.05$).

Excretion of nitrogenous waste

The 32HighLys and 32LowLys diets promoted the highest production of nitrogenous waste ($p \leq 0.05$) (41.87 ± 1.31 and 42.78 ± 0.71 g N kg^{-1} produced fish, respectively). In contrast, the decreased dietary protein in the 29BAL and 22BAL diets resulted in the lowest nitrogen residue values (37.21 ± 0.74 and 23.59 ± 0.88 g N kg^{-1} produced fish, respectively; Figure 1).

Table 3 – Amino-acid ratios of the experimental diets based on their lysine level.

Amino acids, %	32HighLys[1]	32LowLys[2]	29BAL[3]	22BAL[4]	SL[5]	AMINO Tilapia[6]
Arginine	95	126	86	86	82	91
Phenylalanine	65	90	70	70	73	66
Histidine	41	48	35	34	34	41
Isoleucine	63	76	61	61	61	63
Leucine	114	161	103	103	66	84
Lysine	100	100	100	100	100	100
Methionine	47	42	42	42	52	42
Methionine+Cystine	66	68	59	60	63	69
Threonine	66	73	73	74	73	66
Tryptophan	20	22	19	19	20	21
Valine	70	85	57	57	55	70

[1]Formulated to meet the minimum amino acid recommendation of AMINOTilapia® (Evonik) with certain amino acid levels exceeding the recommended ideal ratio. [2]Formulated to meet Santiago and Lovell's (1988) minimum amino acid recommendation with certain amino acids exceeding the recommended ideal ratio. [3]Santiago and Lovell's (1988) amino acid recommendation and 292.1 g kg^{-1} digestible protein, balanced to meet ideal amino acids. [4]Santiago and Lovell's (1988) amino acid recommendation and 221.6 g kg^{-1} digestible protein, balanced to meet ideal amino acids. [5]Santiago and Lovell's (1988) ratio, based on lysine level. [6]Evonik ratio, based on lysine level (AMINOTilapia®).

Table 4 – Growth performance of Nile tilapia juveniles fed diets containing different amino acid profiles for 41 days.

Variables	32HighLys	32LowLys	29BAL	22BAL
Initial weight, g $fish^{-1}$	6.74 ± 0.12	6.77 ± 0.06	6.73 ± 0.06	6.75 ± 0.09
Weight gain, g $fish^{-1}$	81.15 ± 3.89^{a}	73.61 ± 4.22^{b}	74.85 ± 3.49^{ab}	65.12 ± 3.02^{c}
Thermal growth coefficient, %	0.23 ± 0.01^{a}	0.22 ± 0.01^{b}	0.22 ± 0.01^{ab}	0.20 ± 0.01^{c}
Feed intake, g $fish^{-1}$	100.55 ± 2.94^{a}	92.22 ± 5.19^{b}	94.62 ± 4.78^{ab}	83.09 ± 3.96^{c}
Feed conversion ratio, g g^{-1}	1.24 ± 0.02^{a}	1.25 ± 0.01^{ab}	1.26 ± 0.01^{ab}	1.28 ± 0.02^{b}
Protein gain, g $fish^{-1}$	12.63 ± 0.57^{a}	11.52 ± 0.53^{b}	11.67 ± 0.53^{b}	9.96 ± 0.29^{c}
Protein retention, %	39.21 ± 1.90^{c}	38.96 ± 0.59^{c}	42.26 ± 1.26^{b}	54.15 ± 1.59^{a}
Survival rate, %	100 ± 0.00	100 ± 0.00	99 ± 2.24	100 ± 0.00

Values were expressed as mean (± SD) of five replicates (n = 20 fish per replicate). [a,b,c]Values with different superscripts in the same row indicate differences.

Table 5 – Body composition of Nile tilapia juveniles fed different amino acid profiles for 41 days (expressed as wet matter).

Fraction	Initial	32HighLys	32LowLys	29BAL	22BAL
Moisture	77.17	67.50 ± 0.41[a]	67.31 ± 0.60[a]	67.86 ± 0.52[a]	65.79 ± 0.41[b]
Crude protein	13.66	15.44 ± 0.78	15.48 ± 0.21	15.44 ± 0.43	15.15 ± 0.33
Ether extract	5.17	13.15 ± 0.79[b]	13.44 ± 0.89[b]	12.85 ± 0.93[b]	15.31 ± 0.56[a]
Ash	3.75	3.55 ± 0.18	3.37 ± 0.20	3.66 ± 0.30	3.62 ± 0.16

Values were expressed in g 100 g^{-1} as mean (± SD) of five replicates (n = 5 fish per replicate). [a,b]Values with different superscripts in the same row indicate differences.

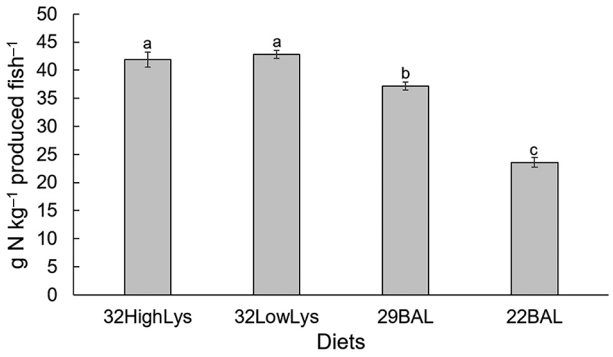

Figure 1 – Nitrogen loss in Nile tilapia juveniles fed diets containing different amino acid profiles for 41 days. Values expressed based on the mean (± standard deviation) of five replicates (n = 5 fish per replicate).

Body composition

Protein and ash contents did not differ ($p > 0.05$) among the fish-fed diets with different digestible protein and AA contents (Table 5). However, lipid and body moisture levels were significantly affected ($p \leq 0.05$). Fish fed the 22BAL diet had the highest body lipid content (15.31 ± 0.56 g 100 g^{-1}) of all diets. Overall, a lipid content ranging from 12.85 to 15.31 g 100 g^{-1} was recorded, regardless of the experimental diet.

Discussion

The formulation technique proposed here aimed to balance the AA profile to maintain protein quality, as the balance of dietary AA in each experimental diet was paramount. To ensure adequate AA balance, the basal diet (22BAL), which met the Nile tilapia AA requirements established by Santiago and Lovell (1988), served as the basis for formulating the 29BAL diet (the proposed original AA profile was not modified). It was possible to increase the dietary protein level without compromising the established AA balance as all AA were increased at the same rate. The dietary AA composition dictates the dietary protein concentration; however, formulating feeds to achieve the required protein and AA levels can lead to imbalances. The approach proposed here was based on meeting AA requirements and keeping the dietary AA balance constant (either in percentage or g kg^{-1} of diet). The 29BAL diet was a product of this approach in the present study. When comparing the AA composition (g kg^{-1}) of the 29BAL diet with the 22BAL diet, the 29BAL diet contained a higher concentration of all EAAs. However, the ratios among AAs remained unchanged between both diets. Maintaining a well-balanced AA profile when increasing protein levels is crucial. All AA should increase at an equal rate to achieve that balance. During such a process, the concentration of AA may differ. For instance, in the 29BAL diet containing 290 g kg^{-1} of digestible protein, the concentration of arginine increased to 17.9, while in the 22BAL diet, with 220 g kg^{-1} of digestible protein, the arginine concentration was 13.9 (Table 2). Despite that difference, the lysine-to-arginine ratio remained the same between both diets, as shown in Table 3 (Lys: Arg 86 vs 86). Increasing protein levels without increasing all amino acids in the same proportion is a common mistake that can lead to imbalances among amino acids at each dietary protein level and promote possible antagonisms among amino acids. This approach allows for the inclusion of arginine to be maintained at an adequate ratio to lysine. Arginine content commonly exceeds lysine content in diets, which could impair lysine absorption, as has been demonstrated in rainbow trout (Kaushik et al., 1988). However, we could not reduce the leucine content in our diets, considering the inclusion of soybean and corn gluten meals, which were the protein sources used here. This made it impossible to maintain an adequate leucine-to-lysine ratio in the 22BAL and 29BAL diets.

In the Nile tilapia production sector, it is common to use commercial feeds containing crude protein levels of 36 to 40 % for juveniles. Previous research has shown that the optimal dietary crude protein level for Nile tilapia juveniles weighing from 17 to 65 g is lower than previously thought, with the ideal level being 35 % (Abdel-Tawwab et al., 2010). In addition, a recent study conducted by Teodósio et al. (2020) found that juvenile tilapia weighing 5.9 to 31 g can be fed with even lower crude protein levels (30 % on an as-fed basis) without a negative impact on growth. Several studies have sought to establish an optimal protein concentration for Nile tilapia to meet its nutritional requirements (El-Saidy and Gaber, 2005; Botaro et al., 2007; Abdel-Tawwab et al., 2010; Teodósio et al., 2020). However, protein composition is a crucial point to consider, and AA profile and digestibility are key factors in determining the quality of a protein source (Ng and Romano, 2013). Diets with an unbalanced AA profile and/or protein-rich ingredients with low digestibility can result in

high estimates of dietary protein requirements (Gous et al., 2018). Different growth results were obtained by the 32HighLys and 32LowLys diets, which contained the same protein level but different AA profiles. Weight gain and TGC were the highest in fish fed the 32HighLys diet. When the protein content was reduced to 29 % digestible protein (29BAL) while applying our proposed formulation technique, productive performances similar to those recorded in fish-fed 32HighLys and 32LowLys diets were observed. The 29BAL diet has proven effective in reducing protein levels without affecting growth. Although the diet contained 290 g kg^{-1} of digestible protein on a dry matter basis, when converted to a crude protein on an as-fed basis of 287 g kg^{-1} it was still lower than the 30 % crude protein found by Teodósio et al. (2020). We emphasize that our experimental diets' protein and amino acid contents are not necessarily lower than what is current practice in the industry. However, the amount of protein or any other nutrient in the diet directly relates to the fish's ability to consume the feed. In situations where fish present reduced feed intake, the diet should be more concentrated in all nutrients to ensure that the daily demand in grams or milligrams is met, allowing fish to reach their maximum growth capacity. A study reported that body protein deposition did not change among Nile tilapia-fed diets with different protein levels, regardless of the increased protein intake of fish fed with higher protein levels (Teodósio et al., 2020). We observed a different pattern in the present study depending on the diet evaluated. For example, the protein gain was similar in the diets formulated following the recommendations of Santiago and Lovell (1988) (32LowLys and 29BAL). However, the protein contents of such diets differed, following the pattern observed by Teodósio et al. (2020). The reduction in protein levels of the 22BAL diet compromised protein gain compared with other treatments that used Santiago and Lovell's (1988) recommendation (Table 4). In contrast, there was a significantly increased deposition of body protein in fish fed the 32HighLys diet despite the protein level being equal to that of the 32LowLys diet. The additional protein gain may be related to the higher feed intake recorded in fish fed the 32HighLys diet (Table 4). When the protein retention was evaluated, which correlated the protein deposition with protein intake, there was no difference between fish fed the 32LowLys and 32HighLys diets. This shows that both diets allowed the same efficiency in depositing the ingested protein. According to the feed intake and growth theory proposed by Emmans (1981), animals tend to grow to reach their genetic growth potential, implying that they need to eat enough feed to maintain the growth dictated by their genetics. Furthermore, the author works on the premise that the animals are fed enough to meet the requirement of the first limiting nutrient in the diet (Emmans, 1987). It is unclear what led to the increased feed intake of fish fed the 32HighLys diet, as all AA reached the AMINOTilapia® recommendations. Another point that must be considered concerning the 32HighLys and 32LowLys diets is the inclusion of Lys and EAA-to-lysine ratio differences between AMINOtilapia® and Santiago and Lovell's (1988) recommendations, which led to distinct protein gains. In addition, the methionine and valine were more concentrated in AMINOtilapia® than Santiago and Lovell's (1988) recommendations. Additionally, a study applying the deletion technique for Nile tilapia juveniles showed that methionine and valine were the most limited essential AA in nitrogen deposition. When these AA were reduced by 45 % in the experimental diets, the nitrogen deposition was reduced by 65 % compared to a balanced diet (Diógenes et al., 2016). Thus, further studies are needed to elucidate any factors interfering with feed intake and how different EAA-to-lysine ratios could improve in Nile tilapia growth.

Fish fed the 29BAL diet had improved protein retention compared to those fed with the highest protein level (32HighLys and 32LowLys). The same pattern found in studies that evaluated graded dietary protein levels for Nile tilapia juveniles also observed increased protein retention when the dietary protein level was reduced (Abdel-Tawwab et al., 2010; Teodósio et al., 2020). In addition to impacting animal growth, it should be noted that protein is also utilized for maintenance purposes, with its surplus being excreted into the environment. Fish fed the 32HighLys and 32LowLys diets had the highest nitrogen loss values, but with a reduction in the protein level in the 29BAL and 22BAL diets, this nitrogen loss decreased significantly. The same pattern was highlighted by Abdel-Tawwab et al. (2010), and Teodósio et al. (2020) as protein levels decreased in the diet. A study evaluating two size classes of tilapia (fingerlings and advanced juveniles) found that fish did not use excess protein (above 35 % crude protein); instead, the excess dietary protein was deaminated and converted to ammonia (Abdel-Tawwab et al., 2010). Similar responses were reported by Teodósio et al. (2020) when increased excretion of nitrogenous compounds registered high catabolism of protein. The high release rates of nitrogenous compounds in the aquatic environment can cause eutrophication (Zhang et al., 2015). In addition, they can increase the emergence of pathogenic microorganisms in the water (Cabral, 2010). Thus, increased regulation of aquaculture effluents has motivated the development of tools that can mitigate the impacts of these effluents on the aquatic environment (Ghasemi et al., 2018). In this context, using environmentally friendly diets that promote lower levels of nitrogenous waste could be an alternative for reducing these impacts. In the present study, the diets with the lowest protein levels reduced nitrogenous compounds in the water by approximately 5 g N kg^{-1} produced fish or 12 % (29BAL) and 19 g N kg^{-1} produced fish or 44 % (22BAL) compared to the 32HighLys and 32LowLys diets. The benefits of the 22BAL and 29BAL diets become relevant when a large production scale is considered. Lowered nitrogenous waste excretions could make fish farming more sustainable. Despite the significant decrease in the excretion of nitrogenous compounds in fish fed the

22BAL diet, only the 29BAL diet showed a similar growth performance to the 32HighLys and 32LowLys diets.

As regards the quality of dietary protein and PR, it is essential to consider dietary energy levels and sources (Schrama et al. 2018; Konnert et al., 2022a, b). Excessive protein and low levels of non-protein energy sources (carbohydrates and lipids) in the diet should be avoided, making it essential to formulate diets with balanced energy and protein levels (Teles et al., 2020). However, excess dietary energy can compromise feed intake. Compared to terrestrial animals, fish have a much lower energy requirement for maintenance, as they do not need to maintain their body temperature and demand less energy when excreting nitrogen in the form of ammonia (Kaushik and Seiliez, 2010). In our study, the productive performance of fish fed the 22BAL diet was affected by the high energy: protein ratio (16.91 kcal g^{-1} of protein; Table 4). At higher energy: protein ratios, feed intake is compromised owing to excessive energy intake, which results in fat depositing in the body (Silverstein et al., 1999). Such response was observed here in fish fed the 22BAL diet, which presented a considerably lower feed intake and a higher body fat content (15.31 $\pm$ 0.56 g 100 g^{-1}) than the other diets.

It is essential to consider that the formulation method proposed here sought to balance the dietary EAA in diets with less protein levels. The study showed that it is possible to reduce dietary protein levels while maintaining a balance among EAA. However, future work should optimize this technique to make it economically viable, considering that we used high-cost AAs such as L-histidine, L-phenylalanine, and L-isoleucine. Thus, for future studies, we propose a dose-response assay aimed at economic viability to determine how much dietary protein can be reduced without compromising growth when the balance of AA is maintained among dietary treatments. In addition, it would be worth investigating to what extent nitrogen excretion can be reduced so that fish farming becomes an activity that is not only profitable but also sustainable for the environment.

The proposed strategy allowed us to obtain a diet with less digestible protein, and maintain a balanced AA profile without compromising Nile tilapia performance. The digestible protein content of that diet was 290 or 260 g kg^{-1}, on a dry or as-fed basis, respectively (320 or 287 g kg^{-1} crude protein on a dry or as-fed basis, respectively), with a digestible energy: digestible protein ratio of 12.48 kcal g^{-1} protein. In addition, the 29BAL diet reduced the excretion of nitrogenous waste by 12 % (5 g N kg^{-1} produced fish), implying a smaller impact on the aquatic environment.

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Author's Contributions

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