Received: 8 September 2022 | Revised: 25 October 2023 | Accepted: 25 October 2023

DOI: 10.1111/jwas.13040

ORIGINAL RESEARCH

Journal of the World Aquaculture Society Open Access | WORLD AQUACULTURE Society | WILEY

Sustainable feed formulation to community-based aquaculture: *Oreochromis niloticus* fingerlings performance and antioxidant status

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Funding information
AKDN - Aga Khan Development Network and FCT - Foundation for Science and Technology, Grant/Award Number: 333204505; FCT - Foundation for Science and Technology, Grant/Award Number: 47175; FEDER/ERDF, COMPETE 2020, CRESC Algarve 2020 and Portugal 2020

Abstract

Community-based aquaculture can reduce dependence on natural resources, promote biodiversity conservation, and improve local economies and food security. However, this activity is highly dependent on local conditions regarding natural resources, such as the availability of produced organisms, adequate feeds, and environmental factors. As ectothermic organisms, fish are more susceptible to temperature fluctuations in culture conditions. A set of raw ingredients (e.g., cassava and local beans) produced or available in villages from Cabo Delgado (Mozambique) with nutritional potential for fish feed were selected to produce an experimental diet. The following objectives were defined: (1) evaluate growth performance of tilapia fingerlings fed a diet produced with local ingredients, compared with a commercial-like diet; and (2) evaluate the response to thermal

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stress (18, 26, and 32°C) by tilapia fingerlings fed with tested diets. Tilapia fed with an experimental diet presented lower growth rates, lower DNA damage, higher neurophysiological, and antioxidant activity, leading to increased oxidative stress. Regarding energy budget, tilapia fed with the experimental diet presented higher protein content at 26°C and lipids at 18°C, leading to greater energy available at these temperatures. Overall, local ingredients can be successfully used as an additional feed source for tilapia production in community-based aquaculture in earthen ponds.

KEYWORDS
antioxidant defenses, genetic damage, growth, sustainability

1 | INTRODUCTION

Fish protein has significant potential to reduce food and nutritional insecurity in Africa. In addition, fish is also an important contributor to further development goals in Africa, promoting socioeconomic growth, alleviating poverty, and improving the livelihoods of marginalized communities. However, aquaculture in Africa encounters several challenges, limiting the capabilities of local governments to ensure the sector's sustainability and profitability. The lack of improved fish breeds, feeds, and technical training; weak research capacity; inadequate human and financial resources; poor market infrastructure and access; and weak governance and regulation represent the major challenges to the aquaculture sector in Africa (Chan et al., 2019).

Community-based aquaculture can assume a fundamental role in food production in developing countries, especially in rural villages far from large urban centers, where animal protein production for human consumption is limited, leading to nutritional problems that affect mainly children (Rocha & Dinis, 2021). Moreover, this activity has been suggested as an alternative income source that can help to improve the livelihoods and food security of the poorer communities (Ateweberhan et al., 2018).

The productivity of fish aquaculture in extensive regimes, without regular feed supply, is low and unsatisfactory, attending to the communities' labor and investment. Because aquatic organisms are healthy foods and can contribute to filling the deficit in animal protein in many populations, sustainable and community-based aquaculture can represent a way to overcome these constraints. The marine and freshwater biodiversity, directly and indirectly, supports food safety, nutrition, and livelihoods that are fundamental for millions of people worldwide. So, preserving aquatic ecosystems is vital to meet the nutritional needs of a sustainable growing global population (FAO, 2020). Despite the interest of the aquaculture industry in species with high economic value, such as marine carnivorous species, the world production of finfish continues to be dominated by freshwater herbivorous species (Chan et al., 2019).

Community-based aquaculture can assume a fundamental role in the production of herbivorous/omnivorous species, including carp, catfish, and tilapia (FAO, 2020). Tilapia are increasingly contributing to the food supply as a source of animal protein for the human population (Ng & Romano, 2013). The major challenge that tilapia farmers face, particularly in developing countries, is the development of cost-effective feeds using locally available and affordable resources (El-Sayed, 2020b). Dietary protein sources and inclusion levels mainly determine the feed cost. Earthen ponds contain natural food that provides a significant amount of protein, so commercial pond diets for tilapias usually contain 25% to 28% protein (Roberts, 2002). On the other hand, in undeveloped regions, commercial diets are unavailable or expensive. At low stocking densities, where natural food can contribute as an important

source of nutrients, complementary feeding with locally available, inexpensive raw ingredients, such as rice bran, copra meal, brewery waste, and coffee pulp, can increase production (Roberts, 2002). Consequently, the evaluation of raw ingredients produced or available in rural villages, that do not compete with human food sources or production, is vital for the development of community-based aquaculture.

Nile tilapia (*Oreochromis niloticus*) has the fastest growth rate among the tilapia species. It can grow up to 1 kg in 12 months compared with *Oreochromis mossambicus*, which would grow about 400 g in the same period (Moyo & Rapatsa, 2021). The widespread tilapia culture is because of its specific attributes, making it attractive to the aquaculture industry. Fast growth, tolerance to an extensive range of environmental conditions (such as temperature, salinity, and dissolved oxygen), resistance to stress and diseases, capability to reproduce in captivity, short generation time, low trophic levels consumers, and acceptance of inert feeds immediately after yolk-sac absorption are some of the most relevant attributes that make this species appealing to aquaculture (El-Sayed, 2020a). Regarding the ideal temperature ranges, this species shows a higher growth rate in higher water temperatures, ranging between 25 and 28°C (Moyo & Rapatsa, 2021). The lower and upper lethal temperatures are <11–12°C and >42°C, respectively (FAO, 2020). However, the water temperature fluctuations seem to affect their feeding behavior, being commonly followed by a complete stop of feeding and a lack of demand for feed (Magouz et al., 2020). High water temperatures have been reported to increase Nile tilapia mortality rate, which can jeopardize their farming (Dawood et al., 2020). This physical stressor can be reflected in decreased fitness, challenging the homeostatic power of fish and threatening its survival (Olivotto et al., 2002). Besides, fish metabolic scope and growth can also be affected, resulting in catabolic or gluconeogenic effects of corticosteroids (Chowdhury & Saikia, 2020).

The stress-induced increase in cortisol levels accelerates glucose metabolism and the production of reactive oxygen species (ROS), which can lead to oxidative stress in organisms through the increased production of ROS and the incapability to detoxify the ROS active species or repair damage (Madeira et al., 2013).

Therefore, the objectives of this work were (1) to evaluate the growth performance of tilapia fingerlings fed an experimental diet produced with local ingredients (from the Cabo Delgado province, Mozambique) in comparison with a commercial-like diet (control) and (2) to evaluate the response to thermal stress (18, 26, and 32°C) by tilapia fingerlings fed both diets. The ultimate goal of this study was to contribute with an adequate feed formula, based on locally available ingredients, that can improve fish production sustainability for local consumption in Mozambican rural villages.

2 | MATERIALS AND METHODS

2.1 | Experimental diets

The diets were produced by SPAROS, Lda (Olhão, Portugal), and the respective formulations are represented in Table 1. A commercial-like formula was used as a control (Ctrl) diet, and an experimental (Exp) diet was formulated with local ingredients collected in Mozambique. Most of the ingredients in the Exp diet are household food waste. The experimental diet also contains 2.5% insect meal to simulate the natural feed present in the earthen ponds. The amino acid profile of each diet is presented in Table 2. Diets were analyzed for chemical (dry matter, ash, crude protein, crude fat, and gross energy) and amino acid composition, as described in Aragão et al. (2020).

2.2 | Fish and growth trial

The trial was carried out in agreement with the Guidelines of the European Union Council (Directive 2010/63/EU) and Portuguese legislation for the utilization of laboratory animals. Nile tilapia (Silver Natural Male Tilapia™)

TABLE 1 Formulation and proximate composition of the control (Ctrl) and the experimental (Exp) diets (%).

Ingredients (%)	Ctrl	Exp
Fishmeal	7.50	2.50
Soybean meal	25.00	10.00
Poultry meal	7.50	-
Wheat meal	10.00	-
Rice bran	10.00	-
Corn meal	14.25	-
Rapeseed meal	7.75	-
Sunflower meal	7.75	-
Insect meal	-	2.50
Cassava leaves MZ	-	13.25
Moringa leaves MZ	-	1.00
Cassava root MZ	-	1.00
"Jugo" beans MZ	-	11.20
Shelled peanuts MZ	-	14.00
"Nhemba" beans MZ	-	9.50
"Pweri" beans MZ	-	13.25
Corn MZ	-	9.70
Sorghum MZ	-	6.00
Soybean oil	6.50	-
Palm oil	-	3.60
Vitamin and mineral premix[a]	1.00	1.00
Binder (guar gum)	1.50	1.50
Mono-calcium phosphate	0.40	-
L-lysine	0.15	-
L-threonine	0.20	-
L-tryptophan	0.05	-
DL-methionine	0.45	-
Proximate composition (% as fed basis)		
Crude protein	32.16	21.12
Crude fat	10.70	10.65
Ash	7.20	4.30
Moisture	7.20	7.30
Gross energy (MJ kg^{-1})	18.72	18.51

[a]Premix—vitamins (mg kg^{-1} diet): DL—alpha tocopherol acetate, 100 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 6.88 mg; DL—cholecalciferol, 0.05 mg; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg; betaine, 500 mg. Minerals (g or mg kg $diet^{-1}$): cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middling's. MZ: local ingredients from Mozambique.

TABLE 2 Amino acid profile of the control (Ctrl) and experimental (Exp) diets and amino acid requirement for Nile tilapia, *Oreochromis niloticus*.

Amino acid (mg g^{-1} dry matter)	Ctrl	Exp	Amino acid requirements for *O. niloticus* (NRC, 2011)
Arginine	32.6	19.9	12.0
Histidine	9.8	5.9	10.0
Lysine	20.9	13.2	16.0
Threonine	17.3	8.3	11.0
Isoleucine	15.1	10.1	10.0
Leucine	24.7	17.7	19.0
Valine	17.4	12.0	15.0
Methionine	12.9	3.0	7.0
Phenylalanine	19.9	15.6	11.0
Cystine	2.9	1.6	-
Tyrosine	18.0	15.1	-
Aspartic acid + Asparagine	25.9	19.2	-
Glutamic acid + Glutamine	47.2	32.8	-
Alanine	16.4	10.2	-
Glycine	22.3	11.3	-
Proline	17.6	10.2	-
Serine	16.5	11.2	-
Taurine	0.9	-	-

fingerlings were acquired from TIL-AQUA International B.V. (Someren, Netherlands), and the trial was performed at the Centre of Marine Sciences (CCMAR) facilities (Faro, Portugal). Nile tilapia fingerlings with an average body weight of 0.5 g were acclimatized for 25 days to the new rearing facilities in a recirculating aquaculture system (RAS) and fed a commercial diet.

Fingerlings were reared in 100 L-cylindrical tanks in a RAS. The system was equipped with a mechanical filter, a biological filter, and a UV sterilizer. Water temperature (25.5 ± 0.5°C), dissolved oxygen (96.5 ± 2.7% saturation), and pH (8.4 ± 0.2) were monitored daily. Fish mortality was also evaluated daily, and dead fish were weighed. The concentration of ammonia and nitrites were monitored weekly, or more frequently, if necessary, with colorimetric tests, and maintained at undetectable levels. Photoperiod was natural (October until December, 37°02′34.9″″N 7°58′15.6″W).

Fingerlings of Nile tilapia with an initial average body weight of 0.75 ± 0.11 g and total body length of 3.8 ± 0.2 cm were randomly distributed into six tanks at an initial density of 0.36 kg m^{-3} (50 fish per tank). Each diet was randomly assigned to triplicate tanks. The fish were fed to visual satiety by hand, three times a day (09 h30, 12 h30, and 16 h00) and the feed intake was recorded daily for 64 days.

2.2.1 | Sampling

Before the beginning of the trial, 15 fish were euthanized using a lethal dose of anesthetic (1.5 mL L^{-1} of 2-phenoxyethanol; Sigma-Aldrich) and stored at −20°C until analysis. To monitor growth and feed utilization, an intermediate weighing was performed where fish from each tank were bulk weighed and counted under moderate anesthesia (0.5 mL L^{-1} of 2-phenoxyethanol) after 27 days.

At the end of the trial (64 days), fish were bulk weighed, and 8 fish per tank were individually weighed and measured under moderate anesthesia. From these, three fish were euthanized with lethal anesthesia and liver and viscera were collected and weighed for analysis of somatic indexes. The fish were deprived of food for 24 h before the initial and the final samplings.

2.2.2 | Key performance indicators

The growth performance and the feed utilization parameters were calculated through the following formulas:

Weight gain (% IBW) = $100 \times (FBW - IBW) \times IBW^{-1}$, where IBW and FBW are the average of the initial and final body weights, respectively

Daily growth index (DGI) = $100 \times (FBW^{1/3} - IBW^{1/3}) \times days^{-1}$ (Glencross, 2020)

The Fulton's condition factor (K) = $(W \times 100) \times L^{-3}$, where W = weight (g) and L = total length (cm)

Feed conversion ratio (FCR): apparent feed intake × wet weight gain^{-1}, where wet weight gain is FBW – IBW

Daily voluntary feed intake (VFI, % day^{-1}): $100 \times$ crude feed intake $\times$ $ABW^{-1} \times days^{-1}$, where ABW is (IBW + FBW)/2

Protein efficiency ratio (PER): wet weight gain × crude protein intake^{-1}

Hepatosomatic index (HSI, %) = $100 \times$ liver weight × body weight^{-1}

Viscerosomatic index (VSI, %) = $100 \times$ viscera weight × body wight^{-1}

2.3 | Temperature trial

A temperature trial was carried out after the growth trial, in which the response to thermal stress (18, 26 and 32°C) by tilapia fingerlings fed both diets was evaluated. Fingerlings of Nile tilapia with an initial average body weight of 11.96 ± 1.99 g and a total length of 8.82 ± 0.47 cm in the control diet (Ctrl) and an initial average body weight of 2.29 ± 0.84 g and a total length of 5.26 ± 0.60 cm in the experimental diet (Exp) were provided at the end of the growth trial.

Fish were acclimated for 96 h (4 days) at 26°C and fed according to their previous nutritional background. After, five fish were randomly distributed in six 250 L RAS with five 10 L tanks each (1 fish per 10 L tank). Each RAS was set for the water temperature tested (18, 26 and 32°C) and one RAS was used for each temperature and diet combination. For thermal stress treatments, the temperature was gradually adjusted for approximately 5 h for 18 and 32°C, respectively. The exposure to the different temperatures was performed for 24 h.

All the RAS were kept simulating a natural photoperiod with a daily water renewal of 70%. Every day the water temperature, dissolved oxygen, and pH were measured. Colorimetric tests were also performed to assess the presence of total ammonia nitrogen (TAN).

2.3.1 | Tissue collection and biochemical parameters

Fish were not fed for 24 h before and during the sampling. At each sampling moment, initial (T0h) and final (T24h) samplings, five specimens were collected per experimental unit (RAS) ($n = 5$), and the biometric parameters such as body weight (g) and total length (cm) were measured. Immediately after collection, fish blood was drawn from the posterior cardinal vein using glass Pasteur pipettes, and blood smears were immediately prepared for the erythrocytic nuclear abnormalities (ENA) assay and erythrocytic maturity index (EMI) assays. For the biochemical analysis, tissue samples of 5 fish per treatment were used ($n = 5$), for the blood analysis (Comet, ENA and EMI), only samples of three fish per treatment were used ($n = 3$).

For the genetic damage analyses, 2 μL of blood was collected using a micropipette, diluted in 1 mL of PBS and DMSO (9:1, v/v) in microtubes and kept on ice. The microtubes were posteriorly kept at −20°C for 1 week and then at −80°C until further analyses. Fish were sacrificed by cervical transection.

For the neurophysiological, oxidative stress and energy budget evaluation, liver, muscle, and whole head were also collected in microtubes (liver and muscle) and in falcon tubes (whole head) and immediately frozen in liquid nitrogen and stored at −80°C until analyses.

Comet assay

The comet assay was performed according to the technique described by Collins (2004) and adapted by Guilherme et al. (2010). A system of six mini-gels per slide was adopted, following a model created by Shaposhnikov et al. (2010) in order to increase the assay output. Each organism is represented by two replicate mini-gels, being three different organisms represented in each slide. Each slide represents a different treatment because three fish per treatment were used ($n = 3$).

Briefly, 20 μL of cell suspension was mixed with 70 μL of 1% low melting point agarose, in PBS, and drops with 7 μL were placed in the precoated slide as two rows of three mini-gels, without coverslips. The slides were kept for ±5 min at 4°C in order to solidify the agarose and then immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X-100, pH 10) at 4°C, and kept for ±1 h in the dark.

After lysis of agarose-embedded cells, slides were then moved to the electrophoresis tank (Sub-Cell® GT, Bio-Rad) and immersed in the electrophoresis solution, for 20 min, for alkaline treatment. DNA migration was performed for 15 min, at a fixed voltage of 25 V, a current of 300 mA (power supply PowerPac™, Bio-Rad), which resulted in 0.7 V cm^{-1} (achieved by adjusting the total volume of buffer). The slides were then neutralized in PBS for 10 min, followed by 10 min in distilled water and 10 min in ethanol (100%) at 4°C, and left to dry overnight at room temperature. For nucleoids counting and DNA damage evaluation, the slides were stained with ethidium bromide (20 μg mL^{-1}) and observed using a Leica DMLS fluorescence microscope (x400 magnification). The DNA damage was quantified through visual classification of nucleoids into five comet classes, according to the tail intensity and length, from 0 (no tail) to 4 (almost all DNA in the tail) (Azqueta et al., 2009).

The total score expressed as a genetic damage indicator (GDI) was calculated by multiplying the percentage of nucleoids in each class by the corresponding factor, according to the following formula:

$$\mathrm{GDI} = \sum \%\text{nucleoids class } i \times i$$

GDI values were expressed as arbitrary units in a scale of 0 to 400 per 100 scored nucleoids (as an average value for the 2 mini-gels observed per fish).

Erythrocytic nuclear abnormalities (ENA) assay and erythrocytic maturity index (EMI)

The assay was carried out in mature peripheral erythrocytes, according to Pacheco and Santos (1996). Previously, one blood smear per animal was fixed with methanol for 10 min and stained with Giemsa (5%) for 30 min. Slides were coded and scored blind. From each smear, 1000 erythrocytes were scored, under ×1000 magnification (Leitz HM-Lux 3 microscope), to evaluate the relative frequency of the following nuclear lesions: kidney-shaped nuclei (K), lobed nuclei (L), segmented nuclei (S), vacuolated nuclei (V), and micronuclei (MN). Results were expressed as the sum of frequencies for all the categories observed (K + L + S + V + MN).

The EMI was determined according to Maceda-Veiga et al. (2010) with the modifications proposed by Castro et al. (2018). Briefly, 10 microscopic fields were randomly selected per slide (one slide per fish and photographed under ×1000 magnification (Leitz HM-Lux 3 microscope). Then, in each microscopic field, 25 random cells were analyzed with ImageJ software, measuring the minor axis of the nuclei and the major axis of the cells (A and B). EMI was calculated for each cell by dividing A by B values for a total of 250 cells. From the values of the ratio, cells were then categorized into one of the 10 maturity classes: [0.0 ≤ Class 1 < 0.1], [0.1 ≤ Class 2 < 0.2], [0.2 ≤ Class 3 < 0.3],

[0.3 ≤ Class 4 < 0.4], [0.4 ≤ Class 5 < 0.5], [0.5 ≤ Class 6 < 0.6], [0.6 ≤ Class 7 < 0.7], [0.7 ≤ Class 8 < 0.8], [0.8 ≤ Class 9 < 0.9], and [0.9 ≤ Class 10 < 1]. Class 1 represents erythrocytes with a higher maturity level, and class 10 corresponds to cells with lower maturity status. Finally, average values for the frequency (%) of cells observed in each maturity class were represented for each experimental group.

Neurophysiological and oxidative stress biomarkers

All the liver and muscle samples were divided into two aliquots and weighed. One set of liver samples was individually homogenized and kept on ice with 1600 μL of 0.1 M K-phosphate buffer, pH 7.4 and sonicated (pulsed mode of 10% for 30 s, 250 Sonifier, Branson Ultrasonics) for the oxidative stress analyses. From the post-mitochondrial supernatant (PMS), aliquots for the measurement of catalase (CAT), glutathione-S-transferase (GST), total glutathione (tGSH), protein carbonylation (PC), and protein content were centrifuged for 20 min at 10,000*g* (4°C).

The set of muscle samples was individually homogenized and kept on ice with 1600 μL of ultrapure water and sonicated (pulsed mode of 10% for 30 s). Three aliquots of 300 μL were separated from these samples for the analyses of the energy budget through the lipid, sugar, and protein contents, as well as the electron transport system (ETS) activity.

The eyes of each sampled head were collected and weighed. The eyes were individually homogenized by sonication (pulsed mode of 10% for 30 s) on ice with 1000 μL of 0.1 M K-phosphate buffer, pH 7.4, to determine the acetylcholinesterase (AChE) activity. Regarding AChE, the post-mitochondrial supernatant (PMS) was obtained through the sample's centrifugation for 20 min at 10,000*g* (4°C).

All biomarkers measurements were performed with the Microplate reader Multiskan Spectrum (Thermo Fisher Scientific, USA). The protein concentration of PMS was determined following the Bradford method (Bradford, 1976) adapted from Bio-Rad's Bradford micro-assay set up in a 96-well flat bottom plate, with a standard of bovine γ-globulin. Catalase (CAT) activity was determined in PMS by measuring the decomposition of the substrate H_2O_2 at 240 nm (Claiborne, 1985). Glutathione-S-transferase (GST) activity was determined in PMS following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). tGSH content was calculated as the rate of TNB^{2-} formation with an extinction coefficient of DTNB chromophore formed, $\varepsilon = 14.1 \times 103\ M^{-1}\ cm^{-1}$ (Baker et al., 1990; Rodrigues et al., 2017).

Acetylcholinesterase activity (AChE) was measured using PMS by following Ellman's method (Ellman et al., 1961) adapted to a microplate (Guilhermino et al., 1996). The method uses acetylthiocholine as a substrate following the increase of absorbance at 412 nm.

The quantification of the energy available (Ea—as the sum of sugars, lipids, and protein) and energy consumption (Ec—measured as ETS activity) were measured following the method labeled by de Coen and Janssen (1997) with slight modifications for microplate reading (Rodrigues et al., 2015). To 300 μL homogenate samples, 500 μL of chloroform (119.38 M; ACS spectrophotometric grade, ≥99.8%) and methanol (32.04 M; ACS reagent, ≥99.8%) were added to separate the total lipid content by centrifugation. The organic phase of each sample was transported to a clean glass tube, where 500 μL of H_2SO_4 was added; these were then incubated at 200°C for 15 min. Samples were cooled down to room temperature, and ultra-pure water (1500 μL) was added to each tube. The absorbance of samples and tripalmitin, as a standard, were measured on the microplate at 375 nm. The carbohydrate and total protein reserves were determined by first adding 100 μL of 15% TCA to 300 μL homogenized samples, succeeding an incubation of 10 min at 20°C. After centrifugation at 1000*g* for 10 min at 4°C, the supernatant was collected and used for carbohydrate measurements. The pellet was then resuspended in 500 μL of NaOH, incubated for 30 min at 60°C and neutralized in 280 μL of HCl. This fraction was used for protein measurements. To quantify carbohydrate content, standards of glucose and samples were incubated at 20°C for 30 min and the absorbance was read on the microplate at 492 nm. Total protein content was quantified following Bradford's (Bradford, 1976) following de Coen and Janssen (1997), ETS activity was measured on 300 μL homogenate samples which were added 150 μL of homogenization buffer (0.3 M Tris base; 0.45% (w/v) polyvinylpyrrolidone; 459 μM $MgSO_4$; 0.6% (v/v) Triton X-100 at a pH 8.5) and centrifuged at 1000*g*, 10 min, 4°C. On the microplate, 50 μL of supernatant was incubated with

150 μL of buffered solution 0.13 M Tris base containing 0.27% (v/v) Triton X-100; 1.7 mM NADH; 274 μM NADPH; and with 100 μL of INT solution (p-iodonitrotetrazolium; 8 mM). The absorbance was measured kinetically over a 3 min period at 490 nm. The fractions of energy available were converted into energetic equivalent values using the corresponding energy of combustion: 39.50 kJ g^{-1} lipid, 17.50 kJ g^{-1} glycogen, and 24.00 kJ g^{-1} protein (de Coen & Janssen, 1997).

The cellular oxygen consumption rate conversion was prepared based on the stoichiometric relationship, wherein for every 2 μmol of formazan formed, 1 μmol of oxygen is consumed. The final energy consumption (Ec) value was found by conversion to energetic values using the specific oxyenthalpic equivalent to an average lipid, protein, and carbohydrate mixture of 480 kJ mol^{-1} O_2. The available energy (Ea) was calculated as the sum of the total lipid, carbohydrate and protein contents. At last, CEA value was determined according to the equation CEA = Ea/Ec (Verslycke et al., 2003).

2.4 | Statistical analysis

All the data were analyzed through Shapiro–Wilk normality tests and for homogeneity of variance through Kolmogorov–Smirnov test. For the growth data, variations were tested using independent samples *t*-test by groups. The data were transformed when necessary for the temperature trials to meet ANOVA assumptions of normality and equal variance. Two-way ANOVA was used, followed by post hoc tests (Tuckey's and Sidak's) in order to examine the influence of two different categorical independent variables, temperature and type of diet, on the DNA damage (Comet assay), erythrocytic nuclear abnormalities (ENA) assay, neurophysiological activity (AChE), antioxidant defenses (CAT, GST, tGSH, and PC), energy reserves (CEA, Ea, and Ec), and the fish condition factor (K). This aims to assess the main effect of each independent variable and if there is any interaction between them. A significance level of $p < 0.05$ was used. The statistical analysis was performed using Graphpad Prism software version 7.0.

3 | RESULTS

3.1 | Growth trial

The overall growth performance of Nile tilapia fingerlings fed with the Ctrl and Exp diets is presented in Table 3 (statistics and *p*-values are presented in Table S1). All fish had an increase in body weight at the end of the trial. The fish fed with Ctrl diet were significantly heavier when compared with the group fed with the Exp diet. Similarly, the fish weight gain and the daily growth index (DGI) showed significant differences between the two groups, with higher values observed for fish from Ctrl group.

The feed conversion ratio (FCR) was significantly higher in the group fed with the experimental diet than in the Ctrl group. On the contrary, the protein efficiency ratio (PER) was significantly higher in the Ctrl fed fish. No differences were observed among the two groups regarding the Fulton's condition factor (K), and the hepatosomatic (HSI), and viscerosomatic (VSI) indexes. Survival was similar among dietary treatments.

3.2 | Temperature trial

3.2.1 | DNA and chromosomal damage (comet and ENA assays) and hematological dynamics (EMI)

No significant differences were observed between the two groups regarding the genetic damage indicator (GDI) values of Nile tilapia (Figure 1a, Table S2). However, the Ctrl fish exposed at 18 and 32°C had significantly higher

TABLE 3 Growth performance of *O. niloticus* fed a control (Ctrl) and experimental (Exp) diet for 64 days.

	Ctrl		Exp	
Parameters	**Mean**	**SEM**	**Mean**	**SEM**
Initial body weight, IBW (g)	0.71[a]	0.00	0.71[a]	0.00
Final body weight, FBW (g)	9.78[a]	0.67	1.98[b]	0.16
Weight gain (% IBW)	1055.02[a]	25.97	179.65[b]	8.78
Daily growth index (DGI)	1.79[a]	0.04	0.61[b]	0.03
Fulton's condition factor (K)	1.75[a]	0.02	1.68[a]	0.03
Feed conversion ratio (FCR)	1.09[a]	0.00	1.87[b]	0.01
Daily voluntary feed intake (% day^{-1})	2.86[a]	0.02	2.79[a]	0.06
Protein efficiency ratio (PER)	2.85[a]	0.01	2.54[b]	0.02
Hepatosomatic index (HSI)	0.67[a]	0.11	1.00[a]	0.19
Viscerosomatic index (VSI)	9.00[a]	0.35	9.90[a]	0.33
Survival (%)	96.0[a]	2.31	92.7[a]	2.40

Note: All values are presented as means ± standard error of the means (SEM, $n = 3$, except for IBW, where $n = 50$, for final body weight and K where $n = 24$ and for HSI and VSI where $n = 9$). Different letters within the same row indicate significant differences ($p < 0.05$) among the dietary groups.

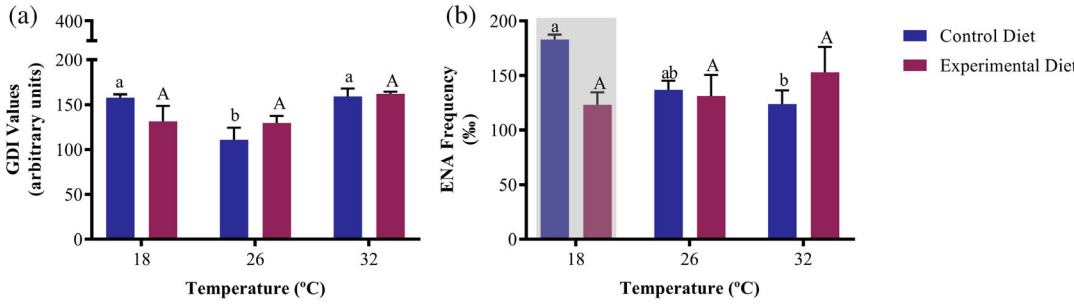

FIGURE 1 (a) Genetic damage indicator (GDI, mean ± SEM), expressed as arbitrary units, in blood cells of *O. niloticus*. (b) Erythrocytic nuclear abnormalities (ENA, mean ± SEM) frequency (‰) in peripheral erythrocytes of *O. niloticus* after 24 h exposure to the tested temperatures (18, 26, and 32°C). Statistically significant differences (Sidak's multiple comparisons test, $p < 0.05$) are represented by shade area—between dietary groups within the same tested temperature; different letters indicate significant differences between temperatures within each dietary group.

GDI values than the fish exposed at 26°C. The fish fed the Ctrl diet presented significantly higher total ENA frequency than the Exp fed fish at 18°C (Figure 1b, Table S2). Furthermore, the Ctrl group had a significantly higher total ENA frequency when exposed at 18°C than at 32°C (Figure 1b, Table S2). Regarding the EMI assay, the fish fed with the Ctrl diet presented a higher percentage of mature classes (class 0 to 5) at all tested temperatures when compared with the Exp fish (Table 4). This is in line with the higher percentage of immature erythrocytes (classes 6 to 10) in the fish fed with Exp diet at all the tested temperatures.

3.2.2 | Neurophysiological and oxidative stress activity

No significant differences were observed regarding the AChE activity measured in the eyes of fish, disregarding the diet or temperature treatments (Figure 2, Table S3).

Fish fed with the Ctrl diet presented a significantly lower CAT activity in the liver than Exp fed fish when exposed to 26 and 32°C (Figure 3a, Table S3). Additionally, CAT activity in the Ctrl fish decreased at 32°C when compared with fish at 18°C. GST activity in the liver of Nile tilapia presented no statistically significant differences between the different dietary groups and temperatures tested (Figure 3b). The tGSH content in the liver was significantly altered because of the diet. Fish fed Exp diet presented significantly higher tGSH than the fish fed Ctrl diet at 32°C (Figure 3c, Table S3). In addition, in the Exp group, tGSH levels increased in fish exposed to 32°C when compared with the group exposed to 18°C (Figure 3c). The PC levels in the liver of Nile tilapia were significantly lower in fish fed the Ctrl diet than the Exp fed fish when exposed to 18 and 26°C (Figure 3d, Table S3).

3.2.3 | Cellular energy allocation assessment

Regarding the sugar content in the muscle tissue of Nile tilapia, no significant alterations were observed between the groups (Figure 4a, Table S3). Fish fed Exp diet presented higher protein content at 26°C compared with the fish fed Ctrl diet (Figure 4b, Table S3). Besides, the Exp group presented a significantly lower protein content at 18 and 32°C compared with the group exposed at 26°C (Figure 4b). The lipid content in the fish fed with Exp diet was significantly higher compared with the fish fed Ctrl diet at 18°C (Figure 4c, Table S3). Further, fish from both dietary treatments presented, at 18°C, significantly higher lipid content than the fish exposed at 32°C (Figure 4c).

The Ea in the muscle tissue of Nile tilapia was significantly higher in fish fed the Exp diet when exposed at 18 and 26°C compared with fish fed Ctrl diet at the same temperatures (Figure 4d, Table S3). Within the Exp group,

TABLE 4 Erythrocytic maturity index (EMI) in *O. niloticus* fed with the Ctrl and Exp diets after 24 h (T24h) exposure to the different temperatures (18, 26, and 32°C).

Groups	Mature classes (1 to 5)	Immature classes (6 to 10)
18°C—Ctrl	99.60	0.40
18°C—Exp	91.07	8.93
26°C—Ctrl	95.20	4.80
26°C—Exp	82.80	17.20
32°C—Ctrl	98.40	1.60
32°C—Exp	87.20	12.80

Note: For each group, average values for the frequency (%) of cells observed are represented in two groups, a group of mature classes (class 1 to 5) and a group of immature classes (class 6 to 10).

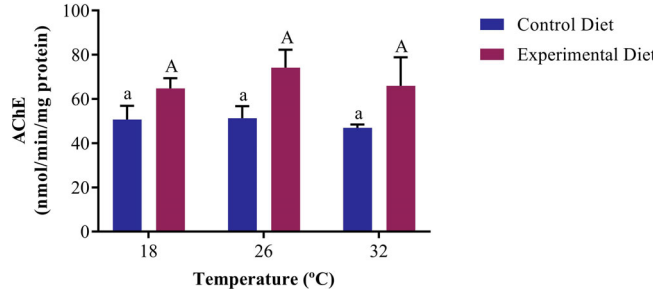

FIGURE 2 Acetylcholinesterase (AChE, mean ± SEM) activity in the eyes of *O. niloticus* after 24 h exposure to the tested temperatures (18, 26, and 32°C). Same letters indicate no significant differences between temperatures within each dietary group (Sidak's multiple comparisons test, $p < 0.05$).

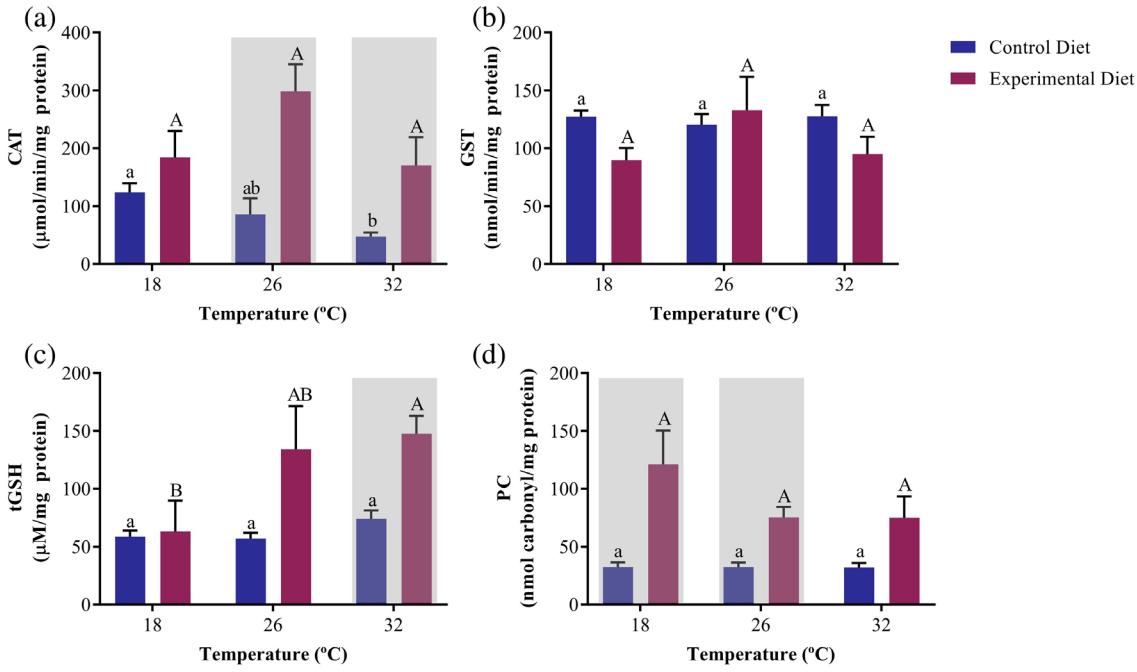

FIGURE 3 (a) Catalase (CAT) activity, (b) glutathione-S-transferase (GST) activity, (c) total glutathione (tGSH), and (d) protein carbonylation (PC) levels in liver of *O. niloticus* after 24 h exposure to the tested temperatures (18, 26, and 32°C). Data are presented as mean ± SEM. Statistically significant differences (Sidak's multiple comparisons test, $p < 0.05$) are represented by shade area—between dietary groups within the same tested temperature; different letters indicate significant differences between temperatures within each dietary group.

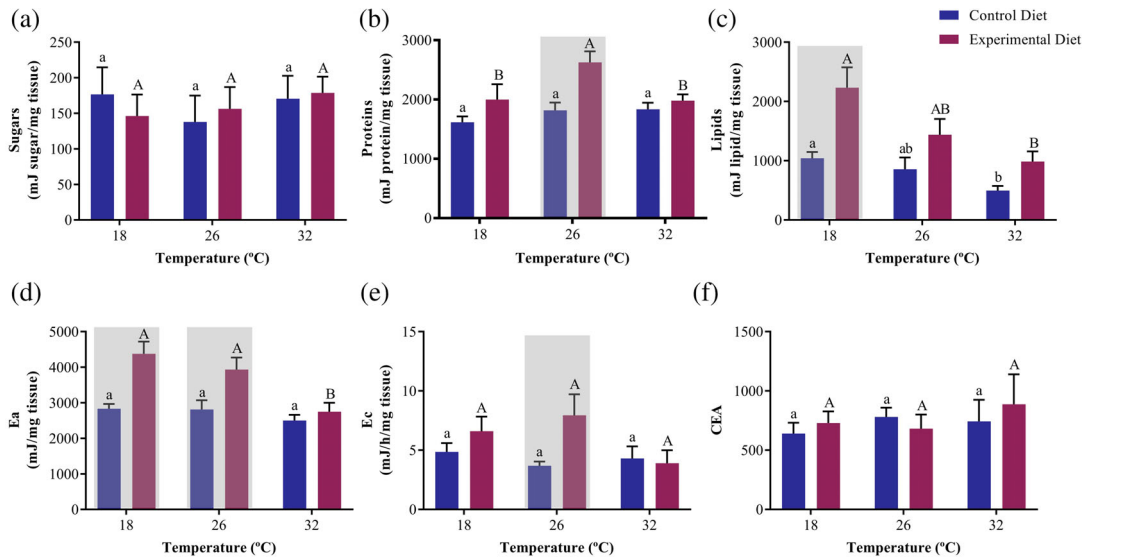

FIGURE 4 (a) Sugars, (b) protein and (c) lipids content and (d) energy available (Ea), energy consumption (Ec), and (f) cellular energy allocation (CEA) in muscle of *O. niloticus* after 24 h exposure to the tested temperatures (18, 26, and 32°C). Data are presented as mean ± SEM. Statistically significant differences (Sidak's multiple comparisons test, $p < 0.05$) are represented by shade area—between dietary groups within the same tested temperature; different letters indicate significant differences between temperatures within each dietary group.

at 32°C, fish presented lower values of Ea when compared with fish exposed to 18 and 26°C (Figure 4d). Regarding the Ec, the fish fed Exp diet presented higher values of Ec at 26°C than the fish fed with Ctrl diet at the same temperature (Figure 4e, Table S3). No differences were observed between the two groups of fish regarding CEA (Figure 4f, Table S3).

4 | DISCUSSION

Community-based aquaculture can assume a fundamental role in the production of food in undeveloped countries, especially in villages far from large urban centers. These communities live on the edge of poverty and do not have the economic capacity to acquire commercial aquafeeds.

This production system is usually carried out in earthen ponds, with no extra feed supply. The fish depends on a variety of natural food such as organic matter, and small invertebrates, like plankton and insects. In some cases, feed based on agricultural by-products such as brans, plant-based oils, vegetation, and manures may be supplied (FAO, 2019). Furthermore, most of the fish feed in these communities is imported at a high cost, which makes it essential to search for and develop diets that the communities can produce with local ingredients. The experimental diet was formulated with several local ingredients that have been used to replace the protein sources in the commercial diet, such as fishmeal, poultry meal, soybean meal, and other plant-based ingredients. This replacement resulted in a diet with a lower protein content than the commercial diet and with some dietary amino acid deficiencies. However, some of the local ingredients used may have the potential to reduce some of these deficits or even bring additional benefits. Indeed, in a study carried out with Nile tilapia, in which moringa (*Moringa oleifera*) was incorporated into the fish diet, it was observed an increase in fish growth performance and immunity of fish against bacteria (*Aeromonas hydrophila*) (Monir et al., 2020). Cassava leaves are a rich source of protein, minerals, and vitamins and also contain a similar amount of essential amino acids to those found in hen's egg and present higher amino acid content than spinach leaves, soybean, oat, and rice grain (Latif & Müller, 2015). Although the experimental diet does not have the optimal protein content and does not completely fulfill the amino acid requirements for the species, fish intake may be compensated with natural food, such as small invertebrates, available in the earthen ponds. Insects are natural food sources for fish, especially continental species, and there is a rising interest in using insects as protein sources (Nogales-Mérida et al., 2019). Insects can represent an interesting aquafeed ingredient (Hua et al., 2019) because they require fewer natural resources (such as land and water) than plants and can be reared on by-products, thus transforming abundant and low-cost organic waste into protein-rich animal biomass suitable for use in aquafeeds (Barroso et al., 2014). In this study, only a small amount of insect meal (2.5%) was incorporated into the experimental diet to mimic the natural feed present in earthen ponds.

4.1 | Growth trial

Considering the growth trial, fish fed with the Exp diet had a lower growth performance, being lighter at the end of the experiment than fish fed with the Ctrl diet. Despite being an omnivorous species, tilapia as well as other fish, require more protein content in their diet at early growth stages. According to El-Sayed (2020a), the protein requirement ranges from 30% to 40% for juvenile tilapia, while adult tilapia requires from 25% to 30% of dietary protein for optimum performance. Therefore, the protein content of the Exp diet was not considered ideal for tilapia fingerlings. It should be noted that the Exp diet was as optimized as possible based on the resources of the Mozambican communities. Although feeding with the Exp diet may have impaired growth performance, both diets were translated into similar fish condition factors and survival. Thus, in both dietary treatments, fish grew proportionally, indicating that the Exp diet meets the minimal requirements, ensuring fish survival and growth. Furthermore, HSI and VSI were not significantly affected, indicating no major metabolic impairments in fish fed the Exp diet.

According to the literature, the incorporation of plant-based ingredients in the fish feed may often result in lower feed intake, nutrient-energy digestibility, and retention, which can affect the growth performance because of the presence of glycoalkaloids, as compared with fish fed with fishmeal-based diets (Fournier et al., 2004; Randazzo et al., 2021; Silva et al., 2010). In a study carried out by Fournier et al. (2004), using a mix of plant feedstuffs (corn gluten meal, wheat gluten, and lupin) as a substitute for FM in diets for juvenile turbot, the voluntary feed intake was low and strongly affected by plant protein incorporation in diets. Another study carried out by Silva et al. (2010), reported low feed intake, growth performance, and protein accretion in Senegalese sole fed a plant protein-based (with soybean meal, corn gluten, wheat meal, and wheat gluten) diet. Another factor influencing feed intake and, consequently, growth performance is the lower palatability that plant-origin ingredients have (Daniel, 2018). Promising results in growth performance and feed conversion ratio were observed in rainbow trout fed plant-based diets with the inclusion of functional ingredients of animal origin, such as black soldier fly (*Hermetia illucens*) and poultry by-product meals (Daniel, 2018; Gaudioso et al., 2021; Randazzo et al., 2021). Also, a study carried out by Tusche et al. (2012) with various combinations of wheat gluten and potato protein concentrate at a fixed FM replacement of 56% in rainbow trout diets demonstrated no significant growth performance differences.

This was considered a potential problem in this experiment, because of the incorporation of local plant ingredients used in the Exp diet that might present some antinutritional factors. Nevertheless, local ingredients were cooked before dietary inclusion, as the Mozambican communities can do, to minimize the potential antinutritional factors. As a result, the daily voluntary feed intake observed in this study reveals no differences between groups, indicating that both diets were readily accepted by the fish. Additionally, there were no observed potential toxic effects associated with the diets, as evidenced by the similar survival rates among all groups. Nevertheless, the feed conversion ratio (FCR) was higher in the experimental groups, meaning that fish fed Exp diet needed more feed to achieve the same level of growth as the Ctrl treatment. The feed conversion ratio for *O. niloticus* may vary significantly because of factors such as the type of diet, the feeding regime, and the fish life stage (Abdel-Tawwab et al., 2010; El-Sayed, 2020a; Magouz et al., 2020; Tusche et al., 2012). In fact, the results of this study are positive. Despite the better results observed for fish fed the Ctrl diet, fish fed the Exp diet had similar results compared with previous studies with the same size-range tilapia (Abdel-Tawwab et al., 2010). Lower FCR values indicate higher feed efficiency, leading to a reduction in production costs (Martínez-Córdova et al., 2017). In some production scenarios in earthen ponds, the FCR is normally low, <1 for tilapia, because of the availability of natural food (Hasan & New, 2013). Thus, the FCR obtained in the experimental groups is expected to decrease in community-based aquaculture in earthen ponds.

The protein efficiency ratio (PER) was higher in the Ctrl fed fish, suggesting that these fish were more efficient using the dietary protein than fish fed with the Exp diet. The lower PER values found in fish fed the Exp diet are probably a result of the low dietary protein content. At this point, it cannot be excluded that nutrient digestibility, especially protein/amino acid digestibility, might have been affected because of the presence of some antinutritional factors in the Exp diet, which would also have an impact on the PER. This aspect deserves attention in further studies. However, in fertilized earthen ponds, naturally occurring food can supply fish with adequate protein levels until a certain limit of fish biomass (Diana et al., 1991), which may be translated into better PER in a community-based aquaculture scenario.

4.2 | Temperature trial

Water temperature represents a critical environmental factor that can affect fish growth directly, controlling feed consumption, nutrient requirements, lipid utilization, and deposition and, overall, can directly affect fish metabolism (Marques et al., 2020). Although this study was carried out to assess the short-term effects of thermal stress, according to Li et al. (2023), Nile tilapia's long-term exposure to thermal stress could, in fact, result in higher mortality rates and decreased production performance. The exposure of organisms to thermal stress is known to cause

alteration of hematological parameters (Kulkeaw & Sugiyama, 2012), resulting in significant alterations in the integrity of the nucleolus, thus affecting the overall state of fish physiology. Comet and ENA assays may reflect different types of genetic damage, comet assay represents the precocious nature of the damaging events, and ENA reflects a delayed appearance of damage (Guilherme et al., 2010). According to Hamed and El-Sayed (2019) moringa leaf extract (20 mL/30 L water) applied to tilapia was revealed to have antioxidant and antigenotoxic properties against the toxicity of the pesticide pendimethalin. In a similar way, these protective characteristics may have a role in the response of tilapia fingerlings to thermal stress observed in our study.

Regarding the DNA damage evaluated through the comet assay, no differences were observed between the two groups yet, the control group presented lower DNA damage at the optimum temperature (26°C). However, the damage evaluated through ENA assay revealed that at 18°C, the control group presented higher damage when compared with the experimental group. In fact, temperatures below the suboptimal have been reported to have a negative impact on Nile tilapia, such as decreasing metabolic rate and growth and, hindering fish immunity which can induce higher mortality (Nobrega et al., 2020). On the other hand, fish fed with the Exp diet seemed to have more tolerance to exposure to 18°C, which might be because of the antioxidant properties of the natural ingredients used in the feed formulation.

The erythrocyte maturity index (EMI) presented slightly higher percentages of immature erythrocytes in the groups fed Exp diet compared with the Ctrl groups. Thus, the experimental diet appears to increase the percentage of immature erythrocytes in circulation, which, according to Maceda-Veiga et al. (2010) and Marques et al. (2020) can be explained by a promotion of the erythropoiesis, that is, the production of new blood cells (erythrocytes), or more broadly, from the increase of hematopoiesis, that is, the formation of blood cellular components (Kulkeaw & Sugiyama, 2012).

Specifically, in teleost fish, the stress response includes a large number of physiological processes, which are regulated through the sympathochromaffin (SC) axis and the hypothalamic–pituitary-interrenal (HPI) axis. The activation of the HPI axis, mediated by cortisol levels, enhances pathways involved in energy-substrate mobilization, including gluconeogenesis, and simultaneously decreases energy-demanding pathways, such as growth and immune function (Chowdhury & Saikia, 2020). According to Olivotto et al. (2002) cortisol plays an adaptative function against stressors since it regulates metabolic energy hydro-mineral balance, oxygen uptake, and immune competence.

The AChE activity showed higher values in the groups fed with the experimental diet, despite not presenting significant differences. Cholinesterases are also responsible for the hydrolysis of the gut hormone ghrelin, known as the "hunger hormone" for its function as a stimulant of hedonic feeding, regulating weight gain and fat metabolism (Santana et al., 2021). Therefore, higher values of AChE activity in the fish fed experimental diet may suggest that this hormone was produced in greater amounts to stimulate fish to feed. As previously mentioned, the optimum dietary values of protein are directly influenced by the size of fish because the demand for dietary protein decreases as fish grow (Yamamoto et al., 2005). Therefore, it was expected that, at early development stages, tilapia fed with the experimental diet would demonstrate higher levels of stress response when subjected to thermal stress.

Overall, CAT activity in the liver showed higher values in the fish fed Exp diet at 26 and 32°C. Considering the nonenzymatic antioxidant capacity (tGSH levels), the groups fed Exp diet revealed higher values of tGSH in the liver at 32°C. It is known that higher metabolic rates of fish should result in increased demand for GSH and might account for high GSH levels (Leggatt et al., 2007). Regarding oxidative damage at protein level (PC), increased PC levels were observed in the fish exposed to 18 and 26°C fed with Exp diet. This can be because of the imbalance between ROS generation and detoxification, resulting in an intensification in protein oxidation that can be promoted thru reactive oxygen species (Suzuki et al., 2010). Indeed, the fish fed Exp diet (with ~1% of *M. oleifera* content), presented increased levels of oxidative stress and damage. Elabd et al. (2019), in a study performed with fingerling Nile tilapia (2.0 ± 0.5 g average body weight) fed with a diet supplemented with 1.5% of *M. oleifera* (with 38.29% of protein content) for a three-month period observed that moringa incorporation in the diet improved stress indices (including antioxidants such as CAT), consequently improving their growth indices (body mass gain, specific growth rate, length rate, and feed conversion ratio) and somatic indices (spleen somatic index, hepatosomatic index, and intestine

somatic index). Yet, the fingerlings had almost twice the body weight and the percentage of the protein in the diet supplemented with 1.5% moringa was higher than those in our Exp diet. Furthermore, it is required to adjust the studied diet as a whole and not only the amount of moringa because our Exp diet is a more complex mixture of natural ingredients that might be interacting in different ways. In order to maintain physiological homeostasis, organisms under stress factors may initiate compensatory adjustments in their energy metabolism, leading to an increase in the energy consumption of some subtracts such as glycogen, lipids, or protein (Rodrigues et al., 2015). Besides, fish's energy budget changes may be translated into impaired growth, or even survival, and decreased fish meat quality (Anacleto et al., 2018). With this in mind, the energy budget of tilapia fingerlings under temperature fluctuations was assessed. The energy available (Ea) in *O. niloticus* tissues presented increased levels in fish fed with the Exp diet and exposed to 18 and 26°C. No significant differences were observed in the carbohydrate content of fish fed with the Exp diet, regardless of the tested temperatures. Tilapias are omnivorous fish, with a relevant vegetal component in their diets. So, they are expected to utilize dietary carbohydrates (35%–40% of digestible carbohydrates) more efficiently than carnivorous fishes (El-Sayed, 2020c). Regarding the protein content, the Exp diet appears to decrease the protein levels of fish at 18 and 32°C, which may indicate that fish are mobilizing their protein content under thermal stress. This is important data because the proteins can play a vital role in maintaining body homeostasis and preventing the leakage of fluid from blood circulation (M. Hamed et al., 2019). However, at a mid-long term, this will impair growth performance. Overall, the lipid content in the tilapia fed Exp diet was higher than in fish fed the Ctrl diet. In fact, dietary lipids are an important source of highly digestible energy (Lim et al., 2011). On the other hand, the energy consumption (Ec) showed increased levels at 26°C in fish fed with Exp diet compared with fish fed Crtl diet, which suggests an increase in aerobic energy usage to metabolize the components of the Exp diet.

As the CEA is determined through the ratio between the Ec and the Ea, no significant alterations between the two groups were observed in all tested temperatures. In the face of environmental changes, such as temperature fluctuations, the maintenance of the energy budget is vital for an organism to be able to implement the needed metabolic adjustments, minimizing possible negative consequences on its fitness (Anacleto et al., 2018).

5 | CONCLUSIONS

This study focuses on a possible diet formulation for community-based tilapia aquaculture in Africa. In this type of fish production, no feed is normally provided, so fish depend exclusively on the natural food present in ponds and occasionally on agricultural by-products. Thus, the development of a fish feed based on locally available ingredients, including agricultural wastes and by-products, can represent a great potential to improve fish production, leading to an increase in animal protein available for these communities.

Since the ultimate goal of this study was to improve fish production in earthen ponds for local consumption and to increase the protein intake of the rural Mozambican communities, the differences in fish growth do not represent a significant constraint, as fish can compensate for dietary protein deficiencies through access to natural food in ponds, such as insect larvae.

Considering the possible effects of thermal stress on Nile tilapia production at genetic damage assessed through comet and ENA assays, the Exp diet appears to contribute to a decrease in genetic damage events, at both precocious and delayed appearance of damage events in the short term. Regarding fish thermal stress responses, the Exp diet appears to increase antioxidant enzymes' activity, but oxidative damage at the protein level was not prevented. Therefore, some adjustments in the amount of local ingredients with known antioxidant properties, such as moringa, should be addressed as they may have the potential to improve these stress indices and consequently improve fish growth performance.

There is a lack of information about some of the local ingredients in the literature, so it would also be important to carry out more studies on fish growth and feed utilization, especially on the nutrient and amino acid digestibility of these ingredients.

For sustainable industrial or community-based aquaculture, it is vital to continue to identify alternative protein sources. Furthermore, the identification of new ingredients, reasonably priced and usually not used in human consumption, should be studied more intensively due to the high price of some plant ingredients such as wheat, and also due to overexploitation of others ingredients such as soy, which affects its sustainability.

ACKNOWLEDGMENTS

The authors are grateful for the financial support provided by FCT—Foundation for Science and Technology and by AKDN—Aga Khan Development Network.

FUNDING INFORMATION

This study received Portuguese national funds from FCT—Foundation for Science and Technology through projects UIDB/04326/2020, UIDP/04326/2020, and LA/P/0101/2020 to CCMAR and contract DL57/2016/CP1361/CT0033 to Cláudia Aragão. We also acknowledge financial support to CESAM by FCT/MCTES (UIDP/50017/2020 + UIDB/50017/2020+ LA/P/0094/2020) through national funds, and Sílvia Pires has a doctoral grant from FCT (2022.13140.BDANA). The project number 333204505, "NutriMo—Development of artisanal diets for community-based tilapia aquaculture in Mozambique" was co-funded by AKDN—Aga Khan Development Network and FCT—Foundation for Science and Technology and project number 47175, FICA, supported by Portugal and the European Union through FEDER/ERDF, COMPETE 2020, and CRESC Algarve 2020, in the framework of Portugal 2020.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; and in the writing of the manuscript, or in the decision to publish the results.

DATA AVAILABILITY STATEMENT

Data will be provided upon request.

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How to cite this article: Pires, S. F. S., Vital, I., Pereira, V., Bustani, H., Aragão, C., Engrola, S., Cabano, M., Dias, J., Soares, A. M. V. M., Rodrigues, A. C. M., & Rocha, R. J. M. (2023). Sustainable feed formulation to community-based aquaculture: *Oreochromis niloticus* fingerlings performance and antioxidant status. *Journal of the World Aquaculture Society*, 1–20. https://doi.org/10.1111/jwas.13040