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Socially Acceptable Feed Formulations May Impact the Voluntary Feed Intake and Growth, but Not Robustness of Nile Tilapia (*Oreochromis niloticus*)

Rodrigo Mendes [1,2,3], Paulo Rema [4,5], Jorge Dias [1], Ana Teresa Gonçalves [1,6], Rita Teodósio [2], Sofia Engrola [2], Francisco J. Sánchez-Vázquez [3] and Luís E. C. Conceição [1,*]

1 Sparos Lda., Área Empresarial de Marim, Lote C, 8700-221 Olhão, Portugal; rodrigomendes@sparos.pt (R.M.); jorgedias@sparos.pt (J.D.); anagoncalves@sparos.pt (A.T.G.)
2 Centre of Marine Sciences (CCMAR/CIMAR LA), Campus de Gambelas, Universidade do Algarve, 8005-139 Faro, Portugal; rteodosio@ualg.pt (R.T.); sengrola@ualg.pt (S.E.)
3 Departamento de Fisiología, Facultad de Biologia, Universidad de Murcia, 30003 Murcia, Spain; javisan@um.es
4 Departamento de Zootécnia, Universidade de Trás-os-Montes e Alto Douro, Quinta de Prados, 5001-801 Vila Real, Portugal; prema@utad.pt
5 CIIMAR—Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, Novo Edifício Do Terminal de Cruzeiros de Leixões, Avenida General Norton de Matos, 4450-208 Matosinhos, Portugal
6 GreenCoLab-Associação Oceano Verde, Campus de Gambelas, Universidade do Algarve, 8005-139 Faro, Portugal
* Correspondence: luisconceicao@sparos.pt

Abstract: Society is becoming more demanding with aquaculture's environmental footprint and animal wellbeing. In order to potentially mitigate these concerns, feed formulations could be based on eco-efficient (circular economy-driven) or organic ingredients. This study aimed to investigate the growth performance, feed utilization, and health status of juvenile Nile tilapia (*Oreochromis niloticus*) when fed with such feeds. The growth trial lasted for 8 weeks, and fish had an initial weight of 31.0 ± 0.5 g (mean ± SD). Fish were fed until visual satiation, in quadruplicate, with one of three isonitrogenous and isoenergetic experimental feeds: a commercial-like feed without fishmeal (PD), a diet based on ingredients compatible with organic certification (ORG), or a feed formulated using circular economy-driven subproducts and emergent ingredients (ECO). Fish fed ECO showed a tendency for decreased feed intake, while ORG fish significantly reduced their intake compared to those fed PD. Consequently, fish fed ECO (62.7 ± 5.4 g) exhibited almost half the growth than those fed PD (107.8 ± 6.1 g), while ORG fish almost did not increase their weight (32.7 ± 1.3 g). ECO and ORG diets had a lower digestibility for protein, lipid, and energy when compared to PD. Feed utilization of fish fed ECO or ORG was also lower than those fed PD. From the health-related genes analyzed, only glutathione reductase (*gsr*) showed statistically significant differences, being more expressed in fish-fed ECO than those fed PD. Thus, even when such novel formulations induced extreme effects on voluntary feed intake, their impact was noted only in fish growth, but not in robustness.

Keywords: feed intake; palatability; eco-efficient feeds; organic feeds; fish welfare; Nile tilapia

Key Contribution: The organic and circular economy-driven feeds had an impact on Nile tilapia growth and/or on feed utilization; however, fish health remained unaffected.

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1. Introduction

Aquaculture provides a vital source of animal protein to the world population. In 2020, 56% of the total aquatic food available for human consumption production was farmed [1].

Nile tilapia (*Oreochromis niloticus*) is one of the most cultivated finfish worldwide and an affordable protein source, being highly relevant for addressing food security, especially in developing countries [2]. To ensure that the global aquatic food demand is met, aquaculture must intensify production while addressing challenges related to its sustainability.

The environmental footprint and animal welfare are pivotal concerns in aquaculture, particularly for the consumer. Consumers are increasingly aware of issues such as resource depletion and environmental intervention [3–5]. Moreover, there are ethical concerns about animal welfare and food security in seafood production, which have led to a strong reduction in the use of antibiotics and other therapeutic drugs in many countries [5–7]. Society expects organisms to be farmed responsibly, prioritizing animal well-being and minimizing the industry's environmental impacts [6,8–10]. Accordingly, optimizing fish nutrition presents one of the potential approaches to improve aquaculture environmental performance and ethical treatment of animals, also enhancing consumer trust and confidence in the industry.

While nutrition plays a crucial role in ensuring the growth, well-being, and health of farmed organisms, aquafeeds have always had an associated environmental impact. Approximately 70% of farmed aquatic animals depend on formulated feeds [1]. Though marine ingredients are more prevalent in aquafeeds for high-trophic-level finfish species (e.g., gilthead seabream), they are also routinely incorporated (inclusion rates of 2–10%) in the diets of low-trophic-level finfish (e.g., Nile tilapia), which are globally produced at larger volumes [1,11]. While common feed ingredients, including marine and plant-based sources, continue to play a significant role in the environmental footprint of the industry, variations exist among regions and production sites [12,13]. Nevertheless, there is still room for improvement and novel aquafeed formulations could focus on sustainable frameworks while reducing the use of ingredients that bring environmental concerns.

To address some of these challenges of aquaculture, there is a growing emphasis on integrating eco-efficient (circular economy) or organic principles to develop socially acceptable feed formulations. Eco-efficiency strategies prioritize resource conservation, waste management, and by-product valorization, while organic production addresses ethical, environmental, and food safety concerns, guided by strict standards, regulations, and certification schemes [9,14–18]. Thus, such feed concepts address criticisms from researchers and NGOs, while fulfilling the requirements of knowledgeable consumers [9,19]. One possible way to implement these concepts could be through the identification of alternative ingredients that would generate novel fish feeds.

Alternative ingredients, such as land animal by-products (LAPs; blood, feather, and poultry meals), insect meals (e.g., black soldier fly, *Hermetia illucens* or mealworm, *Tenebrio molitor*), single-cell microorganisms (e.g., bacteria, cyanobacteria, microalgae, and yeast), and non-traditional plant meals (e.g., sunflower, rapeseed, and lupins) have potential to rise as possible solutions to mitigate some of the current bottlenecks of aquaculture [16,17,20,21]. These alternative ingredients align with principles of circularity, contribute to waste reduction, may require minimal resource consumption, and valorize side streams [22,23]. While their nutritional composition in aquafeeds can vary significantly, some ingredients may offer valuable nutrients such as high crude protein content, balanced amino acid profiles, and essential vitamins [24,25]. Moreover, these alternatives may contain bioactive components (e.g., carotenoids, vitamins, flavonoids, and phytosterol and polyphenolic compounds), which possess antimicrobial, antioxidant, and/or anti-inflammatory properties [26,27]. All these characteristics can promote fish health, immune function, and weight gain, as demonstrated by Ahmed et al. (2020), Aragão et al. (2020), Tippayadara et al. (2021), Velasquez et al. (2016a), and Zhang et al. (2014) [28–32]. Despite these theoretical benefits, it is important to note that the inclusion of alternative ingredients may not be well accepted by the fish, affecting feed intake.

Feed intake can be affected by several variables and has a direct impact on overall farm profitability. Fish have the ability to regulate their feed intake, which is influenced by various factors, including environmental conditions, feed palatability, orosensory proper-

ties, and nutritional composition [33]. Antinutritional factors (ANFs; protease inhibitors, tannins, lectins, and phytates), commonly present in plants, can interfere with feed intake, but also in nutrient absorption and utilization, leading to reduced growth and impaired immune function [34–37]. Studies have shown that some ingredients and combinations above a certain threshold may lead to lower feed intake. For example, the inclusion of rapeseed meal of more than 7% in the diets of *Oreochromis niloticus* fingerlings resulted in a decrease in weight gain and feed intake [38]. Indeed, reduced feed intake may result in insufficient nutrient intake and impact the fish intestine, which is one of the primary targets of dietary changes and has a pivotal role in fish metabolism/digestion [29,39]. In turn, these consequences can affect fish vital physiological functions, impair growth performance, negatively impact welfare, and increase fish susceptibility to diseases [40]. Accordingly, as aquafeed formulations evolve to address sustainability concerns, understanding if feeds are well accepted by the fish becomes of high relevance.

The present work aimed to investigate the impact of novel diets, without fishmeal and wild fish oil (replaced with salmon oil as a by-product from the salmon processing industry), and formulated within eco-efficient (circular economy-driven) or organic frameworks, on the performance, feed utilization, and health of juvenile Nile tilapia (*Oreochromis niloticus*).

2. Materials and Methods

2.1. Experimental Diets

Three experimental diets, practical (PD), organic (ORG), and eco-efficient (ECO), were formulated and produced by SPAROS Lda (Olhão, Portugal). The formulation concept and ingredient selection (Table 1) were based on an eco-efficient and organic framework (ingredients that can be found on the market as organic), market availability, and nutritional composition. Experimental feeds were formulated to meet Nile tilapia's known nutritional, amino acid, and fatty acid requirements. A commercial-like feed, without fishmeal (practical diet, PD), served as control. The organic (ORG) feed was based on ingredients compatible with organic certification and practices, which is an area of growing interest to consumers and the industry due to their sustainable and ethical considerations. The eco-efficient (ECO) was formulated using circular economy-driven subproducts (e.g., poultry and feather meal) and emergent ingredients (e.g., spirulina, insect meal, and quinoa). In particular, spirulina had inclusion levels of 10% and 2.5%, quinoa 5% and 2.5%, rapeseed meal 26% and 13%, and brewer's yeast 10% and 5%, in ORG and ECO, respectively. All diets were formulated to be isonitrogenous (crude protein of ~39.4% as fed) and isoenergetic (gross energy of ~19.2 kJ/g as fed) (Table 1). Amino acid profiles are presented in Table 2. The dietary treatments (PD, ORG, and ECO) were randomly assigned to replicate tanks (n = 4 replicates per dietary treatment).

Initially, all powder ingredients were mixed according to the target formulation in a double-helix mixer (model 500 L, TGC Extrusion, Roullet-Saint-Estèphe, France) and grounded (below 4.00 mm) in a micropulverizer hammer mill (model SH1, Hosokawa-Alpine, Augsburg, Germany). Diets (pellet size: 4.0 mm) were manufactured with a twin-screw extruder (model BC45, Clextral, Firminy, France) with a screw diameter of 55.5 mm. Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, Roullet-Saint-Estèphe, France). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, Sevenum, The Netherlands). Coating conditions were as follows: pressure (700 mbar); spraying time under vacuum (approximately 90 s) and return to atmospheric pressure (120 s). Immediately after coating, feeds were packed in sealed plastic buckets and shipped to the research site where they were stored at room temperature in a cool and aerated emplacement. Representative samples of each diet were taken for proximate composition and amino acid analyses.

Table 1. Diet formulation (% inclusion levels) and proximate composition (% as fed) of the experimental diets (PD, ORG, and ECO) for Nile tilapia (*Oreochromis niloticus*).

Ingredients (% Inclusion Levels)	PD	ORG	ECO
[1] Poultry meal	5.00		2.50
[2] Porcine blood meal			5.00
[3] Feathermeal hydrolysate			5.00
[4] Insect meal			7.50
[5] Microbial biomass			5.50
[6] Brewer's yeast		10.00	5.00
[7] *Spirulina*		10.00	2.50
[8] Soy protein concentrate	5.00		
[9] Pea protein concentrate		5.00	
[10] Corn gluten meal	12.00		
[11] Soybean meal	25.00	12.50	
[12] Rapeseed meal	13.00	26.00	13.00
[13] Sunflower meal	7.50	15.00	15.00
[14] Wheat (whole)	13.90		15.61
[15] Rice bran	9.78	9.78	
[16] Quinoa		5.00	2.50
[17] Whole peas			11.00
[18] Vitamin and mineral premix	1.00	1.00	1.00
[19] Choline chloride	0.20	0.20	0.20
[20] Antioxidant powder	0.20	0.20	0.20
[21] Mono-calcium phosphate	2.55	2.00	2.75
[22] L-Lysine	0.30		0.30
[23] DL-Methionine	0.15		0.22
[24] Yttrium oxide	0.02	0.02	0.02
[25] Salmon oil	2.00	2.00	2.00
[26] Rapeseed oil	2.40	1.30	3.20
Proximate Composition (% as fed)	PD	ORG	ECO
Dry matter (DM)	94.77	93.49	93.93
Ash	7.07	7.32	6.86
Crude protein	38.63	39.65	40.02
Crude fat	8.60	8.58	8.95
Total phosphorus	1.41	1.54	1.48
Gross energy (kJ/g^{-1})	19.24	19.14	19.32

All values are reported as the mean of duplicate analyses. [1] Poultry meal: 62.4% CP, 12.5% CF; SAVINOR UTS, Trofa, Portugal. [2] Porcine blood meal: 89.1% CP, 0.4% CF; SONAC BV, Son, The Netherlands. [3] Feathermeal hydrolysate EM'PAQ: 88.8% CP, 1.6% CF; Empro Europe, Dendermonde, The Netherlands. [4] Insect meal (*Hermetia illucens*), PROTE-IN HP55: 57.8% CP, 8.5% CF. [5] Microbial biomass (*Corynebacterium glutamicum*), Aminopro NT70: 74.1% CP, 3.1% CF, MAZZOLENI SPA, Bergamo, Italy. [6] Brewer's yeast: 38.9% CP, 4.5% CF; PREMIX Lda., Neiva, Portugal. [7] *Spirulina* (*Arthrospira platensis*): 72.1% CP, 1.0% CF, Sopropêche, Wimille, France. [8] Soy protein concentrate, Soycomil P: 62.2% CP, 0.7% CF; ADM, Amsterdam, The Netherlands. [9] Pea protein concentrate, Lysamine GPS: 78.1% CP, 8.3% CF, Roquette, France. [10] Corn gluten meal: 61.2% CP, 5.2% CF, COPAM, São João da Talha Portugal. [11] Solvent extracted soybean meal: 43.8% CP, 3.5% CF, Ribeiro & Sousa Lda., Leiria, Portugal. [12] Solvent extracted rapeseed meal: 34.3%CP, 2.1% CF, Ribeiro & Sousa Lda., Leiria, Portugal. [13] Solvent extracted dehulled sunflower meal, HiPro: 42.9% CP, 3.8% CF, AGP Slovakia, s.r.o, Komárno, Slovakia. [14] Wheat (whole): 11.7% CP, 1.6% CF, Molisur, Sevilla, Spain. [15] Rice bran full-fat: 12.6% CP; 15.5% CF, Casa Lanchinha, Setúbal, Portugal. [16] Quinoa seeds (*Chenopodium quinoa*): 14.0% CP, 5.6% CF, Comfeipas Lda., Olhão, Portugal [17] Whole peas: 19.6% CP, 2.2% CF, Ribeiro & Sousa Lda., Portugal. [18] Vitamin and mineral premix, WISIUM MIX AQUA 1.5%: PREMIX Lda, Neiva, Portugal. Vitamins (IU or mg/Kg diet): DL-alphatocopherol acetate, 100 mg; sodium menadione bisulfate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; 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): cobalt carbonate, 0.65 mg; copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate. 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings. [19] Choline chloride 50%: ORFFA, Breda, The Netherlands. [20] Antioxidant powder, VERDILOX: Kemin Europe NV, Herentals, Belgium. [21] Mono-calcium phosphate, ALIPHOS MONOCAL: 22.7% P, 17.5% Ca, ALIPHOS, Louvain-la-neuve, Belgium. [22] L-Lysine 99%: Ajinomoto EUROLYSINE S.A.S, Paris, France. [23] DL-Methionine 99%: Rhodimet NP99, ADISSEO, Paris, France. [24] Yttrium oxide, Amperit: Höganäs Germany GmbH, Düsseldorf, Germany. [25] Salmon oil: 98.3% CF, 4.6% EPA; 5.2% DHA, Sopropêche, Wimille, France. [26] Rapeseed oil: 98.2% CF, JC Coimbra, Setúbal, Portugal.

Table 2. Amino acid composition (g/100 g fed basis) of the experimental diets (PD, ORG, and ECO) for Nile tilapia (*Oreochromis niloticus*).

Amino Acids (g/100 g Fed Basis)	PD	ORG	ECO
Arginine	2.24	2.57	2.33
Histidine	0.97	0.95	0.89
Lysine	2.03	2.00	2.12
Threonine	1.43	1.60	2.16
Tryptophan	0.42	0.51	0.46
Isoleucine	1.60	1.62	1.62
Leucine	3.54	2.89	2.93
Valine	1.88	1.98	2.13
Methionine	0.80	0.70	0.87
Phenylalanine	1.94	1.75	1.74
Cysteine + Cystine	0.66	0.65	0.70
Tyrosine	1.37	1.30	1.21
Aspartic Acid	3.36	3.48	3.08
Glutamic Acid	7.38	6.77	5.93
Alanine	2.10	2.02	2.11
Glycine	1.82	1.92	2.12
Proline	2.35	1.92	2.20
Serine	1.86	1.82	1.99
Taurine	0.02	<0.002	0.01

All values are reported as the mean of duplicate analyses.

2.2. Fish Husbandry

2.2.1. Growth Trial

The trial was carried out at the University of Trás-os-Montes e Alto Douro (UTAD, Vila Real, Portugal), by trained scientists (following category B FELASA recommendations), according to the European Parliament and European Union Council guidelines on the protection of animals used for scientific purposes [41].

Male juvenile Nile tilapia (*Oreochromis niloticus*) were transferred to the experimental facilities by a duly authorized carrier. During the acclimation period, fish were fed by hand, ad libitum, twice a day, with a commercial diet (Standard 4 Orange, Sorgal, Aveiro, Portugal; 43% CP, 17% CF).

At the start of the study, the animals were randomly distributed into 12 homogeneous groups (CV < 2%), with an initial weight of 31.0 ± 0.5 g (mean ± SD) and condition factor (K) of 1.8 ± 0.2, in indoor fiberglass tanks of 300 L in a recirculating aquaculture system (RAS). Each tank had an initial stocking density of 3.4 kg/m^3 and contained 30 fish. Further, 8 fish from each replicate tank (n = 32 fish per dietary treatment) were anaesthetized (400 mg/L of 2-phenoxyethanol; Sigma-Aldrich, Madrid, Spain) and carefully PIT-tagged under the dorsal muscle to allow identification of individuals for further measurements. All tanks were covered with a net to prevent escapes. The RAS was equipped with a mechanical filter, a submerged biological filter, a UV sterilizer, and an aeration mechanism for oxygenation. Abiotic parameters, feed intake, and mortality were measured and recorded daily, with further removal of dead fish. The average dissolved oxygen in the water was 4.2 ± 1.1 mg/L and the temperature was 24.4 ± 1.3 °C (measured with OxyGuard A/S Probe, Farum, Denmark). A 12 h:12 h (8:00 to 20:00 lights on) light/dark photoperiod was maintained during the study. Experimental diets were supplied daily by hand until apparent visual satiation two times per day (10:00 and 15:00). Distributed feed was quantified throughout the study. The trial lasted for 55 days.

2.2.2. Digestibility Trial

For the digestibility trial, five homogeneous groups of 10 fish (49.4 ± 0.5 g) were distributed in five cylinder-conical tanks of 50 L. Fish were fed by hand, twice a day, during the morning (09:00 and 10:00), until apparent satiation. After an adaptation period of three days, feces collection started. Each day after feeding, tanks were thoroughly cleaned to

remove any uneaten feed, and fish were left undisturbed until the afternoon with clean water (24 °C) and aeration. Further, a recipient was placed in the water outlet at the bottom of the tank, which collected feces through settling decantation. At the end of the day (17:00) the recipient was removed, and the feces was collected and stored at −20 °C until analysis. All feeds were formulated to contain 0.02% of yttrium oxide (Y_2O_3) as an inert marker, which allowed the determination of the apparent digestibility coefficients (ADC's) of the dietary nutrients by an indirect method according to the following formula:

Apparent digestibility coefficients (%), ADC's of dietary nutrients and energy [42]:

$$\text{ADC } (\%) = 100 \times \left[1 - \frac{\text{dietary marker } (\%)}{\text{faecal marker } (\%)} \times \frac{\text{faecal nutrient or energy content}}{\text{dietary nutrient or energy content}}\right]$$

ADC (%) of dry matter (DM):

$$\text{ADC } (\%) = 100 \times \left[1 - \frac{\text{dietary marker } (\%)}{\text{faecal marker } (\%)}\right]$$

2.3. Sample Collection

In all samplings, fish were previously fasted for 24 h before being individually weighed, measured, and euthanized with a lethal dose of anesthetic (900 mg/L of 2-phenoxyethanol; Sigma-Aldrich, Madrid, Spain). Prior to the beginning of the experiment, 10 fish (30.1 ± 8.9 g) from the initial stock were pooled and frozen at −20 °C for subsequent whole-body composition analysis. At the end of the study, a pool of 6 fish from each replicate tank (n = 4 pools per dietary treatment) was sampled and frozen at −20 °C for whole-body composition analysis. Furthermore, the viscera and liver of 3 PIT-tagged fish per replicate (n = 12 fish per dietary treatment) were carefully sampled and weighed for determination of viscerosomatic (VSI) and hepatosomatic (HSI) indexes. From the same fish, the anterior intestine was also carefully dissected and preserved in RNA (Sigma Aldrich, Madrid, Spain) at −80 °C until further genetic analysis.

2.4. Key Performance Indicators

At the start of the experiment, after five weeks, and at the end, fish were counted and bulk-weighted to determine growth performance, feed utilization, and nutrient retention indicators as follows:

$$\text{Weight gain (\%IBW; WG)} = 100 \times \text{wet weight gain (g)} \times \text{initial biomass (g)}^{-1}$$

where wet weight gain (g) = final biomass (g) − initial biomass (g).

$$\text{Relative growth rate (\%.day}^{-1}\text{; RGR)} = 100 \times (e^{g} - 1) \text{ [43]}$$

where g = [ln (final body weight (g)) − ln (initial body weight (g)) × number of feeding days^{-1}].

$$\text{Feed conversion ratio (FCR)} = \text{apparent feed intake (g)} \times \text{wet weight gain (g)}^{-1}$$

$$\text{Voluntary feed intake (\%ABW}^{-1}\text{.day}^{-1}\text{; VFI)} = \text{relative growth rate} \times \text{feed conversion ratio}^{-1}$$

$$\text{Protein efficiency ratio (PER)} = \text{wet weight gain (g)} \times \text{crude protein intake (g DM)}^{-1}$$

$$\text{Viscerosomatic index (\%; VSI)} = 100 \times \text{viscera weight (g)} \times \text{body weight (g)}^{-1}$$

$$\text{Hepatosomatic index (\%; HSI)} = 100 \times \text{liver weight (g)} \times \text{body weight (g)}^{-1}$$

$$\text{Condition factor (K)} = 100 \times \text{body weight (g)} \times \text{total length}^{3}\text{(cm)}^{-1}$$

Nutrient retention (% digestible intake; NR) = 100 × (final whole-body protein, lipid or energy content −initial whole-body protein, lipid or energy content) × (crude protein, crude lipid or gross energy intake^{-1} × ADC% of protein, lipid, or energy)

2.5. Analytical Procedures

Analyses of the diets, whole fish, and feces were performed in duplicates and following the methodology described by AOAC [44]. All samples were freeze-dried and ground until a homogeneous powder was obtained. Dry matter was measured after drying at 105 °C for 24 h; total ash by combustion was measured (550 °C during 12 h) in a muffle furnace (Nabertherm L9/11/B170, Lilienthal, Germany); crude protein (N × 6.25) was measured by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection with a Leco N Analyzer (Model FP-528, Leco Corporation, St. Joseph, MI, USA); crude lipid content was measured by petroleum ether extraction (40–60 °C) using a Soxtec™ 2055 Fat Extraction System (Foss, Hillerod, Denmark), with prior acid hydrolysis with 8.3 M HCl; gross energy was measured in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany); total phosphorus was determined according to ISO 27085:2009 by ICP-AES methodology [45]; phosphorus in the feeds was determined by a colorimetric method involving a wet ashing step followed by phosphorous measurement with 1-amino-2-naphthol-4-sulfonic acid-molybdate in a microplate reader at 660 nm [46]; and yttrium concentration in feed and feces was determined by atomic absorption spectrometry (SpectrAA 220 FS, Varian, Palo Alto, CA, USA) [47].

To determine the total amino acid content of the experimental feeds, samples were initially hydrolyzed in aqueous hydrochloric acid. For cysteine, cystine, and methionine, samples were previously oxidized with hydrogen peroxide and formic acid at cold temperatures. Subsequently, the sample pH was adjusted, brought to volume with a loading buffer, and filtered. Amino acids were separated in an amino acid analyzer and the detection was carried out using post-column derivatization with ninhydrin reagent and 440 and 570 nm. Tryptophan was quantified using high-performance liquid chromatography (HPLC) before being exposed to alkaline hydrolysis. Extraction of free taurine was performed with metaphosphoric acid and protein precipitation with centrifugation. Separation occurred on AAA with a sodium cation-exchange column, post-column derivatization was performed with O-Phtahalic aldehyde (OPA), and detection was carried out via fluorescence at 338/425 nm.

2.6. Reverse Transcription–Quantitative Real-Time PCR (qPCR)

Samples from the anterior intestine of two fish per replicate (n = 7–8 per dietary treatment) were analyzed. To extract total RNA, samples were initially thawed and homogenized using TissueLyser II (Star-Beater, VWR, Radnor, PA, USA) with 1 mL of Tri Reagent (Sigma-Aldrich, Spain), according to the manufacturer's instructions. Total RNA quality and integrity were determined by denaturing agarose gel electrophoresis, while concentration and purity were based on absorbance at 260 nm and ratios at 260:280 and 260:230 nm, using a Nanodrop OneC (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) synthesis was performed by reverse transcription of 1000 ng of total RNA using the RevertAid H Minus First Strand Kit (Thermo Fisher Scientific), according to the manufacturer's protocol. Real-time PCR (RT-PCR) was performed in a CFX384 Real Time PCR detection system (Bio-Rad, Hercules, CA, USA) with PowerTrack™ SYBR™ Green chemistry (Thermo Fisher Scientific), using specific primers (Table 3). Primers for each gene were designed using the Geneious Prime version 2023.1 (https://www.geneious.com, accessed on 4 March 2023) based on sequences from the GenBank database (NCBI; [48]. PCR efficiency was determined using five-point standard curves of a 3-fold dilution series (1:3 to 1:243) of pooled cDNA. For intestinal epithelial integrity, the expression levels of several genes were analyzed: D-amino oxidase (*dao*), occludin (*ocl*), and tight junction protein 2 (*tjp2*). The biomarkers for oxidative status/stress were catalase (*cat*), glutathione peroxidase (*gpx*), glutathione reductase (*gsr*), nuclear factor erythroid 2-related factor 2 (*nrf2*), and heat shock protein 70 (*hsp70*). Genes analyzed for the immune condition were tumor necrosis factor (*tnf-α*), interleukin-1β (*il-1β*), and transforming growth factor β (*tgf-β*). The RT-PCR assays were run in duplicates in a 10 µL volume containing 2 µL of cDNA, 0.625 µL of each specific forward and reverse primers at 10 µM, 5 µL of PowerTrack™ SYBR™ Green Master

Mix (Thermo Fisher Scientific), and 1.75 µL of nuclease-free water. The amplification protocol was set as follows: an initial denaturation step of 2 min at 95 °C, followed by 40 cycles of denaturation for 5 s at 95 °C and 30 s at 58 °C for annealing/extension. Negative controls without sample templates were consistently executed for each primer set. The specificity of reactions was confirmed through the examination of melting curves, using ramping rates of 0.5 °C/5 s, across a temperature span of 60–95 °C. Gene expression levels were normalized using a reference housekeeping gene, the elongation factor 1 *α* (*ef1-α*). The relative mRNA expression of the target genes was calculated according to the Pfaffl method [49].

Table 3. Sequences of forward and reverse primers, along with their accession number, for the reference gene (*ef1-α*) and molecular biomarkers indicators of the intestinal epithelium integrity, oxidative status/stress, and immune condition used in qPCR analysis.

Gene	Forward Primer Sequence (5′ → 3′)	Reverse Primer Sequence (5′ → 3′)	NCBI GenBank Accession Number
dao	CAACCTTTGCAGTGAACCCG	TCACTCCCCTCTTTCGCAAC	XM_005473333
ocl	TCAGATGAGCAGCGCAGAAA	TTCCAGTGCGTCCAACTCTC	XM_005476075
tjp2	GCTACATGGACTCCGGCTAC	GCGATCTGGGCTGTACTCTC	XM_025908597
cat	TCCATTCCCAGAAGCGCAAT	ATTCATGTGACGGTGGCCAT	XM_019361816
gpx	ACTTCCATTCCCCTGCGATG	GCTTGTAAGGTTCCCCGTCA	NM_001279711
gsr	CAGCAGGAAGAGTCAGTGCA	ACCCATCTTGATGGCCACAG	XM_013271309
nrf2	TCTCAGCCCGATGACAGAGA	GTGCTGACCACTGCTCTCTT	XM_003447296
hsp70	CCAAAAGGTGTCCAACGCTG	CCCCACCCAGGTCAAAGATC	NM_001279671
tnf-α	ATGGCAGAAGGATGTGGACC	GACCATGGGATGCGAAGACA	XM_013266976
il-1β	CATGTCTTGCCGCATGGAAG	GTTCAACGGGCTGGTTTTCC	XM_005457887
tgf-β	CACGCTGAAGGACAAATGGC	TCACAGTACCGCCGAAGTTC	NM_001311325
ef1-α	TTGAGAAGGAAGCCGCTGAG	GCTGGTCTCGAACTTCCACA	AB075952

Abbreviations: *dao:* D-amino oxidase; *ocl*: occludin; *tjp2:* tight junction protein 2; *cat*: catalase; *gpx*: glutathione peroxidase; *gsr:* glutathione reductase; *nrf2*: nuclear factor erythroid 2-related factor 2; *hsp70:* heat shock protein 70; *tnf-α*: tumor necrosis factor; *il-1β*: interleukin-1β; *tgf-β*: transforming growth factor β; *ef1-α:* elongation factor 1 *α*.

2.7. Data Analysis and Statistics

All statistical analyses were performed using the computer package IBM SPSS version 26.0 (Armonk, NY, USA). Results are expressed as mean ± standard deviation (mean ± SD). When needed, data were previously transformed using *arcsine* [50] or an arbitrary value was added to ensure values were positive (retention data), and afterward tested for normality and homogeneity using the Shapiro–Wilk and Levene's tests, respectively. Thereafter, data were analyzed by one-way ANOVA followed by Tukey post hoc test or by non-parametric Kruskal–Wallis followed by Dunn's post hoc test (if ANOVA assumptions were not met) to identify differences among the experimental dietary treatments. The level of significance used was $p < 0.05$ for all statistical tests.

3. Results

3.1. Apparent Digestibility Coefficients of Diets

Apparent digestibility coefficients (ADCs) of the dry matter, nutrients, and energy of the experimental diets are presented in Table 4. There were significant differences ($p < 0.05$) regarding all analyzed parameters, except phosphorus ($p = 0.247$), with diet PD presenting higher ADCs, particularly for protein ($p = 0.018$), lipids ($p = 0.005$), and energy ($p = 0.043$). Conversely, diet ECO exhibited the lowest values for dry matter ($p = 0.040$), protein, and energy.

Table 4. Apparent digestibility coefficients (ADCs; %) of nutrients and energy of experimental diets (PD, ORG, and ECO) given to Nile tilapia (*Oreochromis niloticus*) for 55 days.

	Diets			
	PD	**ORG**	**ECO**	***p* Value**
Dry matter (DM; %)	66.0 ± 0.8 [a]	64.0 ± 2.3 [ab]	59.0 ± 4.7 [b]	0.040
Protein (%)	85.3 ± 0.6 [a]	81.0 ± 0.4 [ab]	75.4 ± 4.5 [b]	0.018
Lipids (%)	95.6 ± 0.2 [a]	92.8 ± 0.8 [b]	93.8 ± 1.2 [b]	0.005
Phosphorus (%)	67.8 ± 2.0	68.8 ± 2.2	70.8 ± 2.6	0.247
Energy (%)	74.8 ± 0.9 [a]	73.4 ± 1.5 [ab]	67.1 ± 4.0 [b]	0.043

Data are presented as mean ± standard deviation (n = 4 for diet PD and n = 3 for diets ORG and ECO). Different superscripts within the same row indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments.

3.2. Growth Performance, Feed Utilization and Somatic Indices

The performance indicators of fish fed the experimental diets are presented in Table 5. Fish did not respond in the same way to the diets offered. Although fish were fed until satiation, the voluntary feed intake (VFI) was lower and the feed conversion ratio (FCR) was higher in fish fed ORG compared with those fed PD (p = 0.010 for VFI and p = 0.012 for FCR). Diet PD was well accepted, but diet ECO and especially ORG received a negative response from the fish, which directly affected their performance. Accordingly, final body weight (FBW) and relative growth rate (RGR) were significantly affected by the different experimental diets (p = 0.007 for FBW and $p < 0.001$ for RGR). Fish that were fed diet PD exhibited the highest weight gain (3.5-fold increase), those fed with ECO roughly doubled their initial body weight, and fish fed ORG did not show a noticeable growth. Similarly, the final body weight of fish fed PD was 1.7-fold and 3.3-fold higher than ECO and ORG fish (p = 0.007), respectively. Moreover, the RGR was much higher in fish fed diet PD than in the other two dietary treatments ($p < 0.001$). The protein efficiency ratio (PER) also presented significant differences ($p < 0.001$), being higher in PD fish, followed by ECO and ORG. The viscerosomatic (VSI) and hepatosomatic indices (HSI) were similar in all dietary treatments (p = 0.273 and p = 0.092, respectively), while the condition factor (K) differed ($p < 0.02$), being lower in ORG fish compared to those fed PD. During the study, average survival was high (~99%) and unaffected by the dietary treatments (p = 0.368).

Table 5. Growth performance, feed utilization, and somatic indices of Nile tilapia (*Oreochromis niloticus*) fed with three different experimental diets (PD, ORG, and ECO) for 55 days.

	Diets			
	PD	**ORG**	**ECO**	***p* Value**
FBW (g)	107.8 ± 6.1 [a]	32.7 ± 1.3 [b]	62.7 ± 5.4 [ab]	0.007
RGR (%.day^{-1})	2.3 ± 0.1 [a]	0.1 ± 0.0 [c]	1.3 ± 0.2 [b]	<0.001
VFI (%ABW.day^{-1})	2.1 ± 0.2 [a]	0.02 ± 0.01 [b]	0.9 ± 0.3 [ab]	0.010
FCR	1.1 ± 0.1 [b]	7.1 ± 2.3 [a]	1.5 ± 0.4 [ab]	0.012
PER	2.3 ± 0.2 [a]	0.3 ± 0.3 [c]	1.7 ± 0.4 [b]	<0.001
VSI (%)	7.8 ± 0.7	7.2 ± 0.6	8.1 ± 0.7	0.273
HSI (%)	1.8 ± 0.1	1.0 ± 0.2	1.2 ± 0.5	0.092
K	1.9 ± 0.1 [a]	1.7 ± 0.2 [b]	1.8 ± 0.0 [ab]	0.014

Data are presented as mean ± standard deviation (n = 4 replicates per dietary treatment, except in RGR, VFI, and FCR for ORG, where n = 3). Different superscripts within the same row indicate significant differences (Kruskal–Wallis; $p < 0.05$) between dietary treatments. Abbreviations: FBW: Final body weight; RGR: Relative growth rate; VFI: Voluntary Feed Intake; FCR: Feed conversion ratio; PER: Protein efficiency ratio; VSI: Viscerosomatic index; HSI: Hepatosomatic index, K: Condition factor.

3.3. Whole Body Composition and Retention

Data on the whole-body composition of fish at the beginning and end of the study are presented in Table 6. In all analyzed parameters, except ash, the dietary treatments had an

impact on the body composition, where fish fed diet PD exhibited higher concentrations of all nutrients, dry matter, and energy ($p < 0.004$).

Table 6. Whole-body composition (% wet weight) of Nile tilapia (*Oreochromis niloticus*) fed with three different experimental diets (PD, ORG, and ECO) for 55 days.

		Diets			
(% WW)	**Initial**	**PD**	**ORG**	**ECO**	***p* Value**
Dry matter (DM; %)	26.0 ± 0.8	29.8 ± 1.6 ^{a}	22.0 ± 1.6 ^{b}	25.3 ± 1.9 ^{b}	<0.001
Protein (%)	14.8 ± 0.3	16.7 ± 1.1 ^{a}	13.8 ± 0.5 ^{b}	15.0 ± 0.8 ^{b}	0.003
Lipid (%)	6.3 ± 0.2	9.2 ± 1.4 ^{a}	3.1 ± 1.0 ^{c}	5.9 ± 0.6 ^{b}	<0.001
Ash (%)	4.1 ± 0.3	3.4 ± 0.3	3.9 ± 0.3	3.2 ± 0.7	0.174
Energy (kJ/g)	6.1 ± 0.1	7.3 ± 0.3 ^{a}	4.4 ± 0.4 ^{c}	5.8 ± 0.4 ^{b}	<0.001

Data are presented as mean ± standard deviation ($n = 4$ pools per dietary treatment). Different superscripts within the same row indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments (without considering initial values).

Ash, protein, lipid, and energy retentions of fish fed with the different diets are shown in Figure 1. The rates reflect the tendency of the distinct feed intakes between feeds, and thus all values were higher in fish fed diet PD and lower in those fed ORG ($p = 0.018$ for ash, $p = 0.007$ for protein, $p = 0.007$ for lipids, and $p = 0.007$ for energy). The latter revealed negative results, in particular, −1.7% for protein, −16.5% for energy, and −78.5% for lipid.

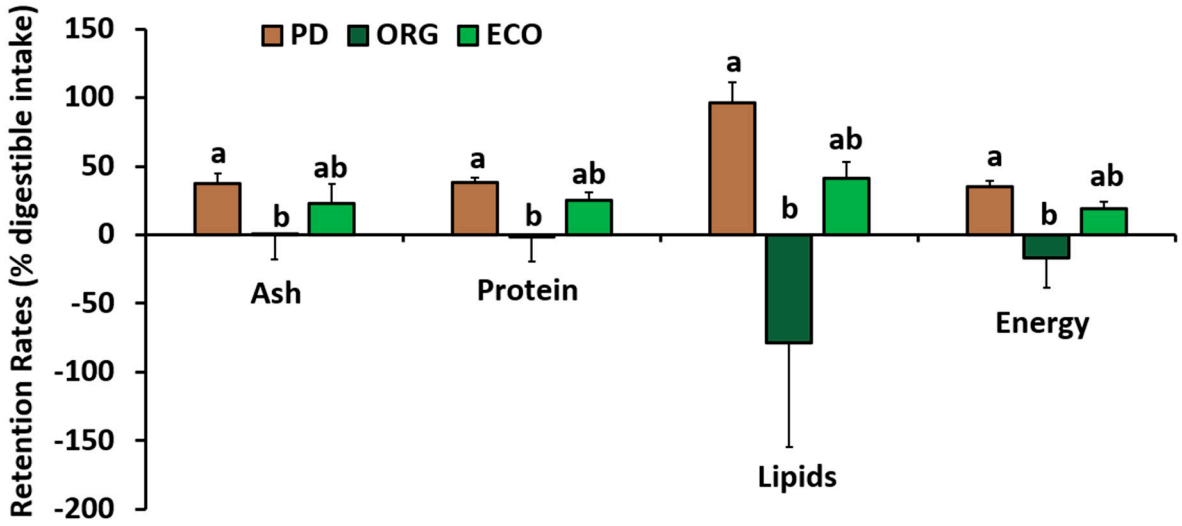

Figure 1. Nutrient or energy retentions (% digestible intake) of protein, lipid, and energy of experimental diets (PD, ORG, and ECO) given to Nile tilapia (*Oreochromis niloticus*) for 55 days. Data are presented as mean ± standard deviation ($n = 4$). Different letters indicate significant differences (Kruskal—Wallis; $p < 0.05$) between dietary treatments.

3.4. Relative Gene Expression

Figure 2 shows the relative expression of genes from the anterior intestine of Nile tilapia juveniles at the end of the experiment. Dietary treatments did not show statistically significant differences between them ($p > 0.05$; ranging from 0.196 to 0.780), with the exception of glutathione reductase (*gsr*), which was more expressed in fish fed diet ECO than the control group ($p = 0.014$).

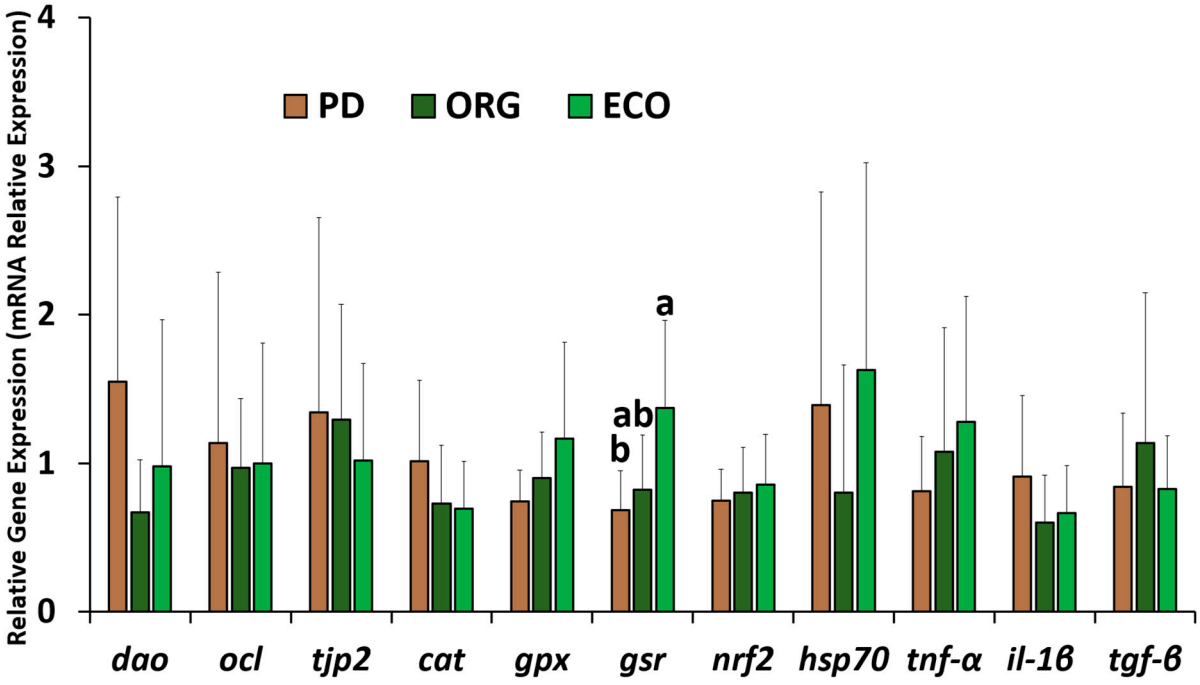

Figure 2. Relative expression (mRNA relative expression) of genes encoding for intestinal epithelial integrity (*dao*, *ocl*, and *tjp2*), oxidative status/stress (*cat*, *gpx*, *gsr*, *nrf2*, and *hsp70*), and immune condition (*tnf-α*, *il-1β*, and *tgf-β*) in juvenile Nile tilapia (*Oreochromis niloticus*) fed with three diets (PD, ORG, and ECO) over 55 days. Data are presented as mean ± standard deviation (n = 7 for CTRL and n = 8 for ORG and ECO). Different letters indicate significant differences (one-way ANOVA; $p < 0.05$) between dietary treatments. Abbreviations: *dao:* D-amino oxidase; *ocl*: occluding; *tjp2:* tight junction protein 2; *cat*: catalase; *gpx*: glutathione peroxidase; *gsr:* glutathione reductase; *nrf2*: nuclear factor erythroid 2—related factor 2; *hsp70:* heat shock protein 70; *tnf-α*: tumor necrosis factor; *il-1β*: interleukin-1β; *tgf-β*: transforming growth factor β.

4. Discussion

4.1. Diet Formulation and Fish Performance

All feeds were formulated without fishmeal and wild fish oil (replaced with salmon oil as a by-product from the salmon processing industry), as they may raise environmental concerns and/or ethical issues, and usually does not compromise feed utilization. This is in line with commercial tilapia feeds, which have low or no inclusion of both ingredients, especially with the goal of limiting production costs and keeping tilapia an affordable food item mainly in developing countries [51]. Such formulations have been tested previously without adverse impacts on tilapia performance, as seen by El-Saidy and Gaber (2003) and Teodósio et al. (2020) [52,53]. All diets were formulated with a mix of distinct inclusions of plant ingredients. A mixture of plant sources can decrease the nutritional imbalances of individual species and improve the nutritional profile of the feeds [20,34,54,55]. Several studies reported partial or full replacements of fishmeal with a plant mixture in tilapia feeds without a negative effect on fish performance and robustness [52,56,57]. Since soy may lead to environmental concerns (e.g., deforestation), soy inclusion was reduced in ORG and ECO diets and replaced with alternative ingredients.

To replace traditional ingredients with alternative ingredients based on organic and circularity frameworks, the inclusion of several constituents from the PD diet had to be modified in ORG and ECO diets. This change has considered the known species' nutritional requirements while maintaining a balanced amino acid profile and proximal composition. Since diets were isonitrogenous and isoenergetic, the dietary macronutrient profiles were most likely not responsible for the distinct performance results. The tested inclusion levels of some alternative ingredients differed between ORG and ECO diets, which was likely the

main cause for the lower fish performance results. Furthermore, while the digestibility of ORG was similar to PD and although ECO exhibited lower digestibility than the other feeds, the differences were marginal and within normal ranges [58]. In this sense, we hypothesize that the lower intake and consequent decreased growth rate of fish fed ORG and ECO diets were mainly related to palatability due to high inclusion levels of (a) particular ingredient(s) above a tolerable threshold for the fish.

4.2. Diet Palatability

Since most of the ingredients were included within known tolerable inclusion levels, the lower palatability was likely affected by specific ingredients. According to the literature, it seems minimal and unlikely that brewer's yeast, peas, and sunflower meal inclusion levels have affected ORG or ECO palatability, as these ingredients are often palatable and their inclusion levels were below the limits by which fish intake can be negatively affected [59–65]. For the same reasons and due to the extensive use in aquafeeds, a possible effect of exclusive ingredients from the ECO diet, including LAPs, insect meal, and microbial biomass, was most likely marginal [21,66–71]. Regarding spirulina, recent work showed that tilapia preferred feeds with spirulina included at 7% rather than at 3.5% [72]. Other studies reported no differences or an increase in tilapia intake when spirulina was included between 2.5 to 12% [73–76]. Since spirulina was included at 2.5% and 10% in diets ECO and ORG, respectively, the effects of this emergent ingredient are unlikely to have affected palatability. Therefore, rapeseed meal and/or quinoa were likely the main reasons for the lower ECO and ORG palatability.

The inclusion levels of rapeseed meal could have affected ORG palatability. Rapeseed meal was present at 26% on diet ORG, double the inclusion from ECO and PD. Literature has shown that rapeseed meal contains ANFs, precisely sinapine and glucosinolates (e.g., progoitrin), which have a bitter taste, decreasing palatability [77–80]. Consequently, this may lead to fish avoiding diets with a higher inclusion of this ingredient as they can discriminate the presence of bitter flavors [81,82]. Zhou and Yue (2010) reported that an inclusion of more than 19% of rapeseed meal in hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) feeds reduced intake, performance, and feed utilization, likely due to the toxic impacts of ANFs [51]. Replacement of fishmeal by rapeseed meal higher than 10% or 20% in the diets of *O. niloticus* fingerlings resulted in a significant decrease in weight gain and feed intake [38]. Indeed, due to feed intake limitations, some authors proposed that rapeseed meal be included in fish diets at levels ranging from 10 to 20% [83,84]. Apart from rapeseed, quinoa also has a bitter nature.

Quinoa was present in both diets ORG and ECO, at inclusion values of 5% and 2.5%, respectively, which could have been above the tolerable limit for the fish. The seed is rich in ANFs (e.g., quinine and saponins), particularly found in the outer coating that serves as a natural defense mechanism against pests [85]. These compounds are often associated with having a bitter nature and can be highly aversive, deterrent, toxic, and harmful for the fish, interfering with quinoa's palatability and significantly limiting the sensory acceptance of quinoa [86–88]. However, in some cases, quinoa was not detrimental to the farmed species. Supplementation up to 30% did not significantly change the FCR in tilapia [89]. Feed intake was not significantly affected by the inclusion of quinoa to replace oat grains in goldfish (*Carassius auratus*) [90]. Distinct results can be related to different quinoa varieties and processing methodologies, such as pressure cooking, that can be used to inactivate ANFs [90,91]. Although in the present study quinoa was heat treated, the temperature used may have not completely eliminated the ANFs, and thus a possible impact of quinoa in palatability must be considered [92,93].

4.3. Diet Digestibility

The ADCs of protein, energy, and lipid were lower in diet ECO compared to PD and although it can be difficult to identify a single cause to justify these values, they are unlikely a concern. The lower digestibility can be attributed to varying proportions of different

ingredients. ECO had lower plant sources than PD, which was likely the main reason for the lower ADCs of energy and protein, as lower trophic level finfish species, such as tilapia, have a well-adapted long gastrointestinal tract to digest plants [94]. The lower ADCs of lipids may be related to several factors, such as phospholipids and fatty acid composition (e.g., chain length, level of incorporation in dietary fat, degree of unsaturation, ands melting points), as well as the proportion of saturated and unsaturated (monounsaturated (MUFA) and polyunsaturated (PUFA)—docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) fatty acids. Although the fatty acid profile was not evaluated, the values of MUFA, PUFA, and EPA+DHA were all estimated and considered during formulation to be similar between dietary treatments. In order to provide more precise reasons for the lower ADCs, it would be needed to analyze the dietary carbohydrates and fatty acid profiles, as well as address the digestibility of raw individual ingredients [16,95,96]. Nevertheless, the protein and lipid ADCs of ECO, ORG, and PD diets were all above 75% and 90%, respectively, which are in line with the recommended values of 75–95% and 85–95% [58]. Hence, the reduced digestibility observed in the ECO diet was unlikely a major factor for the reduced performance, and, possibly, it also did not significantly affect feed utilization.

4.4. Whole-Body Composition and Retentions

Whole-body composition and retentions were negatively affected in fish fed ORG and ECO compared to those fed PD, which are likely related to the distinct feed intakes [97]. Since feed intake was reduced, fish oxidized their lipid storages to obtain energy and maintain their vital processes, structure, and the functionality of cell membranes [98–100]. Since energy was being obtained from fat, it also decreased. Consequently, moisture increased as lipids were replaced with water in the muscle [101]. Similar patterns in body composition were observed in tilapia and other fish species when their intake was reduced [102–106]. However, in most of the studies, body protein composition was not affected, meaning that in the present study, particularly the slight decrease in protein in fish fed ORG was an indication that the feed intake was close to maintenance level and that the animals were only eating to survive [107,108]. As a result, there could have been some impacts on fish health and condition.

4.5. Fish Health and Somatic Indices

Given that from the molecular biomarkers assessed for intestinal epithelial integrity, immune condition, and oxidative status, only glutathione reductase (*gsr*) was significantly affected by the dietary treatments, the overall health status of the fish does not seem to have been compromised. The expression of *gsr*, an important biomarker for oxidative status, was upregulated in fish fed ECO compared to those fed PD. Glutathione reductase is a crucial enzyme that plays a pivotal role in maintaining cellular redox homeostasis and antioxidant defense systems in fish [109]. From the formulation of this feed, the inclusion levels of spirulina and quinoa could have had a higher impact on the antioxidant activity, as both are rich in bioactive compounds with antioxidant properties (e.g., carotenoids, saponins, phycoerythrin, and phycocyanin) [110–112]. Quinoa increased the antioxidant status when included at 10% and 31% in tilapia and rats, respectively [28,113]. Spirulina at inclusion levels ranging from 0.5% to 45% improved the antioxidant capacity of several fish species [73,110,114,115]. The suggestion that fish health was likely not affected in ORG and ECO, despite their lower feed intake, based on gene expression results, is also supported by the somatic indices.

VSI and HSI were similar between dietary treatments, while K was lower in ORG fish, meaning that overall fish robustness was likely not compromised. VSI and HSI are often used to evaluate the nutritional and physiological state of fish metabolism [31]. Since these indexes did not differ between dietary treatments, it indicates that the capacity for nutrient absorption and fish metabolism were not significantly affected, also suggested by the absence of different expressions in health-related genes. The condition factor provides insights into the well-being, nutritional status, and growth of fish [116]. Although K differed

between dietary treatments, the differences were small and the values were considered normal when compared to the ones reported by Asmamaw et al. (2019), Ighwela et al. (2011), and Keyombe et al. (2017) [117–119]. Therefore, it is interesting to note that although fish fed diet ECO and particularly ORG ate much less than those fed PD, their health condition was not affected, meaning that in the case of ORG, they were eating to maintain their body weight and survive.

5. Conclusions

Clearly, more detailed studies are still necessary to optimize organic and eco-efficient formulation frameworks before they are to be implemented commercially for tilapia. When addressing societal concerns on feed formulations, one must consider the balance between environmental and social sustainability with fish performance and well-being. Despite being fed until apparent satiation, the three aquafeed formulations had significantly distinct impacts on juvenile Nile tilapia performance, whereas fish fed ORG and ECO showed considerably lower growth. Diet ORG exhibited reduced palatability likely due to the inclusion levels of specific ingredients on the feeds, most likely quinoa and rapeseed, or their combination with the other ingredients used, which greatly decreased feed intake. Still, fish appeared to have voluntarily chosen feed intake levels close to those required for maintenance as there was no weight loss. The evaluation of the impacts of the diets on gut epithelium integrity, immune condition, oxidative status, and somatic indices revealed no major impacts. Therefore, despite the decreased feed intake and reduced feed utilization, the alternative feed formulations affected tilapia growth but not robustness.

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Data Availability Statement: The data that support the findings of this study will be made openly available in the Zenodo Repository.

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