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Original Article

Fermentation with *Rhizopus* spp. improves *Alternanthera sessilis* protein quality, reduces anti-nutritional factors and promotes Tilapia growth

R Adharyan Islamy[1], Veryl Hasan[2,3,4]*, Fitri Sil Valen[5], Ahmad Syazni Kamarudin[4], Norshida Ismail[4], Michael Czech[6], Nurul Murmainnah[7]
[1]Aquaculture (Kediri City Kampus), Department of Fisheries and Marine Resources Management, Faculty of Fisheries and Marine Sciences, Brawijaya University, Jl. Pringgodani, Kediri City 64111, East Java, Indonesia
[2]Department of Aquaculture, Faculty of Fisheries and Marine Science, Airlangga University. Jl. Mulyosari, Surabaya 60113, East Java, Indonesia
[3]Research Group of Environment and Fisheries Resources Management, Faculty of Fisheries and Marine Science, Airlangga University. Jl. Mulyosari, Surabaya 60113, East Java, Indonesia
[4]School of Animal Science, Aquatic Science and Environment, Universiti Sultan Zainal Abidin, Besut Campus, Besut 22200, Terengganu, Malaysia
[5]Aquaculture Department, Agriculture Fisheries and Biology Faculty, Bangka Belitung University, Gang IV No.1, Balun Ijuk, Merawang District, Bangka Regency, Bangka Belitung Islands 33172, Indonesia
[6]Institute of Hydrobiology and Aquatic Ecosystem Management, BOKU University, Vienna, Austria
[7]Doctoral Program of Agricultural Sciences, Faculty of Agriculture, Brawijaya University, Jl. Veteran, Ketawanggede, Lowokwaru District, Malang City, East Java 65145, Indonesia

**Corresponding author's email:* veryl.hasan@fpk.unair.ac.id
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Abstract

The limited availability and increasing cost of conventional protein sources have encouraged the use of alternative feedstocks, including plant-based materials, for sustainable aquaculture feed production. However, many underutilized plants have low protein content or quality and/or contain high levels of anti-nutritional factors that limit their use in feed. This study aimed to determine the best level of yeast inoculum to use in fermentation of *Alternanthera sessilis* leaves and to evaluate the effects on nutritional composition, essential amino acid availability, anti-nutritional factors, and functional properties. Fermentation using *Rhizopus* spp. was carried out at inoculum levels of 0.0, 0.5, 1.0, 1.5, and 2.0% (w/w), followed by analyses of proximate composition, essential amino acid profile, anti-nutritional factors, fermentation efficiency, and antioxidant activity. The results showed that fermentation significantly increased crude protein content, total essential amino acids, particularly lysine and leucine, as well as degree of hydrolysis, soluble protein, and antioxidant activity. At the same time, phytate, tannin, and oxalate contents were markedly reduced. Most nutritional and functional improvements increased with inoculum level and reached a plateau at higher doses. Based on an integrated overall nutritional score, the 1.5% inoculum level provided the best balance between nutritional enhancement, amino acid bioavailability, and functional quality. These findings indicate that fermentation is an effective approach to improve the nutritional value of *A. sessilis* leaves and supports their potential use as a sustainable plant-based ingredient for aquaculture feed.

Keywords: *A. sessilis*, Anti-nutritional factors, Aquaculture nutrition, Cichlidae, Growth performance, *Rhizopus* spp., Solid-state fermentation

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Introduction

Aquaculture is the fastest-growing way to raise food throughout the world (Garlock et al., 2022). Nile tilapia (*Oreochromis niloticus*) is a widely cultured species due to its rapid growth, high tolerance to diverse environmental conditions, and adaptability to various aquaculture systems (El-Hack et al., 2022). However, the long-term viability of intensive tilapia farming is still constrained by the high cost and unreliable supply of conventional protein sources, especially fishmeal (Hussain et al., 2024). The ongoing reliance on fishmeal not only escalates feed expenses but also heightens environmental and resource sustainability issues (Ndebele-Murisa et al., 2024). These problems have led to the search for different economically viable and locally available plant-based feed ingredients for fish farming.

Leaf meals and agricultural by-products are commonly regarded as a prospective protein source in tilapia diets (Islamy et al., 2025a). But there are two basic reasons why many plant materials can't be used: they don't have a good balance of essential amino acids and they have anti-nutritional factors (ANFs) (Banti and Bajo, 2020). Plant proteins frequently exhibit diminished quantities of important amino acids, notably lysine and methionine, in relation to the nutritional needs of tilapia (Craddock et al., 2021; Furuya et al., 2023). A lack of certain amino acids can inhibit growth, make it harder for the fish to use protein, and make feed less effective. ANFs such phytate, tannin, and oxalate can bind minerals and nutrients, stop digestive enzymes from working, and make nutrients less digestible (Abera et al., 2023). This can challenge fish health and growth (Krogdahl et al., 2010; Samtiya et al., 2020).

The alligator weed *A. sessilis* is a leafy plant commonly found in tropical rice field ecosystems and is widely distributed in Indonesia (Islamy et al., 2025b; Mulyadi et al., 2025). This plant has a lot of biomass and a reasonable amount of nutrients, which means it potentials used as a different type of feed element (Serdiati et al., 2024). But it can't be used directly in aquafeeds yet because of the presence of ANFs and the fact that the nutritional quality can change depending on how the food is harvested and processed. Therefore, appropriate processing methods are needed to improve nutrient availability and reduce ANFs before this plant can be used effectively in aquaculture diets.

Solid-state fermentation (SSF) using filamentous fungi has been widely applied as an effective biological processing technique to improve the nutritional value of plant materials. Fungi such as *Rhizopus* spp. produce extracellular enzymes that can degrade complex carbohydrates, reduce anti-nutritional compounds, and contribute microbial protein and essential amino acids to the substrate (Surya et al., 2024). Previous studies in livestock and poultry nutrition have shown that SSF can increase crude protein content, improve amino acid profiles, and significantly reduce ANFs in plant-based feed ingredients (Yafetto et al., 2023). However, the application of SSF to leafy plant biomass for aquaculture feed is still limited, especially for locally harvested plants such as *A. sessilis*.

In the present study, solid-state fermentation using *Rhizopus* spp. was applied to wild *A. sessilis* collected from rice fields in Mojoroto, Kediri, East Java, Indonesia. The effects of different inoculum levels on nutritional composition, essential amino acid profiles, and anti-nutritional factor content of fermented substrates were evaluated. In addition, these fermented substrates were incorporated into pellet diets to assess their effects on growth performance, survival, feed utilization, and protein digestibility in juvenile Nile tilapia during a 30-day feeding trial. By combining biochemical evaluation with in vivo performance data, this study addresses an important gap in sustainable aquafeed development and demonstrates a practical approach to valorize locally abundant plant biomass for tropical aquaculture systems.

Material and Methods

Substrate collection and preparation

Wild *A. sessilis* foliage were harvested from irrigated rice fields in Mojoroto, Kediri, East Java, Indonesia. The harvested biomass was washed to remove adhering soil and debris, then air-dried in the shade to a constant weight. Dried biomass was chopped into approximately 2–3 cm pieces and milled to pass through a 2 mm sieve prior to fermentation. The resulting substrate was stored in polyethylene bags at ambient temperature until use.

Solid-State Fermentation (SSF)

The Solid-State Fermentation (SSF) process was conducted using *Rhizopus* spp. inoculum obtained from the microbial culture collection at the Laboratory of Aquatic Feed and Nutrition, PSDKU Universitas Brawijaya, Kediri. The *Rhizopus* spp. was cultured on Potato Dextrose Agar (PDA) at 30 °C for 48 h to obtain actively growing mycelia. To prepare the liquid inoculum suspension used for the experiment, the mycelia were harvested and suspended in sterile distilled water to reach a standardized concentration of 10^7 spores/mL. The SSF was performed in triplicate for each treatment, resulting in a total of 15 experimental units. For each unit, 500 g of dry *A. sessilis* substrate was placed into a sterile polyethylene tray. To achieve an optimal substrate moisture content of 60% (w/w), 750 mL of distilled water was added to each tray. The water was added gradually while the substrate was thoroughly mixed by hand using sterile gloves to ensure uniform moisture distribution.

The *R. oligosporus* suspension was then inoculated into the moistened substrate at one of five levels: 0% (control), 0.5%, 1.0%, 1.5%, and 2.0% (v/w, volume of suspension per weight of dry substrate). The 1.0% treatment received 5 mL of suspension per 500 g of dry substrate. Following inoculation, the substrate was mixed again for 5 minutes to ensure the mycelial suspension was evenly distributed throughout the tray rather than concentrated in a single area. The trays were covered with perforated plastic film to allow gas exchange and incubated at 30 ± 1 °C for 72 h without agitation. Temperature and substrate moisture were monitored daily. After the 72 h fermentation period, the fermented materials were oven-dried at 60 °C until a constant weight was reached and subsequently ground to pass through a 1 mm sieve for further analysis.

Pellet feed preparation

Experimental diets were formulated to evaluate the effect of fermentation level of *A. sessilis* on feed quality. The inclusion level of *A. sessilis* meal was fixed at 20% (dry matter basis) in all diets, while the fermentation treatment differed according to inoculum level (R0–R4). The fermented *A. sessilis* meal was used to replace part of the basal plant-based ingredients in the formulation, while maintaining similar ingredient proportions across treatments.

All dry ingredients were weighed according to the formulation (Table 1) and thoroughly mixed using a mechanical mixer for 10–15 min to ensure homogeneity. Water was then added gradually (approximately 25–30% of total feed weight) during mixing to obtain a uniform dough suitable for pellet formation.

Table-1. Ingredient composition (%) of experimental diets containing fermented *A. sessilis* at different fermentation levels.

Ingredient (%)	**R0 (0%)**	**R1 (0.5%)**	**R2 (1.0%)**	**R3 (1.5%)**	**R4 (2.0%)**
Fish meal	15.0	15.0	15.0	15.0	15.0
Soybean meal	10.0	10.0	10.0	10.0	10.0
***A. sessilis* meal**	**20.0**	**20.0**	**20.0**	**20.0**	**20.0**
Corn meal	25.0	25.0	25.0	25.0	25.0
Rice bran	20.0	20.0	20.0	20.0	20.0
Fish oil	3.0	3.0	3.0	3.0	3.0
Vitamin–mineral premix*	2.0	2.0	2.0	2.0	2.0
Binder (e.g., CMC)	5.0	5.0	5.0	5.0	5.0
Total	**100**	**100**	**100**	**100**	**100**

Note: The dietary formulation was kept constant across treatments, with only the fermentation quality of *A. sessilis* differing among diets. * Vitamin–mineral premix supplied essential vitamins (A, D3, E, K, and B-complex) and minerals (Fe, Zn, Mn, Cu, I, and Se) according to manufacturer specifications.

The moist mash was steam-conditioned and pelletized at 70–80 °C using a laboratory-scale pellet mill equipped with a 2 mm die. The resulting pellets were cut into approximately 2–3 mm lengths. The pellets were then dried at room temperature (27–30 °C) for 24–48 h until the moisture content reached approximately 10%. Dried pellets were packed in labeled, airtight polyethylene bags, with labels corresponding to each treatment (R0, R1, R2, R3, and R4), and stored at 4 °C until use to preserve feed quality and prevent microbial deterioration.

Proximate and chemical analyses

Proximate composition of fermented *A. sessilis* and experimental diets was determined on dried and ground samples. Fermented substrates were analyzed after oven drying and milling, while diets were analyzed in pellet form after drying. Proximate parameters, including dry matter, crude protein, crude lipid, ash, and crude fiber, were determined according to standard methods of the Association of Official Analytical Chemists (AOAC, 2016): dry matter (method 934.01), crude protein by Kjeldahl (method 984.13; N × 6.25), crude lipid by Soxhlet extraction (method 920.39), ash (method 942.05), and crude fiber (method 962.09). No modifications were made to these procedures. Nitrogen-free extract (NFE) was calculated by difference as:

NFE (%) = 100 − (crude protein + crude lipid + ash + crude fiber) …… (1)

Samples were hydrolyzed using 6N HCl at 110°C for 24 h prior to analysis, and amino acids were quantified using HPLC following pre-column derivatization. Hydrolyzed samples were filtered, neutralized, and derivatized prior to analysis. Separation was performed using a reverse-phase column with UV detection, and amino acids were quantified using external standards. The HPLC system (e.g., Shimadzu LC-20A, Japan) was operated under manufacturer-recommended conditions.

Anti-nutritional factors were analyzed in fermented plant substrates using established colorimetric methods. Phytate content was determined using the Wade reagent assay, tannin content using the Folin–Ciocalteu method, and oxalate content by acid extraction followed by titrimetric or spectrophotometric determination. Absorbance readings were obtained using a UV–Vis spectrophotometer (UV-1800, Shimadzu). All assays were conducted following standard protocols validated for plant-based matrices. All analyses were performed in triplicate, and results are expressed as mean ± standard deviation.

Experimental fish and rearing conditions

Experimental fish were juvenile Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758), with a total length ranging from 9 to 12 cm and an initial body weight ranging from 15 to 30 g. The mean (± SD) total length and body weight were 10.60 ± 1.15 cm and 23.36 ± 3.28 g, respectively. Fish were obtained from a commercial hatchery and acclimated under laboratory conditions for two weeks prior to the feeding trial. During acclimation, fish were maintained in 100 L fiberglass tanks with continuous aeration at a stocking density of 15 fish per tank. No mortality or abnormal behavior was observed during this period. Acclimation carried out by fish were randomly distributed into 15 fiberglass tanks (100 L capacity), corresponding to five dietary treatments with three replicates per treatment. Each tank contained 15 fish and served as an experimental unit. Continuous aeration was provided throughout the experimental period.

Water quality parameters, including temperature, dissolved oxygen (DO), pH, and total ammonia nitrogen (TAN), were monitored regularly using a digital thermometer, DO meter, pH meter, and spectrophotometric methods, respectively. These parameters were maintained within acceptable ranges for tilapia culture: temperature 26–30 °C, DO > 5 mg L^{-1}, pH 6.5–8.5, and TAN < 0.05 mg L^{-1}. When deviations occurred, corrective measures such as partial water exchange (20–30%) and adjustment of aeration were applied.

The feeding trial lasted 30 days. Fish were fed the experimental diets at a daily ration of 3% of body weight, divided into two equal meals at 09:00 and 16:00. The feeding ration was adjusted weekly based on bulk weighing of fish in each tank to reflect changes in biomass. Uneaten feed was removed 30 minutes after feeding by siphoning from the bottom of the tanks using a flexible hose to maintain water quality. Uneaten feed was not quantified; therefore, feed intake was calculated based on the total feed offered. Fish were batch-weighed at the beginning and end of the 30-day trial, with additional intermediate bulk weighing conducted weekly for feed ration adjustment. Growth performance parameters were calculated based on initial and final biomass per tank. Survival was monitored daily by recording mortalities, and dead fish were removed immediately and their biomass recorded to adjust feed input and biomass calculations accordingly. Final survival rate was calculated at the end of the experiment. Fish handling was minimized throughout the study to reduce stress.

Growth performance and digestibility

Growth performance was evaluated using standard indicators, including weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), and survival rate (SR).

All growth performance parameters were calculated on a tank basis, with each tank considered an experimental unit n = 3 tanks per treatment (biological replicates); analyses were performed in triplicate (analytical replicates).

To minimize variability in growth calculations, experimental fish were size-selected prior to stocking, resulting in a relatively uniform initial size (total length 9–12 cm; body weight 15–30 g; mean ± SD: 23.36 ± 3.28 g), as described in the previous section.

Weight gain (WG) was calculated as the difference between final and initial mean body weight per tank:

WG (g) = Final body weight (g) − Initial body weight (g) ……(2)

Specific growth rate (SGR) was calculated as:

SGR ($\% \text{ day}^{-1}$) = [ln(Final body weight) − ln(Initial body weight)] / Culture period (days) × 100 ……(3)

Feed conversion ratio (FCR) was calculated based on total feed offered per tank divided by biomass gain:

FCR = Total feed offered (g) / Total weight gain (g) ……(4)

Protein efficiency ratio (PER) was calculated as:

PER = Weight gain (g) / Protein intake (g) ……(5)

Survival rate (SR) was calculated as:

SR (%) = (Final number of fish / Initial number of fish) × 100 ……(6)

Apparent protein digestibility coefficient (APDC) was determined during a 15-day digestibility trial conducted after the growth experiment. Diets were supplemented with chromic oxide (Cr_2O_3) as an inert marker. Chromic oxide (Cr_2O_3) was incorporated into each experimental diet at 0.5% as an inert marker for digestibility analysis, and its concentration in feed and fecal samples was determined spectrophotometrically following acid digestion.

Prior to feces collection, uneaten feed was removed by siphoning 30 minutes after feeding to prevent contamination. Fecal samples were collected by observing fish and siphoning freshly excreted feces at regular intervals to minimize nutrient leaching and avoid mixing with uneaten feed.

Collected feces were immediately transferred to clean containers, dried at 60 °C to constant weight, ground, and analyzed for chromic oxide and nitrogen content using standard analytical procedures.

Apparent protein digestibility coefficient was calculated using the following equation:

APDC (%) = 100 − [(Cr_2O_3 in feed / Cr_2O_3 in feces) × (Protein in feces / Protein in feed) × 100] ……(7)

Experimental design and statistical analyses

The study employed a completely randomized design (CRD) consisting of five dietary treatments with three independent replicates per treatment. Each replicate (tank) served as an experimental unit.

The parameters analyzed included growth performance indices (weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio, and survival rate) and apparent protein digestibility coefficient (APDC). All data were expressed as mean ± standard deviation (SD). Prior to statistical analysis, data were tested for normality and homogeneity of variance using the Shapiro–Wilk and Levene's tests, respectively.

Differences among treatments were evaluated using one-way analysis of variance (ANOVA) at a significance level of $p < 0.05$. When a significant treatment effect was detected, post-hoc comparisons were performed using Duncan's Multiple Range Test (DMRT) to identify statistically homogeneous groups of means. In this procedure, treatment means were grouped such that means within the same group were not significantly different from each other, while means in different groups differed significantly at $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics version 25 (IBM Corp., Armonk, NY, USA). Data tabulation and preliminary organization were conducted using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

Results

Proximate composition of fermented *A. sessilis*

The proximate composition of *A. sessilis* was significantly affected by solid-state fermentation with *R. oligosporus* (Table 2).

Table-2. Proximate composition of *A.* sessilis fermented with *Rhizopus* spp. (%, dry matter basis (DM); values are mean ± SD of triplicate analyses per treatment, fermentation time 72 h).

Inoculum (%)	**Dry Matter**	**Crude Protein**	**Crude Lipid**	**Ash**	**Crude Fiber**	**NFE***
0 (R0)	92.0 ± 1.0^a	19.8 ± 1.3^a	2.8 ± 0.3^a	8.6 ± 0.7^a	22.4 ± 1.4^a	46.4
0.5 (R1)	91.7 ± 1.2^a	21.4 ± 1.7^b	3.0 ± 0.4^a	8.9 ± 0.6^a	20.6 ± 1.3^b	46.1
1.0 (R2)	91.5 ± 1.4^a	22.7 ± 1.3^b	3.2 ± 0.4^b	9.0 ± 0.5^a	19.5 ± 1.1^b	45.6
1.5 (R3)	91.3 ± 0.9^a	25.0 ± 1.6^b	3.2 ± 0.6^b	9.2 ± 1.1^a	19.0 ± 1.4^b	43.6
2.0 (R4)	91.1 ± 1.3^a	22.9 ± 1.5^b	3.4 ± 0.8^b	9.1 ± 1.3^a	19.4 ± 1.3^b	45.2

*NFE = Nitrogen-free extract. Superscripts indicate significant differences ($p < 0.05$). All proximate components are expressed on a dry matter basis. NFE was calculated by difference.

Dry matter content was not significantly affected by fermentation, ranging from 91.1 to 92.0% across treatments ($p > 0.05$). In contrast, crude protein content increased significantly with increasing inoculum level, from 19.8% in the control (R0) to a maximum of 25.0% in R3 ($p < 0.05$). Crude lipid content showed a slight but significant increase in fermented treatments, reaching the highest value at R4 (3.4%) compared to the control (2.8%) ($p < 0.05$). Ash content remained relatively stable among treatments, with no significant differences observed ($p > 0.05$). Crude fiber content decreased significantly following fermentation, from 22.4% in R0 to values between 19.0 and 20.6% in fermented treatments ($p < 0.05$). Nitrogen-free extract (NFE) values ranged from 43.6 to 46.4%, with the lowest value observed in R3.

Essential amino acid profile

Fermentation improved the essential amino acid (EAA) composition of the substrate (Table 3).

Table-3. Essential Amino Acid Profile (g/100 g protein) of Fermented *A. sessilis* after fermentation with *Rhizopus* spp. inoculated at five different levels for 72 h.

Essential Amino Acid	**Inoculum (%)**				
	0 (R0)	**0.5 (R1)**	**1.0 (R2)**	**1.5 (R3)**	**2.0 (R4)**
Lysine	3.10 ± 0.14^a	3.44 ± 0.15^b	3.70 ± 0.18^b	3.85 ± 0.19^b	3.82 ± 0.21^b
Methionine	1.05 ± 0.07^a	1.10 ± 0.08^a	1.12 ± 0.07^b	1.15 ± 0.08^b	1.13 ± 0.09^b
Leucine	3.45 ± 0.15^a	3.80 ± 0.17^b	3.95 ± 0.20^b	4.05 ± 0.13^b	4.00 ± 0.26^b
Isoleucine	2.10 ± 0.12^a	2.25 ± 0.17^b	2.35 ± 0.11^b	2.38 ± 0.15^b	2.36 ± 0.13^b
Valine	2.35 ± 0.24^a	2.55 ± 0.19^b	2.60 ± 0.20^b	2.65 ± 0.15^b	2.63 ± 0.17^b
Threonine	1.85 ± 0.09^a	1.95 ± 0.08^b	2.00 ± 0.10^b	2.05 ± 0.10^b	2.03 ± 0.19^b
Phenylalanine	2.50 ± 0.13^a	2.75 ± 0.22^b	2.80 ± 0.20^b	2.85 ± 0.14^b	2.82 ± 0.19^b
Histidine	1.00 ± 0.06^a	1.05 ± 0.08^a	1.08 ± 0.06^a	1.10 ± 0.08^a	1.09 ± 0.12^a
Total EAA	17.40 ± 0.50^a	18.45 ± 0.53^b	19.20 ± 0.59^b	19.88 ± 0.60^b	19.46 ± 1.22^b

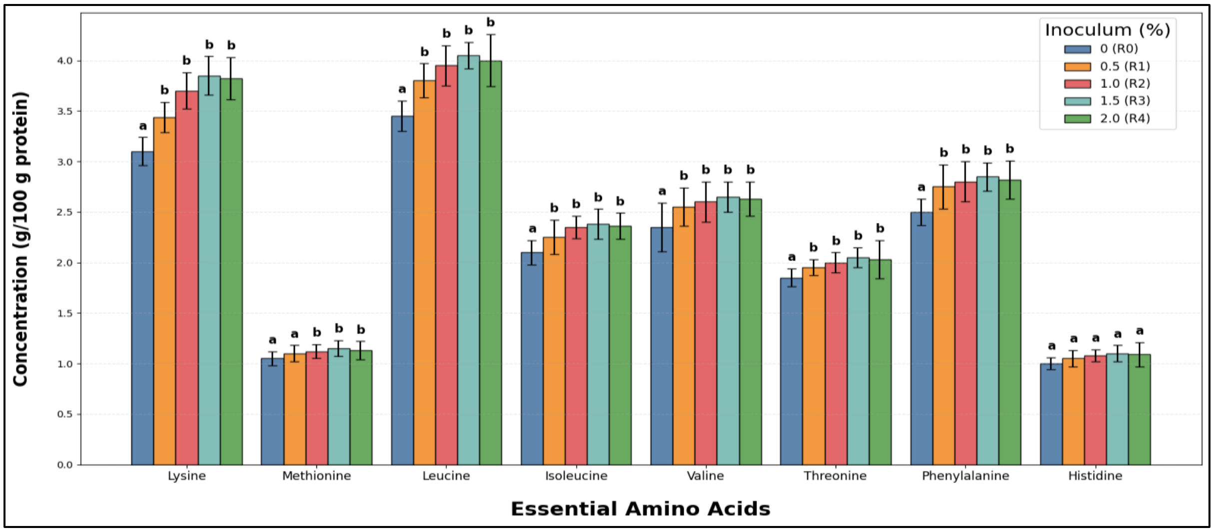

Figure-1. Essential amino acids profile of fermented *A. sessilis* after fermentation with *Rhizopus* spp. inoculated at five different levels for 72 h, error bars show standard deviation.

The essential amino acid profile of *A. sessilis* was significantly affected by fermentation with *Rhizopus* spp. (Table 3). Lysine content increased significantly from 3.10 g/100 g protein in the control (R0) to values ranging from 3.44 to 3.85 g/100 g protein in fermented treatments ($p < 0.05$). Methionine content showed a slight but significant increase, with higher values observed in R2–R4 compared to R0 and R1 ($p < 0.05$). Leucine, isoleucine, and valine contents were significantly higher in all fermented treatments (R1–R4) compared to the control ($p < 0.05$). Threonine and phenylalanine also increased significantly following fermentation, with all treated groups showing higher values than R0 ($p < 0.05$). In contrast, histidine content was not significantly affected by fermentation, remaining relatively constant across treatments ($p > 0.05$).

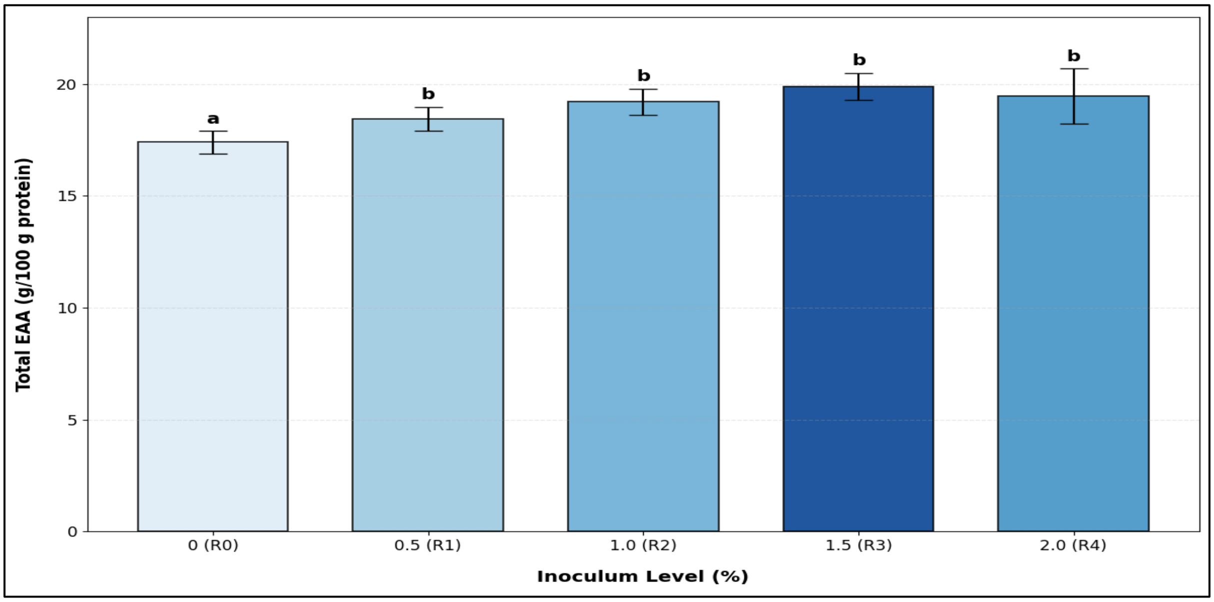

Figure-2. Total essential amino acids in fermented *A. sessilis* after fermentation with Rhizopus oligosporus inoculated at five different levels for 72 h, error bars show standard deviation.

Total essential amino acid (EAA) content increased significantly in fermented treatments compared to the control (Table 3). The total EAA value ranged from 17.40 g/100 g protein in R0 to 18.45–19.88 g/100 g protein in fermented groups ($p < 0.05$), with the highest value observed in R3.

Anti-nutritional factors

Anti-nutritional factors in *A. sessilis* were significantly affected by fermentation with *Rhizopus* spp. (Table 4). Phytate content decreased significantly from 950 mg/100 g DM in the control (R0) to 800–620 mg/100 g DM in fermented treatments (R1–R3) ($p < 0.05$), with no significant difference among R1–R4 ($p > 0.05$).

Tannin content was also significantly reduced from 220 mg/100 g DM in R0 to 150–190 mg/100 g DM in all fermented treatments ($p < 0.05$), with no significant differences observed among R1–R4 ($p > 0.05$). Similarly, oxalate content decreased significantly from 350 mg/100 g DM in the control to 240–300 mg/100 g DM in fermented groups ($p < 0.05$), while no significant differences were detected among the fermented treatments ($p > 0.05$).

Table-4. Anti-Nutritional Factors (mg/100 g DM) of Fermented *A. sessilis* after fermentation with *Rhizopus* spp. inoculated at five different levels for 72 h.

Inoculum (%)	**Phytate**	**Tannin**	**Oxalate**
0 (R0)	950 ± 48^{a}	220 ± 14^{a}	350 ± 20^{a}
0.5 (R1)	800 ± 40^{b}	190 ± 12^{b}	300 ± 16^{b}
1.0 (R2)	680 ± 36^{b}	165 ± 13^{b}	260 ± 14^{b}
1.5 (R3)	620 ± 32^{b}	150 ± 12^{b}	240 ± 13^{b}
2.0 (R4)	635 ± 34^{b}	155 ± 13^{b}	245 ± 14^{b}

Figure 4 shows the changes in anti-nutritional factors following fermentation. All measured parameters, including phytate, tannin, and oxalate, were lower in fermented treatments compared to the control. The reductions were consistent across all inoculum levels, with the lowest values generally observed in R3.

Figure 5 illustrates the trend of reduction in anti-nutritional factors after fermentation. A progressive decrease in phytate, tannin, and oxalate contents was observed from R0 to R3, followed by a slight increase at R4.

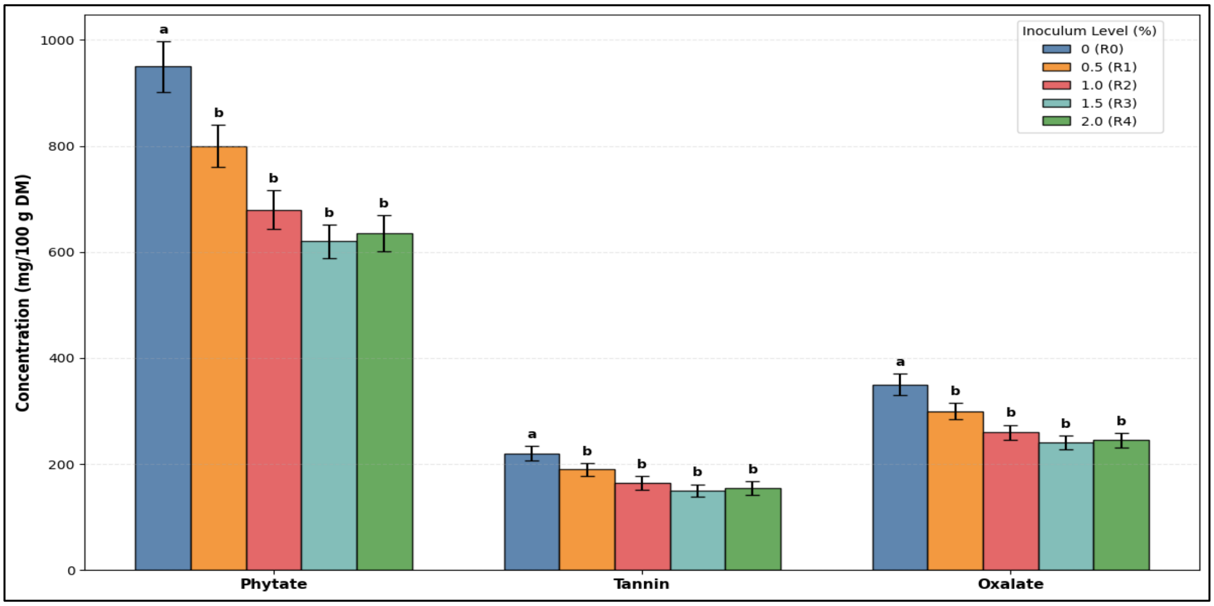

Figure-3. Anti-nutritional factor of fermented *A. sessilis* after fermentation for 72 h with *Rhizopus* spp. inoculated at five different levels, error bars show standard deviation.

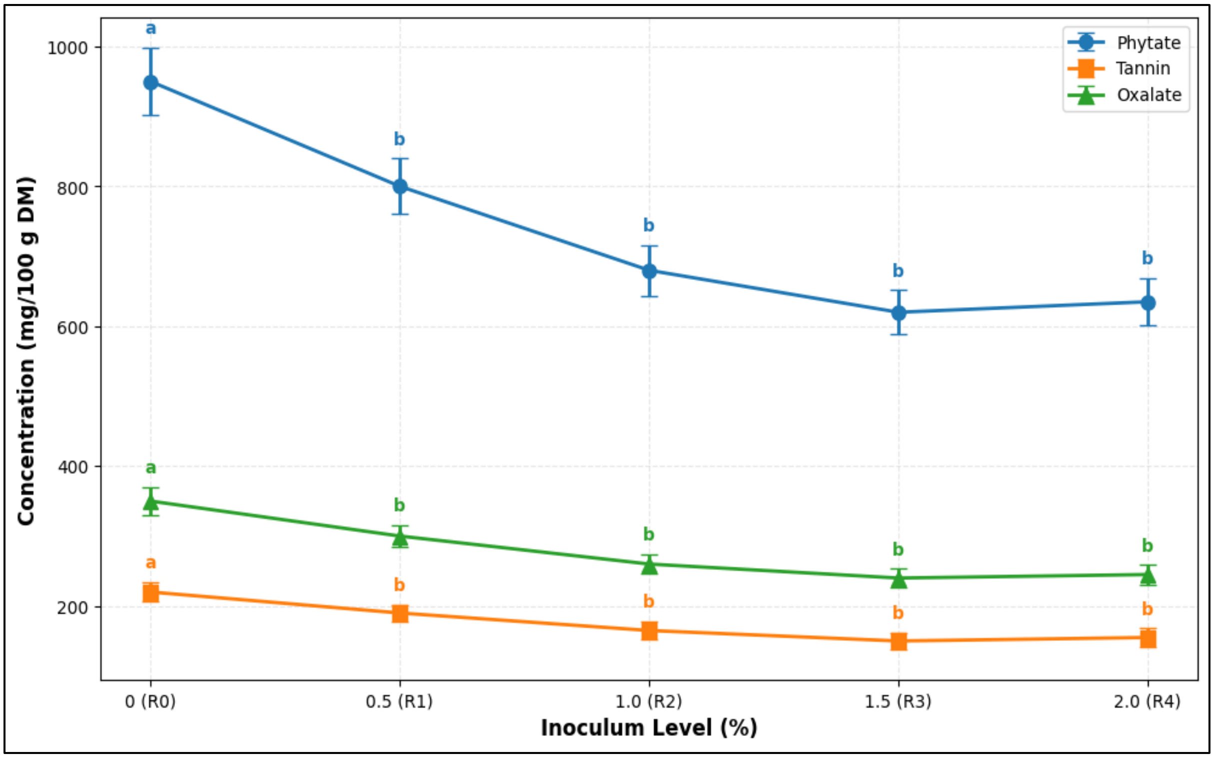

Figure-4. Trend of reduction in anti-nutritional factors in *A. sessilis* after fermentation for 72 h with *Rhizopus* spp. inoculated at five different levels, error bars show standard deviation.

Tilapia growth performance, digestibility, and survival

Tilapia fed diets containing fermented *A. sessilis* showed significant improvements in growth performance and digestibility compared to the control (Table 5; Figure 7).

Table-5. Growth performance, digestibility and 30-day survival rate (SR, %) for tilapia fed on diets with *A. sessilis* after fermentation for 72 h with *Rhizopus* spp. inoculated at five different levels.

Inoculum (%)	**Weight Gain (g)**	**SGR (%/day)**	**FCR**	**PER**	**Apparent Protein Digestibility (%)**	**SR (%)**
0 (R0)	85.0 ± 4.8^a	2.10 ± 0.12^a	1.65 ± 0.09^a	1.20 ± 0.06^a	72.5 ± 3.5^a	95 ± 2^a
0.5 (R1)	92.5 ± 5.0^b	2.24 ± 0.11^b	1.55 ± 0.08^b	1.30 ± 0.05^b	76.0 ± 3.7^b	96 ± 2^a
1.0 (R2)	98.2 ± 5.2^b	2.35 ± 0.12^b	1.48 ± 0.09^b	1.35 ± 0.06^b	78.5 ± 3.8^b	97 ± 1^b
1.5 (R3)	100.0 ± 5.5^b	2.37 ± 0.11^b	1.45 ± 0.08^b	1.38 ± 0.06^b	79.2 ± 3.6^b	97 ± 1^b
2.0 (R4)	99.0 ± 5.7^b	2.36 ± 0.13^b	1.46 ± 0.09^b	1.36 ± 0.06^b	78.8 ± 3.7^b	96 ± 2^b

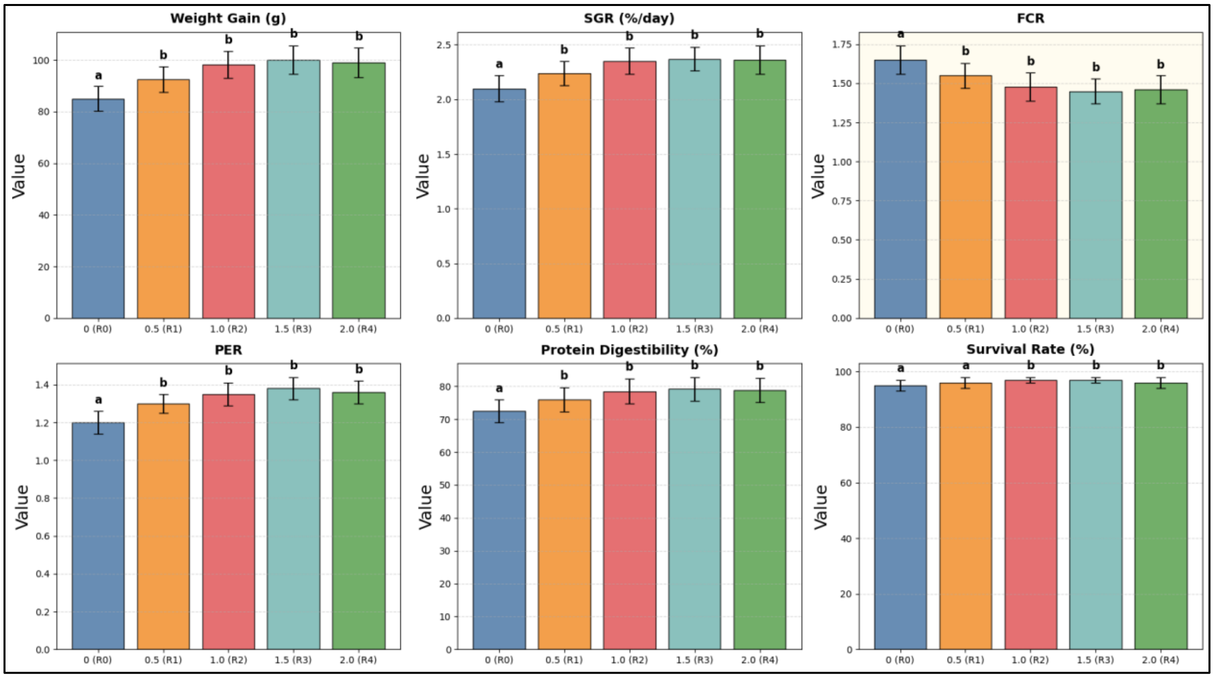

Figure-5. Growth and nutrient utilization performance (30-day feeding trial) of Nile tilapia fed *A. sessilis* after fermentation for 72 h with *Rhizopus* spp. inoculated at five different levels, error bars show standard deviation.

The observed growth trends were consistent with the nutritional improvements in the fermented substrate, particularly the increased crude protein and reduced fiber content observed at higher inoculum levels. Weight gain increased from 85.0 g in R0 to 100.0 g in R3, with a plateau at R4 (99.0 g). Specific growth rate (SGR) followed a similar pattern, rising from 2.10 %/day in R0 to 2.37 %/day in R3, and remaining statistically similar at R4. Feed conversion ratio (FCR) decreased, while protein efficiency ratio (PER) and apparent protein digestibility improved with fermentation, peaking at R3. Survival rate was high across all treatments (95–97 %) and was not significantly affected by fermentation.

Discussion

The present study shows that solid-state fermentation (SSF) of *A. sessilis* using *Rhizopus* spp. can substantially improve its nutritional quality and positively affect the growth performance of juvenile Nile tilapia. The increase in crude protein content observed after fermentation (Table 2) is in agreement with previous studies reporting that *Rhizopus* spp. is able to synthesize microbial protein while simultaneously breaking down fibrous carbohydrates in plant substrates (Zhang et al., 2022). This process leads to a relative concentration of protein in the fermented material, making it more suitable as a feed ingredient. However, the protein and lipid contents tended to plateau at the highest inoculum level (R4), suggesting that above a certain level, increasing fungal inoculum does not further enhance nutrient enrichment. This effect may be related to the depletion of easily fermentable carbohydrates and increased competition between fungal biomass formation and enzymatic activity, which limits additional protein synthesis (Jannathulla and Dayal, 2022; Xiao et al., 2021).

The improvement in essential amino acid composition, such as increases in lysine and leucine content (Table 2), is important for farmed tilapia nutrition. Lysine is commonly the first limiting amino acid in plant-based aquafeeds, and its increase through SSF can directly support improvements in protein utilization and growth performance (Hossain et al., 2023). Leucine plays important roles in protein synthesis and fish muscle maintenance (Rehman et al., 2023); therefore, leucine enrichment enhances the nutritional value of the fermented fish feed. Methionine also increased, consonant with previous findings that amino acid enhancement during SSF depends on fungal metabolism and the chemical composition of the substrate (Li et al., 2023). The plateau in amino acid levels reached under treatment R4 again highlights that optimizing inoculum level is important, as higher fungal doses do not necessarily result in proportional increases in amino acid levels.

SSF also causes a reduction in ANF (Table 3). Reduce of phytate because of activity of fungal phytase produced by *Rhizopus* spp. Lower phytate levels improve the bioavailability of minerals which are essential for fish growth, bone formation, and metabolic processes (Nsabimana et al., 2024). The slight increase in phytate observed at the highest inoculum level may be related to limited enzyme–substrate contact or partial re-binding of residual phytate within the fermented matrix, a phenomenon commonly reported in SSF systems (El-Batal and Karem, 2001). The reductions in tannin and oxalate can negatively affect feed palatability and protein digestibility by forming complexes with dietary proteins and digestive enzymes (Zia-Ur-Rehman and Shah, 2001).

The improvement in growth performance from R0 to R3 corresponds with the progressive increase in crude protein and reduction in crude fiber of the fermented *A. sessilis* (Table 1). These compositional changes likely enhanced nutrient availability and digestibility, thereby improving feed utilization efficiency. The absence of further improvement at R4 suggests that fermentation had reached an optimal level, beyond which no additional nutritional benefit was obtained. The slight decline or plateau in growth performance at the highest inoculum level (R4) may indicate substrate over-fermentation, which can lead to nutrient losses or reduced palatability. Tilapia fed diets containing solid-state fermented *A. sessilis* exhibited higher weight gain, specific growth rate (SGR), and apparent protein digestibility compared to the control group (Table 5). The highest growth performance was observed in treatment R3, while no further improvement was evident in R4, indicating a plateau response at higher inoculum levels. This pattern indicates that increasing fermentation intensity enhances the nutritional value of *A. sessilis* up to an optimal level, beyond which no additional benefits are obtained. The absence of further improvement at higher inoculum levels may be associated with changes in substrate utilization efficiency during fermentation. The improved feed conversion ratio (FCR) in fermented treatments suggests enhanced nutrient utilization, likely due to increased availability of digestible nutrients. These results are consistent with the observed increase in apparent protein digestibility, indicating more efficient protein assimilation and utilization in fish fed fermented diets. The consistency between substrate composition (Table 1) and growth performance (Table 4) supports the conclusion that the observed improvements were primarily driven by enhanced nutritional quality resulting from fermentation rather than differences in diet formulation.

Survival rates remained high across all treatments (95–97%), indicating that fermented *A. sessilis* did not produce toxic effects and was readily accepted by juvenile tilapia.

The results confirm that SSF of locally available, wild-harvested *A. sessilis* is an effective approach to improving protein content, essential amino acid balance, and nutrient digestibility while simultaneously reducing anti-nutritional factors. Inoculum optimization is a key factor, with levels around 1–1.5% providing the greatest nutritional and biological benefits. The use of fermented alligator weed leaf biomass in tilapia feed formulation offers a low-cost and environmentally friendly strategy to reduce dependence on conventional feed sources while still supporting efficient growth in aquaculture systems.

Conclusion

This research shows that solid-state fermentation (SSF) with *Rhizopus* spp. is a good way to make *A. sessilis* more nutritious and help young Nile tilapia grow faster and use protein better. Fermentation greatly boosted crude increased protein content, better critical amino acid profiles (especially lysine and leucine), and lower levels of important anti-nutritional substances including phytate, tannin, and oxalate. These changes made fish that ate fermented diets grow better, use their food more efficiently, and digest protein more easily. The best overall results were achieved with inoculum levels of 1–1.5%, suggesting that excessive inoculum does not confer extra advantages and may result in nutritional plateaus. The primary innovation of our study is in the comprehensive assessment of SSF-treated wild-harvested *A. sessilis* as a beneficial aquafeed component, integrating comprehensive nutritional profiling with in vivo growth and digestibility evaluations in tilapia. This study differs from earlier research that mostly examined laboratory-scale compositional alterations; it establishes a direct correlation between fermentation-induced enhancements in plant biomass and biological performance in fish, offering empirical proof of its use for aquaculture feed formulation. Furthermore, optimizing the inoculum level underscores the significance of process control in solid-state fermentation applications, instead of presuming that increased fungal concentrations invariably yield superior results. This study tackles a pressing issue in aquaculture from both applied and sustainability viewpoints: the rising costs and restricted availability of traditional protein sources like fishmeal. Using locally available leafy biomass through SSF is a low-cost and eco-friendly option that can help tropical aquaculture systems use resources more efficiently and less depend on imported feed materials. This method is especially useful for small and medium-sized farmers in developing areas where it is still hard to get high-quality commercial feeds. Even though these results are promising, more research is needed to support their use on a larger scale. Subsequent research ought to concentrate on prolonged feeding trials to assess growth efficiency, health condition, and nutrient retention during longer cultivation durations. Research is needed on the best amounts of fermented *A. sessilis* to add to commercial diets, as well as how it affects the shape of the gut, the activity of digestive enzymes, and the immune response. Furthermore, economic analysis and pilot-scale fermentation studies are necessary to evaluate production viability and cost-effectiveness in agricultural settings. Investigating the application of different microbial cultures or varying fermentation periods may further improve nutrient accessibility and functional characteristics. Solid-state fermentation of *A. sessilis* offers a potential and scalable way to make plant-based aquafeed ingredients that will last. This method has a lot of promise to help make aquaculture production systems that are more resilient, cost-effective, and ecologically friendly if it is improved and tested more.

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Ethical Approval Statement

All experimental procedures involving Nile tilapia (*O. niloticus*) were conducted in accordance with internationally recognized guidelines for the care and use of aquatic animals in research and complied with institutional standards for animal welfare. Ethical clearance was not required for this study, as the trials involved non-invasive feeding experiments.

Use of Generative AI Tools Statement

The authors declare that artificial intelligence–assisted tools were used in the preparation of this manuscript. ChatGPT was utilized to support language refinement, the structuring of scientific content, and the drafting of preliminary text. Google Colab was utilized for data handling and computational support. SciSpace was used to support literature exploration and reference management, while Grammarly and Gemini were used for grammar checking, clarity improvement, and language polishing. All scientific interpretations, data validation, critical analysis, and final decisions regarding the content remain the sole responsibility of the authors. The use of these tools complies with ethical standards of academic integrity and does not replace human authorship or accountability.

Contribution of Authors

Islamy RA & Hasan V: Conceptualized the study, designed research methodology, supervised experiments and edited the manuscript.
Valen FS & Murmainnah N: Conducted fermentation and feeding experiments, collected data and assisted in laboratory analysis.
Kamarudin AS & Ismail N: Assisted in data analysis, interpretation of results and manuscript editing.
Czech M: Contributed to data interpretation, scientific discussion and critical revision of the manuscript.

All authors read and approved the final draft of the manuscript.

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