Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(4): 2145 – 2167 (2025)
www.ejabf.journals.ekb.eg

Impact of Fermented Azolla on the Monosex Nile Tilapia Fingerlings' Growth Performance and Feed Utilization

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ARTICLE INFO

Article History:
Received: May 28, 2025
Accepted: July 17, 2025
Online: Aug. 4, 2025

Keywords:
Oreochromis niloticus,
Bacillus subtilis,
Saccharomyces cerevisiae,
Fermentation,
Growth,
Azolla

ABSTRACT

Two strains of *Lactobacillus* were used for azolla fermentation in the monosex Nile tilapia (*Oreochromis niloticus*) to determine the optimal conditions for rearing systems in terms of feed replacement ratio. Researchers investigated the impact on growth performance, feed utilization, digestive enzyme activity, antioxidant response, and body composition. The fish had an average initial weight of 15.60 ± 0.12 g. A 90-day feeding trial was conducted to evaluate the benefits of solid-state fermentation using *Saccharomyces cerevisiae* (SFAM), *Bacillus subtilis* (BFAM), or a combination of both (SFAM + BFAM), incorporating fermented azolla as a feed ingredient. Seven isocaloric and isonitrogenous diets (each containing 30% crude protein) were formulated: T1 (control, without azolla), T2 (BFAM-10%), T3 (BFAM-25%), T4 (SFAM-10%), T5 (SFAM-25%), T6 (Mix SFAM + BFAM-10%), and T7 (Mix SFAM + BFAM-25%). Fish were fed twice daily. The results showed that the T3 diet (BFAM-25%) led to the most significant improvements in growth performance, feed intake, and economic efficiency compared to all other treatments. These findings suggest that, under the given experimental conditions, fermented azolla—specifically BFAM at 25% inclusion—can effectively replace up to 25% of soybean meal protein in the diets of the monosex Nile tilapia fingerlings, without compromising performance or profitability.

INTRODUCTION

Egypt's aquaculture industry, which includes the cultivation of species such as the Nile tilapia, catfish, and shrimp, is a significant contributor to employment and food security **(FAO, 2023)**. Despite the growth of the sector, challenges related to disease control, water quality, and the need for improved infrastructure and technology still persist **(Rossignoli *et al.*, 2023)**. In tropical and subtropical countries, one of the most extensively farmed fish species is the Nile tilapia (*Oreochromis niloticus*) **(Ogello *et al.*, 2014)**. It

serves as a major source of animal protein and is one of the primary sources of income in many regions **(Yakubu *et al.*, 2012)**.

Proper nutrition is essential for fish growth, health, and economic viability in aquaculture **(FAO, 2020)**. Feed formulation is typically influenced by nutritional value, cost-effectiveness, and availability **(Robinson & Li, 2008)**. Due to the increasing cost of traditional ingredients and the need for sustainability, alternative fish diets are being explored **(FAO, 2016)**. According to **Gao *et al.* (2024)**, common alternatives include soybean meal, pea protein, corn, cricket and mealworm protein, microalgae, yeast, fermented ingredients, seaweed, and food production byproducts.

Azolla, a free-floating water fern commonly found in tropical and subtropical regions, is gaining popularity as an alternative aquafeed ingredient due to its high protein content, rapid growth, and nutritional benefits **(Hemalatha *et al.*, 2020)**. Research has shown that fish such as tilapia and carp exhibit improved growth performance, feed conversion ratios (FCR), feed intake, and immunity when azolla is partially substituted for conventional feed **(Abdel-Tawwab, 2008; Ghodake & Kalita, 2015)**. Azolla is also cost-effective and accessible for inclusion in fish diets. However, plant-based feeds often present challenges, such as low digestibility, reduced palatability, and the presence of anti-nutritional factors (ANFs). Fermentation is a promising solution to these issues.

Fermenting plant-based fish feed, particularly with the addition of probiotics, can reduce ANFs and improve gut health. Fermented feeds containing beneficial microorganisms like *Lactobacillus*, *Bacillus* and various yeasts have been found to enhance fish growth, immune response, and overall health, while also improving nutrient availability and minimizing the environmental impact of aquaculture **(Mohammady *et al.*, 2024)**.

The objective of this experiment was to investigate the effects of fermented azolla—treated with *Saccharomyces cerevisiae*, *Bacillus subtilis*, and a combination of both (BFAM+SFAM)—on the growth performance, feed efficiency, and total nutrient digestibility in fish.

MATERIALS AND METHODS

This research was conducted at the Cairo Regional Center for Food and Feed, Fish Research Unit. The objective of this experiment was to assess how different fermentation treatments using *Saccharomyces cerevisiae*, *Bacillus subtilis*, and a combination of both (BFAM+SFAM) affected feed efficiency, growth performance, nutrient digestibility, antioxidant status, proximate whole-body composition, and the economic evaluation of the monosex Nile tilapia (*Oreochromis niloticus*) fingerlings.

Tank preparation

Twenty-one 120-liter tanks were utilized for the study. The fish culture medium was freshwater, which had been aerated for 24 hours to elevate dissolved oxygen levels. Water quality parameters were monitored daily using a dissolved oxygen meter (Jenway Ltd., Model 970-DO, Staffordshire, ST15 0SA, UK) and a pH meter (Jenway Ltd., Model 350-pH, Staffordshire, ST15 0SA, UK). Ammonia (NH_3) levels were assessed daily using a Hanna meter, while total ammonia nitrogen (TAN) was measured every two days with a spectrophotometer. Each tank received biweekly partial water replacement using fresh groundwater, amounting to approximately one-third of the tank's volume. According to **Devi and Bhatnagar (2013)**, these water quality parameters fall within the acceptable range for rearing the Nile tilapia.

Solid-state fermentation of *Azolla* meal

Lactic acid bacteria (LAB), yeast, and microorganisms were uniformly mixed with *Azolla pinnata* at a ratio of 1g per 100g of substrate for fermentation **(Surianti *et al.*, 2021)**. The process began with weighing 1kg of *Azolla*, and the fermentation was conducted at the Cairo Regional Center for Food and Feed, Fish Research Unit. The substrate was autoclaved at 121°C for 20 minutes and then divided into three parts for different fermentation treatments:

1. **Yeast fermentation (SFAM)**

Following the method of **Yabaya *et al.* (2009)**, 1.1L of distilled water (50% moisture) and 60.5mg of commercial dry yeast (*S. cerevisiae*, Fermipan®, GB Ingredients, China) were added to 2kg of autoclaved *Azolla* meal. The mixture was homogenized for 15 minutes to reach a cell density of 3×10^6 CFU/g. Fermentation was carried out for 72 hours at 40°C (optimal for *S. cerevisiae*) in a 10-liter glass basin covered with aluminum foil.

2. **Bacterial fermentation (BFAM)**

According to **Joshi *et al.* (2011)**, 2kg of *Azolla* meal was inoculated with 600mL of *B. subtilis* inoculum sourced from the Microbiological Resources Center (MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The bacterial inoculum (E20 strain) was incubated under controlled conditions (65% relative humidity, 37 °C) for 72 hours in a biochemical oxygen demand (BOD) chamber.

3. **Mixed fermentation (SFAM + BFAM)**

Equal portions (1:1) of the SFAM and BFAM products were blended. From each treatment—SFAM, BFAM, and the mixture—10g was taken to analyze the chemical composition and anti-nutritional factors (ANFs).

Following fermentation, all *Azolla* meals were dried at 105°C for 3 hours to terminate microbial activity. Final drying was completed at 70°C until constant weight was achieved for each treatment. The analysis of ash, crude protein, fiber, and lipids was conducted both at the beginning and after 72 hours of fermentation using standard AOAC methods **(AOAC, 2022)**. Table (1) presents the nutritional composition of the fermented azolla before and after fermentation.

Table 1. Effect of fermentation on chemical composition of Azolla meal (% on DM basis)

Chemical composition	**RAM**	**BFAM**	**SFAM**	**MIX(B+S)**
Dry Matter	9.95	8.60	8.50	8.50
Crude protein	29.2	35.8	33.50	34.01
NDF	35.56	30.60	31.50	31.30
Ether extract	3.9	3.8	3.6	3.8
Ash	15.9	16.2	16.1	16.1

RAM azolla meal, BFAM: azolla meal fermented by *Bacillus subtilis*, SFAM: azolla meal fermented by *Saccharomyces cerevisiae*, Mix (B+S): azolla meal fermented by *Bacillus subtilis* + *Saccharomyces cerevisiae.*

Determination of anti-nutritional factors (ANF's)

Antinutritional compounds were identified in both fermented and unfermented plant material samples. Benzoyl-DL-arginine-p-nitroanilide (BAPA) was used as a substrate in the enzymatic colorimetric method to measure trypsin inhibitory activity **(Smith *et al.*, 1980)**. The Folin-Denis reagent was employed in a spectrophotometric method to determine both hydrolyzable and condensed tannins **(Price & Butler, 1977; Earp *et al.*, 1981)**. Phytates (measured as phytic acid activity) were detected at 492nm using an enzymatic-spectrophotometric method (Cat No. K-PHYT, Megazyme International Ireland Ltd., Wicklow, Ireland). Oxalates were identified using HPLC. The results of these analyses are presented in Table (2).

Table 2. Effect of fermentation on anti-nutritional factors of Azolla meal

Anti-nutritional substances	RAM	BFAM	SFAM	Mix(B+S)
Trypsin inhibitor (mg. g-1)	1.84	1.47	1. 56	1.51
Phytates (% phytic ac.)	0.16	0.14	0.15	0.15
Soluble Tannins%	0.44	N d	Nd	Nd
Oxalates%	1.67	0.19	0.20	0.20

RAM azolla meal, BFAM: azolla meal fermented by *Bacillus subtilis*, SFAM: azolla meal fermented by *Saccharomyces cerevisiae*, Mix (B+S): azolla meal fermented by *Bacillus subtilis* + *Saccharomyces cerevisiae.*

Determination of amino acid composition

An automated amino acid analyzer was used to determine the amino acid compositions of Azolla meal (AM), yeast-fermented azolla meal (SFAM), bacteria-fermented Azolla meal (BFAM), and the combined treatment (SFAM + BFAM). Analyses were conducted at the Central Laboratory of Suez Canal University, after samples were hydrolyzed with 6M HCl for 24 hours at 110°C **(Bassler & Buchholz, 1993)**. Prior to acid hydrolysis, sulfur-containing amino acids were oxidized using performic acid. The results of the amino acid analysis are presented in Table (3).

Table 3. Effect of fermentation on amino acid composition of Azolla meal (g/100g-1DM)

Item	RAM	BFAM	SFAM	Mix(B+S)	Amino acid requirement
Arginine	1.14	1.52	1.45	1.48	1.18
Histidine	0.45	0.53	0.52	0.52	0.48
Lysine	0.99	1.10	1.05	1.07	1.43
Methionine	0.44	0.57	0.55	0.56	0.75
Leucine	1.55	1.66	1.60	1.62	0.95
Isoleucine	0.93	1.12	1.10	1.11	0.87
Threonine	0.88	0.95	0.92	0.93	1.05
Phenylalanine	1.01	0.99	0.93	0.95	0.28
Valine	1.19	1.50	1.42	1.45	0.78
Tryptophan	0.50	0.65	0.59	0.60	0.28

RAM azolla meal, BFAM: azolla meal fermented by *Bacillus subtilis*, SFAM: azolla meal fermented by *Saccharomyces cerevisiae, Mix (B+S): azolla meal fermented by Bacillus subtilis + Saccharomyces cerevisiae.*

Experimental diets

Experimental diets were formulated to contain approximately 30% crude protein and a protein-to-energy ratio of around 65mg protein/kcal, with an estimated gross energy of 465 kcal/100g. All diets were isonitrogenous (7 diets in total). The proximate chemical composition of the experimental diets is shown in Table (4).

The control diet (T1) contained 400g/ kg soybean meal without any azolla inclusion. Diets T2 and T3 included fermented azolla with *Bacillus subtilis* at inclusion levels of 10 and 25%, respectively. Diets T4 and T5 included fermented azolla with *Saccharomyces cerevisiae* at 10 and 25% levels, respectively. Diets T6 and T7 contained fermented azolla with a combination of *B. subtilis* and *S. cerevisiae* (Mix B+S), also at 10 and 25% inclusion levels.

Each diet contained 5g/ kg of chromic oxide (Cr_2O_3) as an inert marker to assess nutrient digestibility.

Dry ingredients including fish meal, soybean meal, fermented azolla meal, rice bran, yellow maize, and wheat bran were mixed thoroughly with soybean oil for five minutes. The mixture was then processed using a California Pellet Mill (San Francisco, CA, USA) to form 2-mm diameter pellets. The pellets were dried at 60°C for four hours and stored at −20°C until use.

Experimental fish and culture technique

The monosex Nile tilapia (*Oreochromis niloticus*) fingerlings were obtained from a private hatchery in Damietta Governorate, Egypt. The fish had an average initial weight of 15.60 ± 0.12 g. They were acclimatized for two weeks at the Regional Center for Food and Feed in Cairo, Egypt, prior to the start of the experiment.

The feeding trial lasted for 90 days. Fish were fed the control diet (30% crude protein) twice daily at 9:00 a.m. and 3:00 p.m. to apparent satiation. A total of 315 fingerlings were used in the experiment. The fish were randomly distributed into fiberglass tanks (70 × 60 × 50 cm), with each of the seven treatments assigned three replicates. Each tank was stocked with 15 fish.

Approximately one-third of the water volume in each tank was replaced daily. Uneaten feed was siphoned out 30 minutes after feeding, dried, and weighed to calculate feed consumption. Feed intake per fish over the 90-day period was recorded.

Both diet samples and whole fish samples were analyzed for moisture, crude protein, crude fat, and ash following standard procedures outlined by **AOAC (2022)**.

Table 4. Ingredients and chemical composition of dietary treatments (% DM basis)

Item	T1	T2	T3	T4	T5	T6	T7
Fish meal	9	9	9	9	9	9	9
Soybean meal	40	33	24	33	24	33	24
BFAM	-	10	25	0	0	0	0
SFAM	-	0	0	10	25	0	0
Mix(B+S)	_	0	0	0	0	10	25
Yellow corn	25	16	10	16	10	16	10
Corn Gluten	5	5	5	5	5	5	5
Wheat bran	7	10	10	10	10	10	10
Wheat rice	7	10	10	10	10	10	10
Soybean oil	2	2	2	2	2	2	2
Vitamin premix	2	2	2	2	2	2	2
Mineral premix	2	2	2	2	2	2	2
Binder (CMC)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Chromic oxide	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Chemical composition (on DM% basis)							
Dry Matter DM	90.09	89.87	88.85	89.28	89.30	89.75	89.88
Crude protein(CP)	30.48	30.54	30.45	30.50	30.55	30.54	30.48
Ether Extract (EE)	7.43	7.05	7.10	7.00	7.04	7.03	7.07

Crude Fiber (CF)	5.50	5.78	5.84	6.01	5.90	5.91	5.98
Ash	10.81	11.75	11.97	12.66	12.50	12.01	12.02
NFE	45.22	55.12	55.36	55.41	55.99	55.49	55.55
Gross Energy (GE) (kcal/100g)	465.26	465.71	466.65	466.20	469.23	467.04	467.33
P/E ratio	65.51	65.57	65.25	65.42	65.10	65.39	65.22

BFAM: azolla meal fermented by Bacillus subtilis, SFAM: azolla meal fermented by Saccharomyces cerevisiae, Mix (B+S): azolla meal fermented by Bacillus subtilis + Saccharomyces cerevisiae, CMC: Carboxymethyl cellulose, NFE: Nitrogen free extract = 100 - (%protein +% lipid +% fiber +%ash), GE: Gross Energy based on protein (5.65 Kcal/g), fat (9.45 Kcal/g) and carbohydrate (4.11Kcal/g), P/ E ratio: Protein to energy ratio in mg protein/kcal of gross energy, each Kg vitamin & mineral mixture premix contained Vitamin A, 4.8 million IU, D3, 0.8 million IU; E, 4g; K, 0.8g; B1,0.4g; Riboflavin,1.6g; B6,0.6g, B12,4mg; Pantothenicacid,4 g; Nicotinic acid,8g; Folic acid,0.4g Biotin,20mg, Mn, 22g; Zn, 22g; Fe,12g; Cu, 4g; I,0.4 selenium, 0.4gandCo, 4.8mg **(NRC, 2011)**.

Growth performance parameters

- Body Weight Gain (BWG): Weight gain (g/fish) = Final weight (g) – Initial weight (g) **(Carlos, 1988)**.
- Average Daily Gain (ADG): ADG = (W2 – W1) / T, where W2 = final weight, W1 = initial weight, and T = duration in days **(De Silva & Anderson, 1995)**.
- Relative Growth Rate (RGR): RGR (%) = [(W2 – W1) / W1] × 100, where W1 = initial weight and W2 = final weight **(De Silva & Anderson, 1995)**.
- Specific Growth Rate (SGR): SGR (%) = [ln(final weight) – ln(initial weight)] × 100 / time in days **(El-Sayed & Kawanna, 2004)**.

Feed utilization parameters

- Feed Conversion Ratio (FCR): FCR = Dry feed intake (g) / Wet weight gain (g).
- Feed Efficiency Ratio (FER): FER (%) = Body weight gain (g) / Dry matter feed intake (g) × 100.
- Protein Efficiency Ratio (PER): PER (%) = Body weight gain (g) / Protein intake (g on DM basis) × 100.
- Protein Productive Value (PPV): PPV = (P2 – P1) / Pf, where P1 = protein content in fish carcass at the start of the experiment (g), P2 = protein content at the end (g), and Pf = total protein intake during the experiment (g, DM basis) (**El-Sayed & Mansour, 2003)**.

Survival rate (SR)

SR (%) = (Nt / No) × 100, where Nt = total number of fish that survived by the end of the experiment, and No = total number of fish at the start **(Biswas *et al.*, 2005)**.

Digestibility study

Two months after the experiment began, feces were collected daily from each pond in the morning before feeding, and this was continued for one month. As described by **El-Saidy and Gaber (2002)**, the feces were collected on filter paper and dried. Fecal samples collected over a ten-day period were pooled, and then freeze-dried prior to analysis. Chemical analysis was conducted according to the **AOAC (2022)** standards.

Chromic oxide was used as an inert marker for digestibility studies, as it is a validated method widely used in fish nutrition research. Chromic oxide content was determined using the procedure described by **Furukawa (1966)**. The apparent nutrient digestibility coefficients (ADC) were calculated using the formula proposed by **Schneider *et al.* (2004)**:

ADC dietary nutrient=

$$\textbf{ADC dietary nutrient} = 1 - \left(\frac{\text{marker in diet}}{\text{marker in feces}} \times \frac{\text{nutrient in feces}}{\text{nutrient in diet}} \right)$$

Total digestible nutrients (TDN)

Total digestible nutrients were calculated using the following formula:

% digestible crude protein + % digestible crude fiber + % digestible N-free extract + (2.25 x % digestible ether extract). % TDN = % DCP + % DCF + % DNFE + (2.25 x % DEE)

Where:

- DCP = Digestible Crude Protein
- DCF = Digestible Crude Fiber
- DNFE = Digestible Nitrogen-Free Extract
- DEE = Digestible Ether Extract

The factor 2.25 accounts for the higher energy yield from fat oxidation, as fats provide approximately 2.25 times more energy than carbohydrates.

Proximate chemical analysis

Proximate analysis of the fish body composition and diet was conducted following the **AOAC (2022)** methodology. Whole fish samples collected before and during the experiment were ground and homogenized for analysis.

- Moisture content was determined by drying the samples at 105°C for 24 hours.
- Ash content was measured using a muffle furnace (Nabertherm B150, Bremen, Germany) at 550°C for 5 hours.
- Crude protein was calculated by the Kjeldahl method (N × 6.25) using a VELP Scientifica UDK 149 system (Usmate Velate, Italy).
- Crude lipid content was determined using a Soxhlet extraction system (VELP Scientifica SER 148, Usmate Velate, Italy) with petroleum ether (boiling point 40–60°C).

Economical evaluation

Scientists are continuously exploring low-cost, readily available, and unconventional feedstuffs capable of supporting healthy and high-yield fish production, due to the high

cost and limited availability of traditional aquafeed ingredients. In developing countries, fish farmers must utilize affordable, locally sourced ingredients in aquafeed formulations to counter rising protein costs and increasing demand for conventional feed sources **(Panigrahi *et al.*, 2014)**.

A simple economic analysis was employed to calculate the feed cost required to increase fish biomass. The calculation was based on the local retail market prices of dietary ingredients at the time of the study **(Eid & Mohamed, 2008)**.

The local ingredient prices (in LE/kg) were as follows:

- Herring fish meal: 60.0
- Fermented Azolla by *Saccharomyces cerevisiae*: 12.0
- Fermented Azolla by *Bacillus subtilis*: 11.0
- Fermented Azolla by Mix (*S. cerevisiae* + *B. subtilis*): 11.50
- Wheat bran: 12.0
- Wheat rice: 12.0
- Corn gluten: 60.0
- Soybean meal: 30.0
- Corn meal: 12.0
- Soybean oil: 50.0
- Vitamin premix: 50.0
- Mineral premix: 50.0

Economic Evaluation Formulae:

1. **Cost/kg diet (LE)**
   = Cost per kg of the diet (based on ingredient prices)
2. **Consumed feed to produce 1 kg fish (kg)**
   = Feed intake per fish over the study period ÷ Final weight per fish (kg/kg)
3. **Feed cost per kg fresh fish (LE)**
   = Step 1 × Step 2
4. **Relative % of feed cost/kg fish**
   = (Step 3 result ÷ Highest value in Step 3) × 100
5. **Feed cost per 1 kg gain (LE)**
   = Feed intake per kg gain × Step 1
6. **Relative % of feed cost per kg gain**
   = (Step 5 result ÷ Highest value in Step 5) × 100

Statistical analysis

The mean ± SE of three replicates is accustomed to present the results. SAS was used to examine the average values for every parameter under observation using a one-way ANOVA. According to **Duncan (1955)**, differences were deemed significant for all analyses at $P < 0.05$.

RESULTS

Water quality

Table (5) indicates that a significant difference in pH was observed between the fermented diet groups and the control. Water temperature remained consistent across all tanks. The levels of pH, NH_3, and TAN decreased slightly but significantly ($P< 0.05$) in the fermentation treatment groups compared to the control. All water quality parameters remained within the optimal range for tilapia culture, as established by **Boyd (1990)**.

Table 5. Water quality of tilapia experiment under the influence of different fermentation levels of Azolla (% on DM basis)

Parameters	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**
Temp ^{o}c	28.30± 0.11	28.27± 0.01	28.29± 0.04	28.28± 0.13	28.26± 0.10	28.25± 0.16	28.28± 0.09
PH	7.90± 0.05	7.60± 0.04	7.50± 0.05	7.80± 0.00	7.60± 0.01	7.66± 0.03	7.55± 0.02
Dissolved oxygen	6.90± 0.05	7.20± 0.05	7.20± 0.05	6.95± 0.05	7.15± 0.05	7.15± 0.05	7.20± 0.05
TAN (ppm)	1.02 ± 0.00	0.87 ± 0.001	0.68 ± 0.002	0.76 ± 0.0031	0.89 ± 0.00	0.77 ± 0.002	0.90 ± 0.003
NH_3 (ppm)	0.105± 0.00	0.066± 0.00	0.063± 0.00	0.075± 0.0001	0.065± 0.0002	0.067± 0.000	0.063

Nutritional value of Azolla after solid state fermentation

After both solid-state fermentation (SSF) procedures using *S. cerevisiae*, *B. subtilis*, and their combination (Mix B+S), the chemical composition of Azolla meal (AM) improved significantly at 72 hours, according to the study's findings. Additionally, all nutritional parameters were considerably higher in BFAM compared to the Mix (B+S) and SFAM treatments.

As shown in Table (2), after 72 hours of SSF with *B. subtilis* E20, *S. cerevisiae*, and their combination (SFAM + BFAM), crude protein levels were higher than in raw Azolla meal, while crude fiber levels were lower. After 72 hours, consistent changes were observed in crude protein, crude lipid, fiber, and ash content across all fermentation treatments—BFAM, SFAM, and the mix (SFAM + BFAM).

In this study, crude protein increased from 29.20% in raw Azolla to 35.80% in BFAM, 34.01% in the Mix (SFAM + BFAM), and 33.50% in SFAM. Similarly, NDF% decreased from 35.56% in raw Azolla to 30.60% in BFAM, 31.30% in the Mix, and 31.50% in SFAM. As shown in Table (3), BFAM, Mix (B+S), and SFAM all had higher levels of essential, hydrolyzed, and total amino acids than raw Azolla meal.

Growth performance

Growth variations were observed among the Nile tilapia fed the experimental diets. The control group (Diet 1) exhibited the lowest weight gain. As shown in Table (6), fish fed the T3 diet (BFAM-25%) achieved significantly higher ($P< 0.05$) final body weight

(FBW), weight gain (WG), relative growth rate (RGR), and average daily gain (ADG) compared to the control and other treatment groups.

Fish fed T3 (BFAM-25%) had the highest FBW, WG, and ADG, followed in descending order by T7 (Mix B+S 25%), T5 (SFAM-25%), T2 (BFAM-10%), T6 (Mix B+S 10%), and T4 (SFAM-10%). All experimental groups showed significant improvements ($P< 0.05$) over the control group.

Table 6. Growth performance of the Nile tilapia under the influence of different fermentation levels of Azolla

Item	T1	T2	T3	T4	T5	T6	T7
IW (g/ fish)	15.14±0.31	15.14±0.03	15.53±0.09	15.05±0.1	15.40±0.01	15.04±0.04	15.48±0.5
FW (g/ fish)	86.10±0.39^{f}	89.72±0.07^{d}	95.76±0.0^{a}	86.56±0.7^{f}	91.37±0.0^{c}	88.60±0.3^{e}	92.58±0.6^{b}
WG (g/ fish)	70.97±0.29^{g}	74.58±0.10^{d}	80.23±0.0^{a}	71.51±0.6^{f}	75.97±0.0^{c}	73.56±0.3^{e}	77.10±0.9^{b}
RGR	469.27±9.8c	492.69±1.6^{b}	516.71±3.^{a}	475.30±03^{c}	493.39±0.^{b}	489.08±2.8 ^{b}	498.05±20^{b}
ADG (g/ fish/day)	0.79±0.00^{f}	0.83±0.00^{d}	0.89±0.00^{a}	0.79±0.00^{f}	0.84±0.00^{c}	0.82±0.00^{e}	0.86±0.00^{b}
SGR	1.37±0.09	1.48±0.00	1.51±0.01	1.45±0.00	1.48±0.00	1.47±0.00	1.48±0.00
SR	93.33±0.00	97.78±2.22	97.78±2.22	95.56±2.22	97.78±2.22	95.56±2.22	97.78±2.22

Means in the same row having different upper script letters are significantly different ($P<0.05$).
IW: Initial weight, FW: Final weight, WG: Weight gain, RGR: Relative growth rate, ADG: Average daily gain, SGR: specific growth rate, SR: Survival (%).

Feed efficiency parameters

As shown in Table (7), the indicators of feed consumption in the Nile tilapia fed the experimental diets varied significantly ($P< 0.05$). Fish fed the T3 diet (BFAM-25%) achieved the highest protein productive value (PPV), feed efficiency ratio (FER), protein efficiency ratio (PER), and the best feed conversion ratio (FCR). These were followed, in order, by fish fed diets T7, T5, T2, T6, and T4. Overall, fish receiving the T3 diet outperformed those fed T7 and T5 in terms of feed efficiency and growth performance across all replacement levels. Compared to all other treatments, the T3 group showed significantly ($P< 0.05$) greater values in PPV, FER, PER, and FCR.

Table 7. Feed utilization of the Nile tilapia under the influence of different fermentation levels of Azolla

Item	T1	T2	T3	T4	T5	T6	T7

(FI) (g/ fish)	105.51±0.5^{e}	109.01±0.1^{c}	113.30±0.06^{a}	105.66±0.1^{e}	110.27±0.62^{b}	107.97±0.28^{d}	110.50±0.17^{b}
(PI) (g/ fish)	32.16±0.17^{d}	33.29±0.04^{c}	34.50±0.02^{a}	32.23±0.03^{d}	33.69±0.19^{b}	32.97±0.09^{c}	33.68±0.05^{b}
(FCR)	1.49±0.01^{a}	1.46±0.00^{bc}	1.41±0.00^{e}	1.48±0.00^{a}	1.45±0.01^{c}	1.47±0.00^{b}	1.43±0.00^{d}
(PER)	2.21±0.01^{f}	2.24±0.00^{cd}	2.33±0.00^{a}	2.22±0.00^{ef}	2.26±0.01^{c}	2.23±0.01^{de}	2.29±0.00^{b}
(FER)	67.27±0.33^{f}	68.42±0.08^{cd}	70.81±0.03^{a}	67.68±0.01^{ef}	68.90±0.32^{c}	68.13±0.17^{de}	69.77±0.08^{b}
(PPV)	52.02±0.04^{e}	53.34±0.29^{cd}	56.97±0.19^{a}	52.11±0.34^{e}	53.78±0.68^{c}	52.43±0.10^{de}	55.31±0.28^{b}

Means in the same row having different upper script letters are significantly different (P<0.05).
FI: Feed intake, PI: Protein intake, FCR: Feed Conversion Ratio, PER: Protein Efficiency Ratio, FER: Feed Efficiency Ratio
PPV: Protein Productive Value

Anti-nutritional factors before and after fermentation

Anti-nutritional factors, as shown in Table (2), were significantly reduced by fermentation. The level of trypsin inhibitor decreased from 1.84 to 1.47mg/ g in BFAM, 1.56mg/ g in SFAM, and 1.51mg/ g in the mix (SFAM + BFAM). Phytates (phytic acid %) were also reduced from 0.16% in raw azolla to 0.14% in BFAM, and 0.15% in both the mix (SFAM + BFAM) and SFAM. Tannins were reduced from 0.44% to non-detectable levels. Oxalate content dropped significantly from 1.67 to 0.19% in BFAM, 0.20% in SFAM, and 0.20% in the mix.

Apparent digestibility coefficient and total digestible nutrient (TDN)

As shown in Table (8), there were significant differences (P< 0.05) among treatments for the apparent digestibility coefficients of dry matter, organic matter, crude protein, crude fiber, ether extract, nitrogen-free extract, and ash. The T3 group exhibited the highest improvement, with a 25% increase in both digestibility coefficient and overall nutrient digestibility. This was followed in order by T7 (Mix B+S 25%), T5 (SFAM 25%), T2 (BFAM 10%), T6 (Mix B+S 10%), and T4 (SFAM 10%).

Table 8. The apparent digestibility co-efficient and total digestible nutrient (TDN) of the Nile tilapia under the influence of different fermentation levels of Azolla

Item	T1	T2	T3	T4	T5	T6	T7
DM	66.68±0.43^{c}	69.10±0.35^{ab}	70.25±0.42^{a}	68.60±0.59^{b}	69.36±0.36^{ab}	68.88±0.41^{ab}	69.61±0.43^{ab}
OM	68.10±0.4^{d}	70.54±0.25^{bc}	71.92±0.34^{a}	70.15±0.33^{c}	71.03±0.29^{abc}	70.15±0.26^{c}	71.54±0.36^{ab}
CP	68.07±0.56^{c}	69.71±0.58^{bc}	72.10±0.61^{a}	69.23±0.66^{bc}	70.88±0.51^{ab}	69.59±0.63^{bc}	71.58±0.41^{a}
CF	60.58±0.56^{d}	62.58±0.64^{c}	65.30±0.53^{a}	61.58±0.59^{cd}	63.28±0.46^{bc}	61.93±0.41^{cd}	64.82±0.50^{ab}
EE	65.92±0.20^{d}	67.75±0.17^{c}	69.82±0.12^{a}	67.68±0.16^{c}	69.09±0.12^{b}	67.72±0.13^{c}	69.20±0.19^{b}
NFE	73.620.31^{d}	76.99±0.24^{bc}	78.09±0.27^{a}	76.24±0.33^{c}	77.18±0.31^{abc}	76.51±0.27^{c}	77.54±0.29^{ab}
Ash	64.05±0.24^{d}	66.60±0.22^{b}	67.72±0.19^{a}	65.65±0.17^{c}	67.36±0.16^{a}	66.07±0.19^{bc}	67.40±0.19^{a}
TDN	68.80±0.37^{c}	70.21±0.18^{b}	71.70±0.12^{a}	68.89±0.34^{c}	70.30±0.31^{b}	69.53±0.11b^{c}	71.17±0.28^{a}

Means in the same row having different upper script letters are significantly different (P<0.05). DM: Dry matter, OM: organic matter, CP: crude protein, CF: crude fiber, Either extract, NFE: N-free extract, TDN: Total Digestible Nutrient

Body chemical composition

The protein content increased significantly (P< 0.05) by the end of the experiment; however, as shown in Table (9), there were no significant changes in the fish's proximate composition in terms of dry matter or ash. The total body crude lipid content did not differ significantly from the control, although treatment T3 showed the most effective reduction.

Table 9. Body chemical composition of the Nile tilapia under the influence of different fermentation levels of Azolla (% on DM basis)

Treatments	Dry matter	crud protein	crude fat	Ash
Initial	22.40±0.10	50.30±0.09	18.71±0.07	22.18±0.07
T1	28.28±0.08	53.58±0.09^{c}	22.26±0.14	14.98±0.14
T2	29.05±0.15	54.95±0.14^{c}	21.13±0.14	15.59±0.14
T3	29.21±0.14	58.56±0.14^{a}	21.91±0.15	15.63±0.12
T4	28.80±0.12	53.93±0.18^{c}	22.19±0.14	15.44±0.14
T5	29.06±0.14	55.78±0.14^{c}	22.08±0.12	15.59±0.11
T6	28.83±0.10	54.11±0.10^{c}	22.15±0.15	15.47±0.16
T7	29.10±0.08	56.92±0.14^{b}	21.98±0.14	15.63±0.13
p-values	0.589	0.0021	0.958	0.579

Means in the same row having different upper script letters are significantly different (P<0.05).

Economical evaluation

Table (10) presents the financial efficiency calculations of the experimental diets, based on feed cost, cost per kilogram of weight gain, and the relative percentage compared to the control group. The lowest feed cost per kilogram of weight gain (35.53 LE) and the lowest relative percentage (84.92%) were recorded in the group fed T3 (BFAM-25%). This was followed, in order, by T7 (Mix 25%), T5 (SFAM-25%), T2 (BFAM-10%), T6 (Mix 10%), and T4 (SFAM-10%).

Table 10. Economical evaluation of the Nile tilapia under the influence of different fermentation levels of Azolla

Parameter	CON T1	T2	T3	T4	T5	T6	T7
Cost/ Kg Diet (L.E)	28.08	26.72	24.95	26.82	25.20	26.77	25.08
Feed intake (Kg)	0.105	0.109	0.113	0.105	0.110	0.107	0.110
Feed intake cost per Kg fresh (LE)	2.95	2.91	2.81	2.81	2.77	2.82	2.76
Relative to feed cost %	100	98.64	95.25	95.25	93.89	96.94	93.55
FCR	1.49	1.46	1.41	1.48	1.45	1.47	1.43
Feed cost/ 1kg gain (EGP)	41.83	39.16	35.53	39.69	36.54	39.42	36.03
Relative % of feed cost/ kg fish	100	93.59	84.92	94.86	87.33	94.22	86.11

DISCUSSION

The study's findings showed that at 72 hours, the chemical composition of Azolla meal (AM) improved following both SSF treatments by *S. cerevisiae*, *B. subtilis*, and the mix (B+S) (Table 2). In BFAM, every nutritional metric was noticeably greater than the mix (B+S) and SFAM treatments. The results in the study are shown in Table (2) and showed that after solid-state fermentation (SSF) for 72 hours by *B. subtilis* E20, *S. cerevisiae*, and mix (SFAM + BFAM), the crude protein was higher than in raw Azolla meal. After 72 hours, there were consistent changes in the amounts of crude protein, crude lipid, fiber, and ash in BFAM, SFAM, and the blend (SFAM + BFAM). Crude fiber was also lower than in raw Azolla meal.

In the current study, protein increased from 29.20 in raw Azolla to 35.80 in BFAM, 34.01 in mix (SFAM + BFAM), and 33.50 in SFAM. While NDF% diminished from 35.56 to 30.60 in BFAM, 31.30 in mix (SFAM + BFAM), and 31.50 in SFAM. The addition of microbial protein during the biological process of fermentation, as well as increases in the total amount of accessible amino acids in BFAM, SFAM, and mix (SFAM + BFAM), may be responsible for the study's notable rise in crude protein content of AM following hydrolysis **(Hassaan *et al.*, 2015)**. These findings concur with those of **Ismail *et al.* (2021)**, who discovered that fermentation had an impact on *A. pinnata*'s CP and fiber content. While NDF% decreased from 35.55 to 31.43, CP% effectively increased from 21.5 to 22.3%. Additionally, following hydrolysis with rumen digestive fluid, *Azolla pinnata* meal showed a satisfactory increase in protein content from 33.83 to 34.03 while reducing crude fiber by up to 7.80%. Similarly, the gross energy of BSFA enhanced by 1.54%. According to Table (3), the total hydrolyzed essential and amino acid content of BFAM, Mix (B+S), and SFAM was more than that of raw Azolla meal. Additionally, BFAM had higher levels of methionine and lysine than Mix (B+S) and SFAM.

Ismail *et al.* (2021) stated that protease enzymes produced following fermentation contributed to the improvement of the amino acid profile. In addition, fermentation raised the quantities of the amino acids valine, arginine, methionine, and threonine in comparison to crude *A. pinnata*. Our investigation found that the fermentative activity of SSF by *S. cerevisiae* or *B. subtilis* caused the fiber content to decrease from 35.56 to 30.60, 31.50, and 31.30. The fibrinolytic enzymes (cellulase, xylanase, amylases, hemicellulose, β-glycosidase, pectinases, and α-galactosidase) generated during fermentation may be responsible for the reduction in crude fiber content, as stated by **(Hassaan *et al.*, 2017)**.

The anti-nutritional components in Table (4) were significantly reduced by fermentation. In the current study, SSF generated by *B. subtilis* or *S. cerevisiae* had the greatest effect on reducing the quantity of ANFs in Azolla meal. These results could most

likely also be the result of various enzymes being secreted throughout the fermentation process. These findings corroborate those of **Cruz *et al.* (2011)**. In fermented aquatic macrophytes, they reported that the levels of phytates (less than 1.5 g/kg phytic acid), tannins (not detectable), oxalate (almost nonexistent), and trypsin inhibitor decreased from 1.86 to 1.37mg/ g. Additionally, following SSF treatments, **Hassaan *et al.* (2017)** discovered that ANFs in soybean meal and Jatropha seed meal dramatically decreased. Furthermore, SSF employing several fungal species significantly reduced the amounts of phytate, lectin, trypsin inhibitors, and saponin **(Belewu & Sam, 2010)**.

Based on these results, we may conclude that Azolla meal fermentation enhances the product's nutritional value as a feed ingredient in addition to its physiochemical quality.

Additionally, growth parameters—when compared to the Nile tilapia fed the control diet and other experimental diets—were significantly ($P< 0.05$) greater in T3 (BFAM-25). Our increasing results may be explained by **Liu *et al.* (2012)**, who found that the exoenzymes (lipases and proteases) secreted by *B. subtilis* improve growth by promoting nutrient digestibility in grouper fish (*Epinephelus coioides*) fed a diet containing *B. subtilis*. Additionally, several *B. subtilis* species produced vitamins and amino acids, which are necessary nutrients. This consequently enhances feed intake and growth.

Our results agree with those of **Laila *et al.* (2021)**, who used raw Azolla (RA) and fermented Azolla (FA) to feed the monosex Nile tilapia fingerlings (*Oreochromis niloticus*) at RA levels of 10% (10RA), 20% (20RA), 30% (30RA), and 40%. Fish fed diets with 20RA, 20FA, and 30FA outperformed the control group regarding growth performance. Fish fed with FA had a higher survival percentage following the feeding trial (74%). Similarly, fermented Azolla was employed at different levels in the diet of tilapia fry **(Hundare *et al.*, 2018)**, including 0, 10, 20, and 30% fermented Azolla. Length gain, weight gain, specific growth rate, average daily growth, and survival were all significantly ($P< 0.05$) greater in the 20% fermented Azolla treatment. The effects of a 25% *Azolla pinnata* diet on the Thai silver barb development were examined by **Das *et al.* (2018)**. They found that average growth rate, net output rate, and specific growth rate did not significantly differ between the experimental and control groups. All groups showed survival rates above 97% with no appreciable differences, indicating that fermented Azolla (FA) had no effect on fish mortality.

Additionally, the feed utilization indices of the monosex Nile tilapia fingerlings given the experimental diets varied considerably ($P< 0.05$); fish fed at T3 had the greatest PPV, FER, PER, and best FCR, followed by T7, T5, T2, T6, and T4. Fish fed a diet containing T3 generally outperformed those fed diets containing T7 and T5 regarding growth performance and feed utilization across all replacement levels. Compared to other treatments, the T3 (BFAM-25) diet group showed considerably ($P< 0.05$) greater PPV, FER, PER, and the best FCR. These favorable outcomes may be explained by the more

easily digested protein in Azolla that has been fermented by *Bacillus subtilis*. It promotes somatic growth and enhances the fish's capacity to take up and process nutrients.

Our findings concur with those of **Hundare *et al.* (2018)**, who found that after 60 days of rearing, *O. niloticus* fed 20–30% FA improved WG, SGR, and FCR. Dry Azolla was suggested at a 25% level in the diet of *Tilapia zillii* by **Abdel-Tawwab (2008)**, with no effect on growth or feed consumption. Furthermore, *Tilapia zillii*'s feed intake, protein productive value (PPV), FCR, and protein efficiency ratio (PER) were enhanced when 25% of *A. pinnata* was added. Thus, Azolla can be regarded as a nutrient-dense feed source for herbivorous or omnivorous fish **(Hossiny *et al.*, 2008)**.

By enhancing digestion, synthesizing nutrients, generating growth-promoting chemicals, modifying gut microbiota, and lowering pathogen competition, fermenting plant components with *Lactobacillus* spp. enhances fish development and feed utilization **(Liu *et al.*, 2023)**. Other fish species, such as *Barbonymus gonionotus* **(Das *et al.*, 2018)**, *O. niloticus* **(Yousif *et al.*, 2019)**, *Oncorhynchus mykiss* **(Davies *et al.*, 2021)**, *Clarias gariepinus* **(Irabor *et al.*, 2022)**, and *Labeo rohita* **(Ferdous *et al.*, 2023)** have also shown encouraging outcomes when supplemented with fermented plant protein.

In the current study, the ability to digest total nutrients and digestibility coefficient increased in T3 (B25%), followed sequentially by T7 (Mix B+S 25%), T5 (S25%), T2 (B10%), T6 (Mix B+S 10%), and T4 (S10%). These findings match those of **Ismail *et al.* (2021)**. One of the most crucial elements affecting how well fish use their feed is the characterization of digestive enzymes, which gives insight into the fish's ability to hydrolyze lipid, protein, and carbohydrate components of feed. For the best amylase and protease activity, the ideal BSFA levels were 25.66 and 22.85%, respectively. Similarly, **Magouz *et al.* (2020)** found that the activity of digestive enzymes (lipase, amylase, and protease) enabled fish to easily digest Azolla at 20% inclusion without reducing feed intake.

The body chemical composition of the Nile tilapia changed significantly as the amount of BFAM, Mix (B+S), and SFAM in their diet increased, according to the tilapia's chemical body composition profile. The higher protein content in tilapia fed with fermented Azolla by *Bacillus subtilis* in T3 could be connected to the higher dietary essential amino acids in the diets, which stimulated protein synthesis—followed by T7, T5, T2, T6, and T4. Fermentation could improve protein digestibility and bioavailability, aiding in protein production and absorption. The current study found no significant variations in whole-body lipid content ($P > 0.05$), which indicates a decrease in lipid accumulation in whole-body fish.

The latest study's ash content increased when dietary levels of BFAM, Mix (B+S), and SFAM increased. This suggests that diets include adequate amounts of dietary minerals

and that they are absorbed. A similar pattern of ash concentration in whole-body fish was also demonstrated by **Zhang *et al.* (2012)**. Our findings concur with those of **Nancy and Amalarani (2016)**, who found that the Nile tilapia given Azolla meal had higher body weights, higher protein content, and lower lipid content. Increased protein content, improved amino acid profiles, and decreased amounts of cellulose, crude fiber, non-starch polysaccharides, and ANFs are probably responsible for the growth performance gains.

Fermented Azolla paste, at around 20%, can be used to partially replace fish meal, according to **Olukomaiya *et al.* (2019)**. For the Nile tilapia, **Utomo and Ekasari (2011)** discovered that the best outcomes are obtained when Azolla meal is fermented for two days. Integration levels for other species, including *Tilapia zillii*, can reach 25%, as reported by **Abdel-Tawwab (2008)**. Fish species such as *Tor grypus* (10%) and *Labeo fimbriatus* (40%) can also use Azolla as a protein substitute **(Gangadhar *et al.*, 2015)**.

CONCLUSION

Under these experimental conditions, fermented Azolla was found to effectively replace up to 25% of soybean meal protein in terms of economic evaluation, feed utilization, and growth performance of the monosex Nile tilapia fingerlings.

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