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Utilization of Tissue-Cultured *Kappaphycus alvarezii* Seaweed as a Raw Material for Fish Feed via Fermentation Technology

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ABSTRACT

This study aimed to evaluate the effects of incorporating fermented *Kappaphycus alvarezii* powder, derived from tissue-cultured biomass and processed using various fermentative agents, into fish feed formulations. The research investigates its impact on feed protein quality, amino acid profile, as well as fish growth performance and survival rate. The research employed an experimental method comprising two stages. Stage 1: *K. alvarezii* seaweed flour fermentation was carried out using three fermenter treatments: EM4 at 2mL/ 100g, yeast at 1.5%, and a combination of EM4 + yeast (1 mL + 0.75% per 100g). The resulting fermented seaweed flour was then used to produce fish feed with four different concentration treatments: 4%, 8%, and 12%, in addition to a control feed for comparison. The parameters analyzed included crude protein content and amino acid profile of feeds. Data were analyzed descriptively. Stage 2: A biological feed trial using the stage 1 feeds was conducted on the Nile tilapia (*Oreochromis niloticus*) over a 40-day culture period. The experimental design followed a factorial completely randomized design (CRD) with two factors: fermenter type (F) at three levels (EM4, yeast, and EM4 + yeast), and concentration of fermented *K. alvarezii* flour in the feed (K) at three levels (4%, 8%, and 12%), each replicated three times, resulting in a total of 27 treatment combinations plus a control feed treatment. The measured parameters included growth performance, feed conversion ratio (FCR), feed utilization efficiency (FUE), and survival rate. Data were analyzed using ANOVA followed by Duncan's multiple range test. The results showed that adding 12% *K. alvarezii* flour fermented with EM4 significantly improved feed protein content (50.1%) and total amino acid levels (35.71%). A significant interaction between fermenter type and concentration was observed for fish growth, while FCR and FUE were significantly influenced by fermenter type alone. The best treatment was observed in fish feed with diets containing 12% EM4 fermented *K. alvarezii* flour.

INTRODUCTION

Recent advancements in seaweed cultivation—particularly through the use of tissue-cultured *Kappaphycus alvarezii* have led to a significant increase in biomass production. This improvement is attributed to several advantages of tissue-cultured

propagules, including enhanced disease resistance, genetic uniformity with the parent stock, the ability to mass-produce under space-limited conditions, consistently high quality, and year-round availability **(Reddy *et al.*, 2003; Reddy *et al.*, 2009)**. These characteristics enable a more reliable and sustainable supply of seaweed biomass.

The expansion of seaweed production through tissue culture over the past few decades has highlighted its potential for broad applications across various sectors. One such promising area is aquaculture, where seaweed serves as an alternative plant-based raw material for fish feed, owing to its unique nutritional profile **(Mwendwa *et al.*, 2023)**. This potential is further supported by previous studies demonstrating that seaweeds are rich in secondary metabolites, including vitamins **(Pinto *et al.*, 2003)**, carotenoids, phenolics **(Dethier *et al.*, 2005)**, fatty acids **(Alamsjah *et al.*, 2007; Wang *et al.*, 2008)**, functional carbohydrates **(Karsten *et al.*, 2008)**, mycosporine-like amino acids **(Carreto & Carignan, 2011)**, peptides **(Harnedy & FitzGerald, 2011)**, functional proteins **(Cruces *et al.*, 2012)**, and other secondary metabolites **(Oliveira *et al.*, 2013; Svensson *et al.*, 2013)**. These bioactive compounds have beneficial effects when included in animal diets **(Christaki *et al.*, 2012; Rae *et al.*, 2012; Ibañez & Cifuentes, 2013; Garcia-Vaquero & Hayes, 2015)**. These findings suggest that seaweed may not only serve as a nutritional component in fish feed but may also enhance fish health.

One of the most widely cultivated red seaweed species propagated through tissue culture is *K. alvarezii* (syn. *Eucheuma cottonii*). This red macroalga is notable for its high nutritional value, with essential amino acids comprising nearly 50% of its total amino acid content. Additionally, red seaweeds are rich in aromatic amino acids like threonine, though they contain relatively low sulfur-containing amino acids like lysine. This indicates that red algae (Rhodophyta) can serve as complementary protein sources for human and animal nutrition **(Kumar & Kaladharan, 2007; Xiren & Aminah, 2017; Lumbessy *et al.*, 2019)**. Findings by **Lumbessy *et al.* (2025)** indicate that extracts from tissue-cultured *K. alvarezii* propagules contain chlorophyll a, a pigment known for its beneficial effects on health. In addition, seaweeds tend to have a relatively higher total fiber content than terrestrial plant-based ingredients such as tubers, fruits, cereals, and legumes.

The high fiber content in seaweed is largely due to its carbohydrate composition, which may range from 33–50% dry weight **(Rupérez, 2002)**. The type and amount of fiber vary among seaweed groups and are influenced by environmental conditions where the seaweed is cultivated. Therefore, to utilize seaweed effectively in fish feed, technological interventions are required to reduce its fiber content—one such method being fermentation. According to **Andriani *et al.* (2021)**, fermentation is a process in which complex chemical structures are broken down into simpler compounds through enzymatic activity derived from microbial secondary metabolites. This bioconversion can enhance the nutritional quality of feed ingredients by reducing crude fiber content and increasing crude protein content, thus improving feed digestibility **(Sandra *et al.*, 2020)**.

Previous studies have shown that fermentation can enhance the protein content of seaweed. **Brindo and Felix (2014)** reported an increase in the protein content of *Padina*

tetrastomatica fermented using *Lactobacillus* sp. and *Saccharomyces cerevisiae*. Similarly, **Choi *et al.* (2018)** found that fermentation of *Undaria pinnatifida* with *Bacillus subtilis* and *Sargassum fusiforme* with *Aspergillus flavus* also increased protein levels. Furthermore, **Suraiya *et al.* (2018)** demonstrated significant increases ($P< 0.05$) in protein content in *Undaria pinnatifida* and *Saccharina japonica* when fermented with *Monascus purpureus* or *M. kaoliang*.

Despite these promising findings, there remains limited information on the potential of fermented seaweed meal to enhance protein quality in aquafeeds, particularly with respect to its amino acid profile. Therefore, this study was conducted to evaluate the effects of incorporating fermented seaweed powder—derived from tissue-cultured *K. alvarezii*—using various fermentative agents into fish feed formulations, with particular focus on feed protein quality, especially amino acid profile, as well as fish growth performance and survival rate.

MATERIALS AND METHODS

This research was conducted from June 2021 to December 2021 at the Laboratory of Fish Nutrition, University of Mataram. The materials used in this study included *K. alvarezii*, Effective microorganism (EM4), yeast, fish meal, soybean meal, corn flour, wheat flour, fish oil, corn oil, premix and nile tilapia. This study employed an experimental method consisting of two stages.

Stage 1: Fermentation and feed formulation

The first stage involved the fermentation of *K. alvarezii* seaweed flour using three different fermenter treatments to serve as raw materials for fish feed: **(1)** EM4 at 2mL per 100g of seaweed flour, **(2)** yeast (1.5%), and **(3)** a combination of EM4 (1mL) and yeast (0.75%) per 100g of seaweed flour. Subsequently, fish feed was formulated by fortifying it with fermented *K. alvarezii* flour at four concentration levels: 0% (control), 4%, 8%, and 12%. These were combined with the three fermenter types, resulting in a total of 10 experimental feed treatments:

A. Control feed (without *K. alvarezii* flour)
B. Fortification of *K. alvarezii* flour using a 4% EM4 fermenter
C. Fortification of *K. alvarezii* flour using an 8% EM4 fermenter
D. Fortification of *K. alvarezii* flour using a 12% EM4 fermenter
E. Fortification of *K. alvarezii* flour using a 4% yeast fermenter
F. Fortification of *K. alvarezii* flour using an 8% yeast fermenter
G. Fortification of *K. alvarezii* flour using a 12% yeast fermenter
H. Fortification of *K. alvarezii* flour with a 4% fermenter consisting of EM4 and yeast
I. Fortification of *K. alvarezii* flour with an 8% fermenter consisting of EM4 and yeast
J. Fortification of *K. alvarezii* flour with a 12% fermenter consisting of EM4 and yeast

Stage 2: Feeding trial

A biological feeding trial was conducted to evaluate the effects of the formulated feeds (from Stage 1) on the performance of the Nile tilapia (*Oreochromis niloticus*) cultured for 40 days. The experiment was arranged in a factorial Completely Randomized Design (CRD) with two factors:

1. Fermenter types (F)
   - F1: EM4
   - F2: Yeast
   - F3: EM4 + Yeast
2. Concentrations of fermented *K. alvarezii* flour added to the feed (K)
   - K1: 4%
   - K2: 8%
   - K3: 12%

Each of the nine treatment combinations (3 fermenters × 3 concentrations) was replicated three times, yielding a total of 27 treatments. One additional treatment, the control feed (0% seaweed), was included, resulting in 28 experimental units.

1. Seaweed fermentation procedure

The fresh *K. alvarezii* used in this study was cultivated in Teluk Ekas, East Lombok, for 45 days using tissue-cultured biomass. The fresh seaweed was then sun-dried for approximately 3 days. Once dried, the seaweed was cleaned to remove residual salt and impurities, ground into powder, and sieved through an 80-mesh screen to obtain a fine flour texture. The seaweed flour was then subjected to fermentation based on the method described by **Aslamyah *et al*. (2017)** using the following fermenter treatments:

- EM4 Fermenter: 2mL of EM4 was dissolved in 20mL of molasses and evenly sprayed onto 100g of seaweed flour.
- Yeast Fermenter: 1.5g (1.5%) of yeast was dissolved in 20mL of molasses and evenly sprayed onto 100g of seaweed flour.
- EM4 + Yeast Fermenter: 1mL of EM4 and 0.75g of yeast were co-dissolved in 20mL molasses and evenly sprayed onto 100g of seaweed flour.

After treatment, the flour was placed in airtight dark containers and fermented anaerobically for 72 hours. Post-fermentation, the seaweed flour was steamed in boiling water for 1–2 minutes to inactivate fermenting microorganisms. The final fermented products were then subjected to proximate composition analysis.

2. Feed formulation

Fermented *K. alvarezii* seaweed flour was incorporated into fish feed formulations at 0%, 4%, 8%, and 12% for each fermenter treatment (Table 1). All feed ingredients, already in powder form, were weighed according to the predetermined formulation and mixed thoroughly, starting with ingredients in smaller quantities and gradually

incorporating those in larger amounts. The uniformly mixed feed was then steamed for 20 minutes and pelletized using a manual pelletizer. The resulting pellets were sun-dried until fully dehydrated and ready for use.

3. Protein and amino acid profile analysis

Dried feed samples were first analyzed for crude protein content using the Kjeldahl method **(AOAC, 2005)**. Amino acid profiles were determined using High-Performance Liquid Chromatography (HPLC, Shimadzu LC-6A), following the procedure by **AOAC (2005)** and **Herawati *et al.* (2017)**. A 1g feed sample was hydrolyzed in 4mL of 6 N HCl under reflux for 24 hours at 110°C. The hydrolysate was cooled, neutralized to pH 7 using 6 N NaOH, and filtered through a 0.2µm Whatman filter paper. A 10µL aliquot of the filtered sample was mixed with 300µL of OPA (o-phthalaldehyde) reagent. The sample was left to stand for 1 minute to allow the derivatization process to complete. Subsequently, 20µL of the sample was injected into the HPLC column and was left until all amino acids were separated. Sixteen standard amino acids were used as references: aspartic acid, glutamic acid, asparagine, serine, histidine, glycine, threonine, arginine, alanine, tyrosine, methionine, valine, phenylalanine, isoleucine, leucine, and lysine. Quantification was performed using calibration curves created for each amino acid. The amino acid score was calculated using the following formula:

$$Amino\ Acid\ Score = \frac{Essential\ Amino\ Acid\ Content\ of\ Sample}{Reference\ patterns\ of\ FAO/WHO}$$

Table 1. Feed formulation used in the study (per 200g)

Ingredients	Feed (g)			
	0	**4%**	**8%**	**12%**
Fish meal	86	82	78	74
Fermented *K. alvarezii* flour	0	8	16	24
Soybean meal	60	56	52	48
Corn flour	26	26	26	26
Wheat flour	13	13	13	13
Fish oil	7	7	7	7
Corn oil	5	5	5	5
Premix	3	3	3	3
TOTAL	200	200	200	200

4. Biological feed trial on the Nile tilapia (*O. niloticus*)

The test fish used in this study were the healthy juvenile Nile tilapia (*O. niloticus*) measuring 4–6cm in length. Prior to the experiment, the fish were acclimatized for one week. Fish were then transferred to 30L containers filled with 20L of water, each representing one of the 10 experimental feed treatments formulated previously. Each container housed 10 fish, with a stocking density of 1 fish per 2 liters.

The fish were cultured for 40 days and fed at 3% of their total body weight per day, divided into three feeding times: morning, noon, and afternoon. Daily siphoning was performed to maintain optimal water quality throughout the rearing period. The following performance parameters were monitored: 1) growth, including absolute weight gain, absolute length gain, and specific growth rate (SGR), 2) feed utilization efficiency (FUE), 3) feed conversion ratio (FCR), and 4) survival rate (SR) as well as water quality parameters, including temperature, pH, and dissolved oxygen (DO).

5. Data analysis

Protein content and amino acid profile data were analyzed descriptively by comparing the results across treatments and referencing relevant literature. The findings are presented in narrative and tabular forms. Biological performance data were subjected to analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) to determine significant differences among treatment means.

RESULTS

1. Crude protein content of fish feed fortified with fermented *K. alvarezii* seaweed flour

The protein content of the fish feed fortified with *K. alvarezii* powder, derived from tissue-cultured biomass and processed using various fermentative agents, exhibited varying values (Table 2).

Table 2. Crude protein composition of fish feed fortified with fermented *K. alvarezii* seaweed powder cultivated through tissue culture

Test	Treatments (% b/b)									
	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
Protein (%) (dry weight)	46	47.5	48.9	50.1	44.3	42.1	41.1	44.5	45.8	48.4

As shown in Table (2), the protein levels in the experimental feeds varied considerably. However, all treatments produced feed protein levels that met the Indonesian National Standard (SNI 01-7242), which requires a minimum protein content of 25% for

the Nile tilapia grow-out feeds. Among the treatments, the inclusion of *K. alvarezii* flour fermented with EM4 consistently yielded higher protein content across all concentration levels compared to the control and other fermenter treatments, indicating a positive impact of EM4 fermentation on the nutritional enhancement of seaweed-based feed.

2. Amino acid composition of fish feed

The amino acid composition of the fish feed fortified with fermented *K. alvarezii* seaweed powder cultivated through tissue culture varied depending on the type of fermentative agent used (Table 3). HPLC analysis revealed that all fortified feed treatments contained a total of 15 amino acids: 7 essential amino acids (EAAs) and 8 non-essential amino acids (NEAAs). The identified essential amino acids were Histidine, Leucine, Threonine, Methionine, Valine, Phenylalanine, and Isoleucine. The total essential amino acid (EAA) content was higher in nearly all treatments containing fermented *K. alvarezii* compared to the control (A); however, the magnitude of increase varied with the type of fermenter and the concentration of seaweed flour used.

Table 3. Amino acid composition of fish feed fortified with fermented *K. alvarezii* seaweed powder cultivated through tissue culture

No.	Amino Acids	Treatments (% b/b)									
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
	Essential Amino Acids (EAA)										
1	Histidine	1.46	1.64	1.75	1.66	1.63	1.67	1.52	1.35	1.26	1.25
2	Threonine	5.50	5.83	6.08	6.06	5.90	5.77	5.79	5.32	5.49	5.35
3	Methionine	3.31	3.32	3.47	3.55	3.06	3.46	3.28	3.07	3.13	3.17
4	Valine	7.11	7.20	7.27	7.35	7.23	7.40	7.36	7.09	7.24	7.38
5	Phenylalanine	3.37	3.36	3.36	3.34	3.35	3.40	3.37	3.38	3.36	3.34
6	Isoleucine	5.37	5.52	5.55	5.59	5.60	5.70	5.80	5.56	5.52	5.79
7	Leucine	7.85	8.15	8.12	8.16	8.15	8.30	8.28	8.03	8.03	8.11
	Total EAA	**33.97**	**35.02**	**35.60**	**35.71**	**34.92**	**35.70**	**35.40**	**33.80**	**34.03**	**34.39**
	% form Total AA	**34.31**	**35.25**	**35.84**	**35.97**	**35.20**	**36.12**	**35.75**	**34.42**	**34.45**	**34.93**
	Non Essential Amino Acids (EAA)										
8	Aspartic acid	13.70	13.52	13.54	13.57	13.49	13.60	13.67	13.21	13.71	13.71
9	Glutamic acid	17.36	17.11	16.89	16.73	17.00	16.56	16.27	17.40	17.18	16.95
10	Serine	6.06	5.87	5.91	5.78	5.92	5.76	5.65	5.99	5.83	5.72
11	Glycine	9.18	8.49	8.17	8.23	8.59	8.10	8.71	8.64	9.27	8.94
12	Arginine	5.65	5.42	5.51	5.45	5.52	5.18	5.35	5.47	5.37	5.22
13	Alanine	8.82	9.21	9.12	9.25	9.11	9.20	9.44	8.94	9.09	8.98
14	Tyrosine	2.54	2.69	2.63	2.62	2.63	2.61	2.57	2.61	2.43	2.53
15	Lysine	1.73	2.01	1.95	1.94	2.03	2.13	1.97	2.15	1.86	2.00
	Total Non- EAA	**65.04**	**64.32**	**63.72**	**63.57**	**64.29**	**63.14**	**63.63**	**64.41**	**64.47**	**64.05**
	% Form total Non-EAA	**65.69**	**64.75**	**64.16**	**64.03**	**64.80**	**63.88**	**64.25**	**65.58**	**65.55**	**65.07**
	Total AA	**99.01**	**99.34**	**99.32**	**99.28**	**99.21**	**98.84**	**99.03**	**98.21**	**98.77**	**98.44**
	EAA/Non EAA	**0.52**	**0.54**	**0.56**	**0.56**	**0.54**	**0.57**	**0.56**	**0.52**	**0.53**	**0.54**
	EAA/Total AA	**0.34**	**0.35**	**0.36**	**0.36**	**0.35**	**0.36**	**0.36**	**0.34**	**0.34**	**0.35**

3. *In vivo* evaluation of feed on the Nile tilapia (*O. niloticus*)

The *in vivo* feeding trial conducted over a 40-day rearing period of the Nile tilapia (*O. niloticus*) revealed that the interaction between the type of fermenter and the concentration of fermented *K. alvarezii* seaweed flour had a statistically significant effect on fish growth parameters ($P< 0.05$). The highest values for average absolute weight gain, absolute length gain, and specific growth rate (SGR) were observed in fish fed diets fortified with *K. alvarezii* fermented using EM4 across all concentrations. Absolute weight gain ranged from 7.66 to 11.74g (Fig. 1), absolute length gain ranged from 2.38 to 8.66cm (Fig. 2), and specific growth rate ranged from 3.24 to 3.33% per day (Fig. 3). All these values were superior to the control group across all growth parameters.

Further analysis using Duncan's Multiple Range Test indicated that the treatment with a 12% EM4 fermenter produced the highest absolute weight gain (11.74g). Meanwhile, the interaction of the 12% EM4 fermenter treatment demonstrated a comparable effectiveness to the interaction of the 12% yeast fermenter treatment in increasing the absolute length of the Nile tilapia. The EM4 fermenter treatment also resulted in consistently high specific growth rates, with no significant differences among the 4%, 8%, and 12% concentrations, demonstrating its effectiveness in promoting growth across all tested levels.

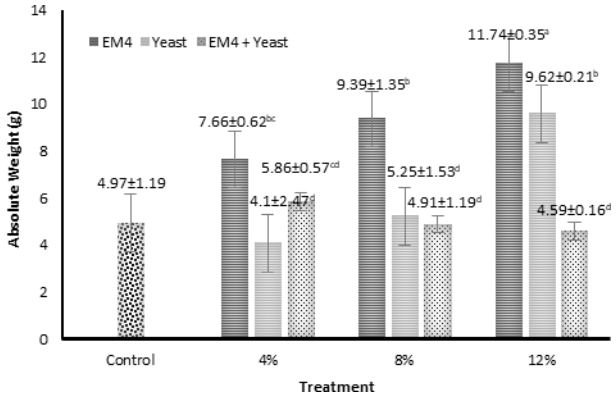

Fig. 1. Absolute weight of the Nile Tilapia (*O. niloticus*) during the rearing period

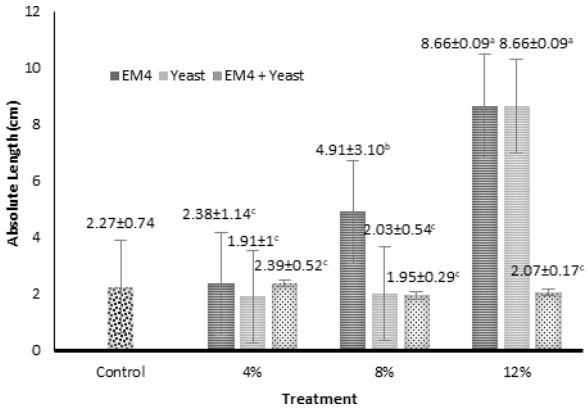

Fig. 2. Absolute length of the Nile tilapia (*O. niloticus*) during the rearing period

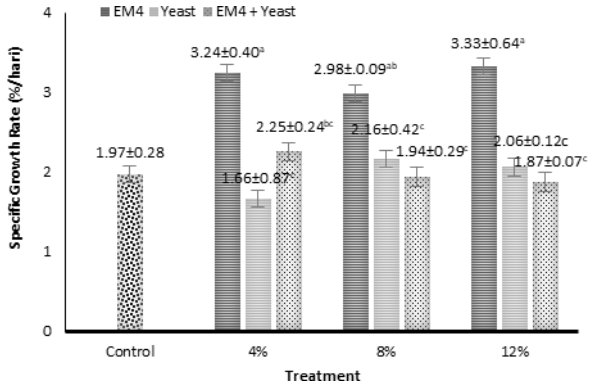

Fig. 3. Specific growth rate of the Nile tilapia (*O. niloticus*) during the rearing period

The *in vivo* feeding trial over 40 days indicated that feed conversion ratio (FCR) and feed utilization efficiency (FUE) in the Nile tilapia were significantly affected only by the type of fermenter used in the diet. The best results for both FCR and FUE were observed in the treatment group fed with feed fortified with *K. alvarezii* fermented using EM4, which achieved an FCR of 1.29 (Fig. 4) and a feed utilization efficiency of 78.43% (Fig. 5). These values were also superior when compared to the control treatment. Further analysis using Duncan's Multiple Range Test showed that the EM4 fermenter treatment significantly improved feed utilization efficiency in the Nile tilapia compared to other fermenter treatments. However, EM4 and yeast fermenters demonstrated comparable effectiveness in reducing the feed conversion ratio, suggesting both can contribute to improved feed efficiency, though EM4 had a slight advantage overall.

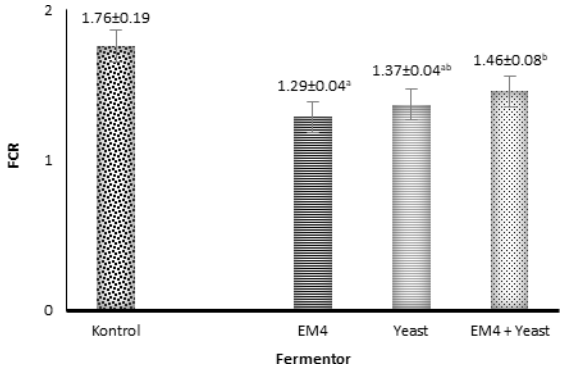

Fig 4. Feed conversion ratio (FCR) of the Nile tilapia (*O. niloticus*) during the rearing period

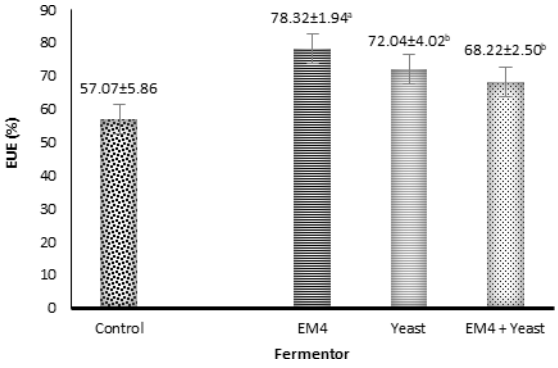

Fig 5. Feed utilization efficiency (FUE) of the Nile tilapia (*O. niloticus*) during the rearing period

The results of the *in vivo* feeding trial over a 40-day period indicated that the survival rate (SR) of the Nile tilapia was not significantly affected by the concentration of fermenters, the type of fermenters, or the interaction between fermenter type and concentration ($P > 0.05$). The average survival rate ranged from 50 to 73.3% (Fig. 6), with all treatment groups showed better survival than the control.

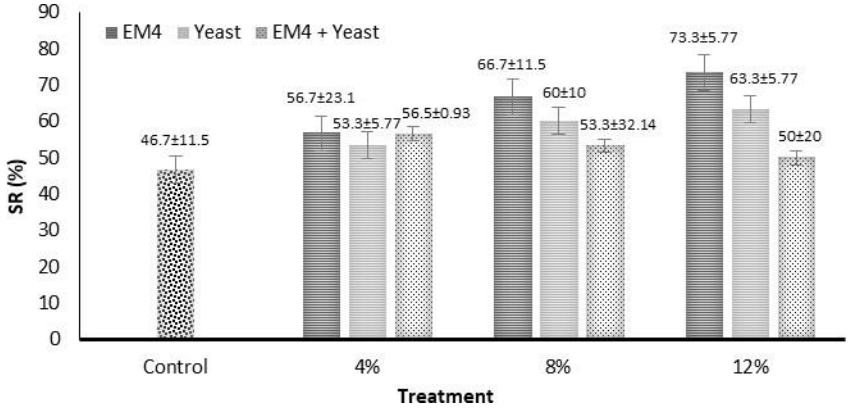

Fig. 6. Survival rate of the Nile tilapia (*O. niloticus*) during the rearing period

The measured water quality parameters across all treatments remained within the optimal range, supporting the growth and survival of the Nile tilapia (*O. niloticus*) throughout the rearing period (Table 4).

Table 4. Water quality parameters

Parameter	Unit	Observed Values	Source: **Fujaya *et al.* (2022)**
Temp.	˚C	27.8 - 28.8	27.4 – 29.6
pH	-	7.4 - 7.8	6.2 – 8
DO	mg/L	4.9 - 7.4	$\geq$ 3

DISCUSSION

Seaweed can serve as a promising alternative raw material in fish feed due to its high protein content, particularly rich in essential amino acids, compared to other plant-based protein sources such as soybean **(Mwendwa *et al.*, 2023)**. Protein is a critical nutrient in fish feed formulation, as fish utilize protein more efficiently than carbohydrates as an energy source **(Wilson, 2003)**. Several previous studies have reported that incorporating seaweed meal into fish feed can enhance fish growth performance. However, at higher inclusion levels, seaweed may adversely affect growth due to its high crude fiber content **(Nakagawa & Montgomery, 2007; Roy *et al.*, 2011; Ragaza *et al.*, 2012; Serrano *et al.*, 2015; Mwendwa *et al.*, 2023)**. According to **Felix and Brindo (2008)**, fermentation is one of the most effective methods to reduce fiber content and improve the protein quality of seaweed,

making it more suitable as a feed ingredient.

In line with this, the fermented *K. alvarezii* seaweed powder cultivated through tissue culture applied in the present study significantly enhanced the protein content of the experimental feeds, particularly those fortified with fermented seaweed using the EM4 fermenter across all treatment levels (Table 2). This improvement may be attributed to the nature of EM4, a liquid microbial consortium that acts as an optimal substrate for various fermentative microorganisms such as lactic acid bacteria, which facilitate rapid decomposition of organic materials **(Dewi *et al*., 2022)**. Among the dominant bacteria in EM4 is *Lactobacillus* sp., which can digest cellulose, starch, sugars, proteins, and fats—thereby supporting microbial colony growth. The proliferation of these microbial colonies leads to increased crude protein content, as microorganisms act as single-cell protein sources **(Naidu & Devi, 2005)**. This mechanism is supported by **Oboh and Elusiyan (2007)**, who reported that microbial biomass growth during fermentation, along with the secretion of extracellular enzymes and microbial proteins, contributes significantly to the rise in protein content. This assumption is in line with **Aslamyah *et al.* (2017)**, who stated that the increase in protein during the fermentation process results from the growth of the fermenter cells.

Additionally, EM4 contains fermentative fungi such as *Saccharomyces* sp. According to **Oboh (2006)**, *Saccharomyces* sp. can reduce fiber content by hydrolyzing complex carbohydrates like starch into simpler sugars (maltose and glucose), which serve as energy sources for fungal growth. The *K. alvarezii* seaweed used in this study is known for its high fiber content. Previous research by **Lumbessy *et al.* (2020)** reported that *E. cottonii* (syn. *K. alvarezii*) contains relatively low protein (approximately 3.11%) but high crude fiber (up to 15.22%), which may impair digestibility when directly used in fish feed. Furthermore, **Lumbessy *et al*. (2023)** found that fermentation using EM4 increased the protein content of *E. cottonii* (syn. *K. alvarezii*) meal to 9.04% while reducing its fiber content to 3.38%.

The presence of both *Lactobacillus* sp. and *Saccharomyces* sp. in the EM4 fermenter plays a crucial role in reducing the fiber content of *K. alvarezii* during fermentation, as both microorganisms can break down fibrous compounds. **Cahya *et al.* (2023)** stated that *Lactobacillus* sp. can digest fibrous materials such as cellulose, while *Saccharomyces* sp. can digest starch-based fibers. These microorganisms contribute significantly to increased protein content by breaking the bonds in fibrous organic matter, thus making nitrogen more available for microbial growth and the production of single-cell proteins during fermentation.

The higher the concentration of *K. alvarezii* seaweed meal fermented using EM4 added to the fish feed formulation, the higher the protein content of the experimental feed. The highest protein content in this study was observed in the feed fortified with 12% EM4-fermented seaweed meal (D), which reached 50.1% (Table 2). This result is consistent with the previous study by **Lumbessy *et al.* (2023)**, which showed that fish feed fortified with fermented *E. cottonii* (syn. *K. alvarezii)* seaweed using EM4 also yielded the highest protein

content at 49.5%. However, the EM4 concentration used in that study was lower, at 8%, compared to the concentration used in this study.

Meanwhile, the experimental feed with *K. alvarezii* seaweed fermented using yeast as the fermenter showed a lower protein content across all concentrations. This may be because yeast fermenters predominantly contain the *Rhizopus* sp. fungi, particularly *R. oryzae*. This fungus produces biomass with lower protein content than fermentation bacteria. This is supported by **Ritala *et al.* (2017)**, who demonstrated that *Rhizopus* sp. can function as a single-cell protein in fermentation but has limitations due to its slower growth rate and lower protein content than other microorganisms.

The better quantity of protein in the experimental feed with the addition of fermented seaweed flour using the EM4 fermenter is also in line with the improvement in its protein quality, specifically the amino acid content (Table 3). According to **Chen *et al.* (2010)**, the amino acid content is linearly dependent on protein content, meaning that lower protein content results in a lower amino acid profile. Therefore, factors influencing protein content also impact amino acid content. The findings of this study show that all experimental feeds with fortified fermented seaweed contain a higher total essential amino acid content than the control feed (A). This indicates that fermentation can enhance the amino acid profile of fish feed, likely due to the proteolytic activity of the microorganisms in the fermenter, which breaks down protein into simpler forms, such as amino acids **(Omafuvbe, 2006; Oboh & Elusiyan, 2007)**.

Furthermore, it was observed that the treatment involving 12% EM4-fermented *K. alvarezii* seaweed flour (D) resulted in a higher total essential amino acid content in the feed, reaching 35.71%, compared to the other fermenter treatments (Table 3). This aligns with the earlier assumption that the greater and more diverse microbial population in the 12% EM4 fermenter produces higher amounts of exogenous enzymes such as proteases, amylases, lipases, and cellulases during the fermentation process. According to **Dewi *et al.* (2022)**, exogenous enzymes play a key role in breaking down complex compounds into simpler forms, including proteases that degrade proteins into amino acids, which are then used by other microorganisms for colony growth. The larger microbial population during fermentation thus leads to higher levels of amino acids derived from protein breakdown by proteases **(Naidu & Devi, 2005)**.

The increased amino acid content resulting from the hydrolysis of protein by proteases during fermentation improves the protein digestibility of the feed. Protein digestibility is an important factor in determining protein quality, as it indicates the biological availability of amino acids. High protein digestibility means the fish's body can effectively utilize the amino acids in the feed. This finding is supported by the results of the *in vivo* trial in this study, where the feed fortified with fermented seaweed meal was tested on the Nile tilapia for 40 days. The *in vivo* tests revealed a significant improvement ($P< 0.05$) in fish growth, as indicated by absolute weight, absolute length, and specific growth rate, across all fermenter treatments and concentrations. The best growth performance was observed in the fish fed with EM4-fermented seaweed at all concentrations (Figs. 1–3). This

finding is consistent with the improved protein and amino acid content observed in the EM4 treatments. Additionally, fish growth was superior in these treatments compared to the control feed (A). **Felix and Brindo (2014)** stated that fermentation processes can enhance nutritional content through microbial synthesis. Microbial proteins are believed to contribute significantly to the protein content of the fermented product.

Meanwhile, feed utilization efficiency (FUE) and feed conversion rratio (FCR) in the Nile tilapia were influenced only by the type of fermenter used. The best FUE and FCR values were observed in treatments fortified with *K. alvarezii* seaweed meal fermented with EM4 (Figs. 4, 5). These results further support the assumption that *K. alvarezii* seaweed meal fermentation can serve as a viable alternative feed ingredient to reduce the use of more expensive fishmeal, without negatively affecting growth performance or feed efficiency. These findings are consistent with those of **Felix and Brindo (2014)**, who reported that fermented *K. alvarezii* could be added to shrimp feed at up to 30%. In a previous study, **Felix and Brindo (2014)** also showed that *Ulva lactuca* seaweed fermented meal could be included in shrimp feed at similar levels without affecting meat quality.

The improved growth performance observed in this study in the EM4 treatments may be attributed to changes in the physical properties of the seaweed meal during fermentation, specifically reductions in particle size and structural alterations. **Cahya *et al.* (2023)** stated that fermentation can lead to reductions in particle size and changes in morphology due to enzymatic activity by microbes. **Murtini *et al.* (2016)** explained that the enzymatic breakdown of organic bonds during fermentation alters particle structure, making the meal more suitable for fish feed application, easier to digest, and capable of enhancing feed quality. **Andriani *et al.* (2019)** further suggested that increasing the distance between particles allows water to penetrate more easily into the feed, making it more rapidly decomposable in water, thereby improving FCR values.

In contrast, the survival rate of the Nile tilapia did not differ significantly across treatments ($P > 0.05$), regardless of fermenter type or concentration used (Fig. 6). This indicates that adding fermented *K. alvarezii* seaweed meal in formulated diets has no harmful or toxic effects and performs comparably to the control treatment. This condition was further supported by the water quality measurements, which remained within optimal ranges throughout the rearing period (Table 4).

Several studies have reported that fermented feed ingredients can enhance immune competence, improving fish disease resistance, growth performance, feed utilization, and gut health **(Siddik *et al.*, 2018; Zhang *et al.*, 2020)**. In this context, **Ang *et al.* (2021)** reported that macroalgae fermentation can produce functional products such as organic acids, antioxidants, phenolics, and flavonoids. Organic acids, in particular, have been shown to significantly enhance growth and health in fish and shrimp **(Ng & Koh, 2016)**.

CONCLUSION

The inclusion of fermented *K. alvarezii* powder, derived from tissue-cultured biomass and processed using EM4 as the fermentative agent, as a fishmeal substitute in feed formulation resulted in a higher protein content of 50.1%, along with a superior total essential amino acid composition of 35.71%. These values were higher compared to those obtained using yeast fermentation alone or a combination of EM4 and yeast. The results of a 40-day in vivo feeding trial with the Nile tilapia showed that the addition of fermented *K. alvarezii* seaweed powder cultivated through tissue culture to fish feed influenced growth performance, feed conversion ratio (FCR), and feed utilization efficiency (FUE), but had no effect on the survival rate. The interaction between fermentative agent type and concentration had a significant effect on fish growth, while FCR and FUE were influenced solely by the type of fermentative agent used. The best improvement in growth, feed utilization efficiency, and feed conversion was observed in the feed treatment containing 12% EM4-fermented *K. alvarezii* powder derived from tissue-cultured biomass.

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