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Title: Evaluation of in vitro Protein Digestibility of Different Feed Ingredients for Tilapia

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ABSTRACT

Protein is a crucial component of fish feed, especially during early developmental stages, as it directly influences growth, health, and immunity. However, due to the rising cost and limited availability of fish meal, a primary protein source, there is growing demand for sustainable alternatives in aquaculture. This study explores the potentials of plant-based proteins as substitutes by evaluating their *in vitro* protein digestibility for tilapia (*Oreochromis mossambicus*). Crude gut enzymes from *O. mossambicus* were used in the pH drop method to measure relative protein digestibility (RPD). Protein content across samples varied from 19% to 61%, with fish meal showing the highest content (61.7%) and wheat bran the lowest (18.6%). *In vitro* digestibility, expressed as RPD% with casein as a reference, ranged from 62.7% to 83.3%. Soybean meal (83.3%) and wheat bran (78.8%) demonstrated significantly ($P \leq 0.05$) higher RPD compared to the reference diet (62.7%), fish meal (6%), and meat and bone meal (63.5%). These findings suggest that plant-based ingredients offer superior protein digestibility, supporting their use as effective, sustainable alternatives in tilapia feed formulations.

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INTRODUCTION

Aquaculture is now one of the fastest-growing sectors in Bangladesh and considered as a major source of animal protein for the global population. In 2022, about 90% of total aquatic animal production was used for human consumption, equivalent to approximately 20.7 kg per capita (FAO, 2024).Aquatic foods provide high-quality proteins, accounting for15% of animal-based proteins and 6% of total proteins worldwide. Aquaculture products are essential sources of key nutrients, including omega-3 fatty acids, minerals and vitamins (FAO, 2024).

In Bangladesh, tilapia (*Oreochromis mossambicus*) has become one of the most popular aquaculture species due to its adaptability to local climatic conditions, high market demand, nutritional value, and simple production techniques (Rahman et al., 2021; Siddique et al., 2022). It is now the third most important fish species in the country after "pangas" (*Pangasius hypophthalmus*) and "rohu" (*Labeo rohita*), providing a crucial protein source for the low-income people (Rahman et al., 2021; Siddique et al., 2022).Despite its benefits, tilapia

production faces several challenges, including high feed and production costs, low-quality fish seed, limited processing facilities, and extreme climatic events (Rahman et al., 2021; Siddique et al., 2022;Mzengerezaet al., 2014; Wang et al., 2021). These factors can hinder farmers' ability to remain competitive and profitable (Yasumaru et al., 2014). To reduce production costs, many tilapia farmers have historically relied on locally available feed ingredients to supplement their fish diets.

Feeding costs are among the highest in aquaculture production, depending on protein content and source. Therefore, protein quality in commercial feeds is a crucial nutritional factor, influencing growth and body composition in aquaculture species (Chisty et al., 2009). Feed digestibility and assimilation are also vital to minimize the conversion of feed into water pollutants, which can negatively impact ecosystems. Efficient feeding relies on the feed's nutritional characteristics, digestibility, and feeding strategy, as these are key elements for delivering the nutrients and energy necessary for optimal growth in cultured species (Carrillo-Farnés et al., 2007; Chisty et al., 2009). Digestibility study is most important for suitable feed formulation (Wang et al., 2021). Protein digestibility reflects the bioavailability of energy and essential nutrients (Moyano et al., 2015), and the digestive capacity of aquaculture species depends on the activity of digestive enzymes in their digestive tracts (Ali et al., 2009). Research into digestive enzymes and nutrient digestibility is essential for understanding digestion mechanisms and selecting ingredients with high nutritional value for each species (Cruz-Suárez et al., 2002).

Since *in vivo* digestibility methods are lengthy and costly, *in vitro* methods have become necessary. These methods, which use the digestive enzymes of the species of interest, are faster and reliable for assessing protein digestibility (Nolasco et al., 2006). *In vitro* digestibility studies simulate the digestive process and environment in laboratory conditions, making them quicker, more affordable, and species-specific compared to *in vivo* tests (Wang et al., 2021; Yasumaru et al., 2014). *In vitro* protein digestibility tests are useful for preliminary screening, particularly when assessing large numbers of test samples across different species (Sousa et al., 2020). In contrast, *in vivo* digestibility tests are labor-intensive, complex, time-consuming, and expensive (Yasumaru et al., 2014).

Among *in vitro* digestibility methods, the pH drop method is simple, effective, and suitable for preliminary screening of large sample sets, providing digestibility values in a short time. Conducting an *in vitro* digestibility study of experimental feed using fish enzyme extract from specific species and ages can be a practical, quick, and reliable method for evaluating feed quality in growth trials (Torrissen et al., 2002). While *in vitro* digestibility methods are commonly used for species like shrimp, salmonids, and carp, research on Tilapia is limited. This study aims to assess the chemical composition (protein and moisture) of commonly used local feed ingredients and evaluate their *in vitro* digestibility for potential use in Tilapia feed formulation using the pH drop method.

MATERIALS AND METHODS

Collection of experimental animals and feed ingredients

Tilapia (*O. mossambicus*) fry were collected from the BRAC Hatchery, Dumuria, Khulna, Bangladesh, and transferred to the wet laboratory of Fisheries and Marine Resource Technology Discipline, Khulna University. Fish were acclimatized in large tanks (1000L) to reduce the mortality. During acclimatization, fish were given commercial diets (CP, 35% protein) for a week. Locally available feed ingredients, such as fish meal, soybean meal, meat, and bone meal, wheat bran, rice bran andrice polish were selected for feed formulation (Table1). All the

ingredients were collected from local market and homogenized separately by grinding.

Proximate composition of feed ingredients

The level of crude protein (%) in samples was determined according to AOAC (1980). In brief, the sample was digested with concentrated sulphuric acid (H_2SO_4) in the presence of a catalyst, followed by distillation with 40% NaOH. Finally, the crude protein was determined by multiplying the total N_2in the feed ingredients by 6.25, where nitrogen was estimated by the advanced Kjeldahl method using an automated nitrogen estimating system.

On the other hand, the moisture content (%) of sample was determined by complete drying of the sample at 105°C for 24 h in an electronic moisture determination oven (Model no. MA 30-000V3, SARTORIUS AG Gottingen, Germany) (Pearson and Eggum, 1976).

Table1. Proximate composition of different feed ingredients

Feed ingredients	Proximate composition (%in DMB)		Cost (BDT/Kg)
	Protein (%)	Moisture (%)	
Fish meal (FM)	61.56 ± 0.85	7.30	80
Soybean meal (SM)	44.08 ± 1.15	12.10	39
Meat and bone meal (MB)	54.13 ± 0.45	8.71	72
Wheat flour (WF)	10.68 ± 0.41	12.23	25
Rice polish (RP)	13.20 ± 0.67	8.41	23
Wheat bran (WB)	18.57 ± 1.08	16.09	26

Feed Formulation using the Selected Ingredients

Five different types of diets comprising one reference and four test diets were formulated by using 'Pearson Square' method (De Silva and Anderson, 1995) and prepared by using hand pellet machine. The reference diet was formulated and prepared that contained 35% crude protein (Table 3). Chromic oxide (Cr_2O_3) was used as an inert marker at a concentration of 0.50% in reference diet. Four test ingredients were selected to determine their apparent protein digestibility. Four test diets were prepared using a combination of70% reference diet and 30% of the test ingredients (Table 2) (Cho and Slinger, 1979).

Table 2. Formulation of test diets

Feed ingredients		Test diets (% inclusion of ingredients)			
		Test diet 1 (Soybean meal)	Test diet 2 (Meat and bone meal)	Test diet 3 (Fish meal)	Test diet 4 (Wheat bran)
Reference diet	Fish meal	9.835	9.835	9.835	9.835
	Soybean meal	19.67	19.67	19.67	19.67
	Meat & bone meal	9.835	9.835	9.835	9.835
	Rice polish	11.655	11.655	11.655	11.655
	Wheat flour	7	7	7	7
	Wheat bran	11.655	11.655	11.655	11.655
	Cr_2O_3	0.35	0.35	0.35	0.35
Test Ingredients	Soybean meal	30	-	-	-
	Meat & bone meal	-	30	-	-
	Fish meal	-	-	30	-
	Wheat bran	-	-	-	30

Table3. Formulation for reference diet and test diets

Ingredients	% Inclusion (dry matter basis; DMB)	Protein (%)	Composition (%)
Formulation for 35% protein rich reference Diet (% in DMB)			
Fish meal	14.05	8.66	
Soybean meal	28.10	12.39	
Meat & bone meal	14.05	7.69	
Rice polish	16.65	2.20	
Wheat flour	10.00	1.07	
Wheat bran	16.65	3.09	
Cr_2O_3	0.50	0	
Composition of the test diets (% in DMB)			
Reference diet (RD)			70%
Test Ingredients (Soybean meal/Meat and bone/Fish meal/Wheat bran)			30%
Total	100.00	35.00	100%

Experimental conditions

About 170 tilapia fries (± 2 inch) were stocked in seven glass aquaria (20×9×12 $inch^3$) with seventeen individuals in each. Fish were fed a laboratory-prepared, formulated diet (35% protein) for one month and reared with continuous aeration at room temperature (approximately 26°C). About 20%water was exchanged every alternative day, and uneaten feeds and feces were removed every day by siphoning.

At the end of the rearing, fish were sacrificed by dissection to collect the guts. The guts of the species were pooled together, kept at chilled condition (≤4°C), and weighted using an electronic balance (Electric balance, AND, GF 300H). The guts were homogenized in a Potter Thomas tissue grinder with a Teflon pestle at cool temperature (≤4°C) by keeping the tissue grinder into ice and diluted with cool distilled water (4°C) at a ratio of 1:10 ($^{W}/_{V}$). The homogenates were transferred to 1.5ml microfuge tubes and immediately centrifuged at 12000 rpm for 15 minutes at 4°C in a refrigerated centrifuge machine (Micro High Speed Refrigerated Centrifuge, VS-15000 CFN 11, Vision, Korea). The upper lipid layer of the supernatant was discarded, and the aqueous supernatant was collected in a previously cooled glass bottle, frozen, and stored at -20°C for further use. All the procedures were conducted at a cool temperature (≤4°C).

Determination of in vitro protein digestibility using fish enzyme

The *in vitro* digestibility assay was determined using the pH drop method. An equivalent amount of each ingredient that provided 240 mg of crude protein was mixed with 30ml of distilled water to produce a suspension of 8.0 mg of crude protein ml^{-1}. The mixture was kept overnight at refrigerated temperature. Approximately 30 ml of crude protein solution and fish enzyme extract were taken into two bikers separately, and the pH was adjusted to 8.0 with 0.1 N NaOH and 0.1 N HCl, and 10 ml of solution and 1 ml of fish enzyme extract were added to the test tube and mixed by vortexing (Vision Scientific Co. LTD) immediately. The hydrolysis reaction started with the addition of enzyme extract, and the pH was recorded at every minute interval for 10 minutes by a pH meter (EZODO, pH-5011).The hydrolysis was done three times for each of the ingredients. Casein was used as a reference

protein for comparing the digestibility. The protein digestibility was estimated as the percentage of magnitude of pH drop (Δ pH) ratio of the ingredients to that of casein. The relative protein digestibility (RPD) was calculated as the percentage of magnitude of pH drop (-ΔpH) of the ratio of ingredient and casein (Lazo, 1994). The RPD of different feed ingredients was calculated by the following equation.

$$RPD\ (\%) = -\Delta\text{pH of ingredients} \times 100 - \Delta\text{pH of casein}$$

Statistical analyses

Normality and homogeneity of the data were tested by the Shapiro-Wilk test and the Levene test, respectively. The similarity of proximate composition, pH change, and relative digestibility data between the feed ingredients and treatments were analyzed by one-way ANOVA at the 5% level of significance using SPSS version 28.0. A Tukey-HSD post-hoc test was done to understand the significant difference between the treatments.

RESULTS AND DISCUSSION

The search for new, nutritious, and affordable protein sources has been an ongoing priority in developing aquaculture feed (García-Galano et al., 2007). *In vitro* digestibility methods simulate the enzymatic phase of digestion, allowing researchers to evaluate ingredient composition effects on nutrient availability. Thus, *in vitro* digestibility is useful for ranking ingredients as potential candidates for feed formulations (Savoie, 1994; Moyano et al., 2015). Results from the *in vitro* digestibility study indicate that plant-based ingredients offer superior protein digestibility, supporting their use as effective, sustainable alternatives in tilapia feed.

Table 4. Percent protein content, moisture content, and relative protein digestibility (mean ± standard deviation) of different feed ingredients by using gut enzyme extract of *O. mossambicus.*

Ingredients	Protein (%)	Moisture (%)	Relative protein digestibility (RPD)	
			RDP (%)	Test-statistics
Wheat Bran	18.56 ± 1.1	11.3	78.8 ± 0.6[a]	$F_{(4,5)} = 31.2$, P <
Soybean meal	44.08 ± 1.2	10.0	83.3 ± 5.6[a]	0.001,
Reference Diet	30.94 ± 1.2	6.7	62.7 ± 0.0[b]	Size effect =
Meat & Bone meal	54.13 ± 0. 5	10.2	63.5 ± 0.0[b]	0.961
Fish Meal	61.56 ± 0.9	7.9	63.1 ± 0.6[b]	

Different superscript letters indicate significant difference between different diets (one-way ANOVA, P<0.05).

In this study, we measured the crude protein content of the test samples and found that fish meal (61.56%) contained the highest level of crude protein, followed by meat and bone meal (54.13%), soybean meal (44.08%), and wheat Bran (18.56%). The combination used in the reference diet contained 30.94% crude protein (Table 4). The higher protein levels in fish meal and meat and bone meal reflect their superior nutritional value. However, the highest costs of these ingredients pose a challenge to sustainable use in aquaculture, highlighting the need for cost-effective alternative feed ingredients that maintain high digestibility and nutritional quality.

Table 5. Change of pH (mean ± standard deviation) in casein, different feed ingredients and reference diet. Different superscript letters indicate significant difference between treatments (one-way ANOVA, P<0.05)

Time (Min)	Casein	M & B	FM	WB	SM	RD	Test Statistics
0	8.0±0.0^{a}	8.0±0.0^{a}	8.0±0.0^{a}	8.0±0.0^{a}	7.9±0.1^{a}	8.0±0.0^{a}	$F_{(5,11)}$ = 0.693; P = 0.640; size effect = 0.239
1	7.7±0.2^{a}	7.8±0.1^{a}	7.8±0.1^{a}	7.9±0.1^{a}	7.8±0.1^{a}	7.8±0.0^{a}	$F_{(5,11)}$ = 1.05; P = 0.435; size effect = 0.324
2	7.5±0.3^{a}	7.6±0.1^{a}	7.7±0.1^{a}	7.7±0.1^{a}	7.6±0.2^{a}	7.7±0.1^{a}	$F_{(5,11)}$ = 0.982; P = 0.471; size effect = 0.309
3	7.3±0.3^{a}	7.6±0.1^{a}	7.6±0.1^{a}	7.6±0.1^{a}	7.5±0.3^{a}	7.6±0.1^{a}	$F_{(5,11)}$ = 1.313; P = 0.327; size effect = 0.374
4	7.1±0.2^{b}	7.6±0.1^{a}	7.5±0.1^{ab}	7.5±0.1^{ab}	7.4±0.3^{ab}	7.5±0.1^{ab}	$F_{(5,11)}$ = 3.251; P = 0.048; size effect = 0.569
5	7.0±0.1^{b}	7.5±0.1^{ab}	7.5±0.1^{a}	7.4±0.1^{ab}	7.3±0.3^{ab}	7.4±0.2^{ab}	$F_{(5,11)}$ = 3.367; P = 0.043; size effect = 0.605
6	6.9±0.1^{b}	7.4±0.2^{a}	7.4±0.5^{a}	7.3±0.1^{ab}	7.23±0.3^{ab}	7.4±0.1^{ab}	$F_{(5,11)}$ = 4.261; P = 0.021; size effect = 0.659
7	6.9±0.1^{a}	7.36±0.1^{a}	7.36±0.5^{a}	7.15±0.1^{a}	7.2±0.3^{a}	7.3±0.1^{a}	$F_{(5,11)}$ = 3.223; P = 0.049; size effect = 0.594
8	6.8±0.1^{b}	7.33±0.2^{a}	7.33±0.5^{a}	7.05±0.1^{ab}	7.16±0.3^{ab}	7.2±0.2^{ab}	$F_{(5,11)}$ = 4.043; P = 0.025; size effect = 0.648
9	6.8±0.2^{b}	7.3±0.1^{a}	7.26±0.5^{a}	7.0±0.0^{ab}	7.16±0.3^{ab}	7.2±0.2^{ab}	$F_{(5,11)}$ = 4.436; P = 0.019; size effect = 0.668
10	6.7±0.2^{b}	7.23±0.1^{a}	7.23±0.5^{a}	7.0±0.0^{ab}	7.06±0.3^{ab}	7.1±0.1^{ab}	$F_{(5,11)}$ = 4.533; P = 0.017; size effect = 0.673

[M & B = meat & bone meal, FM = fish meal, WB = wheat bran, SM = soybean meal, RD = reference diet]

All ingredients and casein solutions were hydrolyzed with the crude gut enzyme extract of *O. mossambicus* for 10 minutes at room temperature, and the pH changes were recorded. The initial pH of casein and other feed ingredients were similar within0 to 3 mins at room temperature. A significantly lower pH was recorded in casein compared to meat and bone and fish meal from 4 to 5 mins, continuing until the end of 10 mins period, except at the 7thmin (Table 5). This trend of pH drop could be further explained by the continuous increase in effect size, indicating a meaningful difference between the treatments. The pH drop during the *in vitro* digestibility test corresponds to the release of protons due to peptide bond hydrolysis by protease in the crude extract (Hsu et al., 1977).

The *in vitro* protein digestibility of different feed ingredients was found significantly different (*P≤0.05*) by using crude enzyme extract of *O. mossambicus*. The relative protein digestibility of soybean meal and wheat bran was notably higher (83.3 and 78.8%, respectively) than the reference diet (62.7%), meat and bone meal (63.5%) and fish meal (63.1%) (Table 4). The effect size of 0.96 indicates that 96% of the total variance is accounted for by the treatment effect, suggesting a large, meaningful difference between the groups.

Previous studies suggest that plant-based proteins, such as soybean meal and wheat bran, are promising substitutes for fish meal in aquaculture. The highest protein digestibility observed in plant proteins aligns with findings by Abdel-Latif et al. (2022), who reported that plant-based crude proteins, such as *Moringa oleifera*, offer a cost-effective source with similar nutritional benefits. Garcia-Carreno et al. (2004) reported a higher apparent protein digestibility of soybean meal (94.63%) in the

diet of *Penaeus vannamei*. The superior digestibility of soybean meal in this study may be due to species-specific variations in enzyme activity, which can play a crucial role in protein digestion. In contrast, Ali et al. (2009) reported an identical relative protein digestibility for soybean meal (76.08%) and fish meal (78.08%) in *Anabas testudineus*, suggesting that apparent protein digestibility is highly dependent on fish species' dietary habits.

The efficiency of protein digestibility largely depends on the species' ability to break down nutrients, emphasizing the role of digestive enzymes that catalyze protein conversion into absorbable amino acids (Lagler and Bardach, 1962; Fisher, 1982).For example, plant-based proteins, such as *Lemna minor*, have exhibited good digestibility (> 50%) and promoted growth performance in Grass Carp (*Ctenopharyngodon idella*) (Srirangam et al., 2016), while they showed reduced digestibility and higher protease inhibition in pink shrimp (*Farfantepenaeus paulensis*) (Lemos et al., 2004).Our findings on soybean meal digestibility are also supported by Sultana et al. (2018**),** who reported about 78% protein digestibility in Nile tilapia, though they reported approximately 23% higher protein digestibility of fish meal (86%) in Nile tilapia compared to this study. Similarly, Eid and Matty (1989) found higher protein digestibility for fish meal (91.3%) in carp (*Cyprinus carpio*) using gut enzymes, while Ezquerra et al. (1998) reported digestibility ranging from 72.52% to 83.59% for fish meal of various origins. In this study, we also observed a higher protein digestibility for wheat bran (78.8%), which indicated that the plant-based proteins can be potentially used as alternatives to fish meal. Such substitutions can support high tilapia juvenile survival rates (90 – 100%) when fish meal is replaced with plant-based proteins, like soybean meal and cottonseed meal (González-Félix et al., 2010).

Despite higher RPD in plant-based ingredients compared to animal proteins, it is important to validate these findings through *in vivo* assays. As noted by Fernandes et al. (2021), *in vitro* methods are relevant for preliminary evaluations of protein digestibility but may be influenced by the buffering capacity of feed components. Additionally, Wang et al. (2021) emphasized that the interactions between protein hydrolysis and other feed components should be considered. Therefore, comparing *in vitro* and *in vivo* test results is crucial for confirming protein digestibility in tilapia feed.

CONCLUSION

In conclusion, this study underscores the superior digestibility of plant-based protein sources, such as soybean meal and wheat bran, over animal-based sources like fish mean and meat and bone in *Oreochromis mossambicus*. The *in vitro* protein digestibility data provide valuable insights into protein quality, essential for developing nutritionally effective feed formulations. The high digestibility of plant-based ingredients makes them as promising alternatives to traditional animal protein sources in *O. mossambicus* diets. However, to confirm these *in vitro* results, comparative studies with *in vivo* digestibility data are essential. Further research integrating both *in vitro* and *in vivo* digestibility could improve feed formulation, ultimately benefiting aquaculture practices for *O. mossambicus* and potentially other fish species.

Competing interest

The authors declare no conflict of interest regarding the publication of this paper.

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