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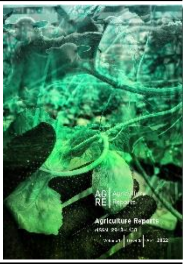

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# Effect of papaya leaf extract inclusion on growth performance and haematological parameters of red hybrid tilapia, *Oreochromis mossambicus* × *Oreochromis niloticus* fed diets formulated with Hermetia meal and Azolla

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**Keywords:** Azolla, Black soldier fly larva, Growth performance, Papaya leaf extract, Red hybrid tilapia

**Abstract:** This research aimed to explore the impact of incorporating papaya leaf extract into diets for red hybrid tilapia prepared with alternative ingredients: black soldier fly larvae (BSFL) and Azolla. Using a 4 x 2 factorial design, eight diets were formulated and fed to the fish in triplicate for eight weeks. All diets were designed to have similar protein, lipid and energy levels, 40%, 8.5%, and 355 (kJ/100 g), respectively. The study used fish with an average initial weight of 2.29 ± 0.06 g. The results showed that the fish fed with the combination of BSFL and papaya leaf extract had the highest final weight of 31.84 ± 1.32 g, compared to other treatments ($p<0.05$), except for the basal diet containing papaya leaf extract. The addition of BSFL meal improved growth parameters, but Azolla appeared to have adverse effects. Incorporating 1.95% papaya leaf extract into the formulation also helped enhance growth performance. However, neither alternative ingredients nor papaya leaf extract affected the FCR and PER of the treatment groups. The VSI and HSI of the fish studied were unaltered across the experimental diets. The addition of alternative ingredients and papaya leaf extract to the fish feed did not affect its moisture and protein content. However, papaya leaf extract treatments had a significant impact on the fish's lipid and ash content. The haematological parameters of the experimented fish were generally unaffected by the dietary treatments. However, the levels of leucocytes, HGT, HCT, and erythrocytes were notably lower in all groups treated with papaya leaf extract. except for leucocytes, HGT, HCT and erythrocytes, the levels were significantly lower in all groups treated with papaya leaf extract. The BSFL meal can serve as a viable substitute ingredient in fish feed. However, caution should be exercised when incorporating Azolla. Also, adding 1.95% papaya leaf extract to the practical diet for tilapia, which was prepared using alternative protein sources, can enhances growth performance without any negative impact on the fish's overall well-being.

## INTRODUCTION

Aquaculture is undeniably an important player in satiating the escalating need for fish protein demand while simultaneously alleviating the strain on our natural fish populations. Its rapid growth is a testament to its significance (Diwan, Harke, & Panche, 2022; Mohan et al., 2022). However, the industry's sustainability depends on the availability of sustainable and cost-effective protein sources for feed production, as current formulations heavily rely on fishmeal from wild stocks (Campanati, Willer, Schubert, & Aldridge, 2022; Little, Newton, & Beveridge, 2016). There is a growing demand for aquaculture to increase productivity to support the growing human population globally. The intensification and diversification of aquaculture have led to the continued growth and expansion of the industry (Campanati et al., 2022; Rohani et al., 2022). Consequently, increased the market demand for fishmeal, an essential ingredient for aquaculture feed production. The inclusion of fishmeal in aquafeed formulations has been claimed to cause ocean resource manipulation and increased aquafeed production costs.

*Hermetia illucens* (black soldier fly larvae, BSFL), and *Azolla pinnata* (mosquito fern, Azolla) are two materials that have the potential to be used as alternative ingredients for aquafeed. The effect of BSFL meal has been widely studied as the fishmeal substitute in many farmed fish because it has been shown to be nutritionally suitable due to its high crude protein content (Ewald et al., 2020; Meneguz et al., 2018; Spranghers et al., 2017). Meanwhile, Azolla is an aquatic macrophyte that has gained attention to be included as ingredient in herbivorous farmed fish such as rohu (Panigrahi, Choudhury, Sahoo, Das, & Rath, 2014) and Nile tilapia (Lumsangkul et al., 2022). Azolla contains moderate protein content and has good digestibility (Kc, Yb, Sb, & Shilpashree, 2016; Kumar et al., 2019). Both, BSFL meal and Azolla can be easily reproduced in a wide range of environmental conditions.

*Carica papaya* leaf extract contains exogenous enzymes, such as papain, chymopapain, and protease, which help to break down complex proteins into polypeptides and amino acid, thereby facilitating digestion and the absorption of nutrients (Alorkpa, Boadi, Badu, & Saah, 2016). Papaya leaf extract has been used as a functional additive in feeds to improve the nutrient utilisation of feed in red hybrid tilapia (Hamid et al., 2022).

Tilapia is farmed for its high-quality protein source at a competitive price (Canonico, Arthington, McCrary, & Thieme, 2005). In general, tilapia can tolerate various food resources with lower protein requirements than carnivorous fish (Ringø, 2020). Red hybrid tilapia generally requires between 25% and 35% protein, so feed costs are lower (El-Sayed, Ezzat, & Mansour, 2004; Twibell & Brown, 1998). Therefore, it is one of the preferred farming species for small farms as the operating costs, especially for feed, are low (Mohammadi et al., 2022). Thus, the current study assessed the effect of papaya leaf extract as a functional feed additive in red hybrid tilapia feed diet containing BSFL meal and Azolla on the growth performance, feed stability, haematology, blood biochemistry

## MATERIALS AND METHODS

### *Animal Ethics*

This study followed the National Institutes of Health's guide for the care and use of laboratory animals and was approved by the Universiti Sains Malaysia's Animal Ethical Committee. (Ref: USM/IACUC/2021/ (127)1128).

### *Experimental design and feed preparation*

A total of eight experimental diets were formulated using 4 x 2 factorial design (4 protein sources, 2 levels of papaya leaf extract). The protein sources were standard (control), azolla, black soldier fly larva, and the combination of azolla and black soldier fly larva. Each diet had 39% crude protein and 8% crude lipid. The diets contained 0 % papaya leaf extract and 1.95 % papaya leaf extract obtained from BioNutricia (Table 1)

**Table 1**. Ingredients and proximate composition of experimental diet (g/100g dry matter).

| Ingredients | Con (-) | Azolla (-) | BSFL (-) | Azo+ BSFL (-) | Con (+) | Azolla (+) | BSFL (+) | Azo+BSF (+) |
|---|---|---|---|---|---|---|---|---|
| Fishmeal[1] | 37.40 | 37.45 | 29.50 | 29.95 | 37.40 | 37.45 | 29.50 | 29.95 |
| Soybean meal[2] | 27.30 | 21.85 | 27.35 | 21.85 | 27.30 | 21.85 | 27.35 | 21.85 |
| Corn starch[3] | 21.00 | 20.65 | 21.50 | 21.20 | 21.00 | 20.65 | 21.50 | 21.15 |
| BSFL[4] | 0.00 | 0.00 | 10.70 | 10.70 | 0.00 | 0.00 | 10.70 | 10.70 |
| Azolla | 0.00 | 11.10 | 0.00 | 11.10 | 0.00 | 11.10 | 0.00 | 11.10 |
| Palm oil[5] | 2.50 | 1.75 | 2.49 | 1.75 | 2.50 | 1.75 | 2.49 | 1.75 |
| Fish oil | 2.00 | 2.05 | 0.54 | 0.55 | 2.00 | 2.05 | 0.54 | 0.55 |
| Vitamin mix[6] | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Mineral mix[7] | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Celite | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Cellulose[8] | 10.30 | 5.70 | 6.50 | 1.95 | 8.30 | 3.70 | 4.51 | 0.00 |
| Papaya leaf extract | 0.00 | 0.00 | 0.00 | 0.00 | 1.95 | 1.95 | 1.95 | 1.95 |
| Proximate composition (%) | | | | | | | | |
| Moisture | 5.59 | 5.51 | 5.27 | 5.63 | 5.59 | 5.51 | 5.27 | 5.63 |
| Crude protein | 39.40 | 39.70 | 38.90 | 39.59 | 41.00 | 39.10 | 41.40 | 39.50 |
| Crude lipid | 8.41 | 8.61 | 8.60 | 8.55 | 8.41 | 8.61 | 8.60 | 8.55 |
| Crude fibre | 4.29 | 5.25 | 5.25 | 4.45 | 4.29 | 5.25 | 5.25 | 4.45 |
| Ash | 13.62 | 12.38 | 12.63 | 11.08 | 13.62 | 12.38 | 12.63 | 11.08 |
| Nitrogen-free extract[9] | 34.28 | 33.86 | 34.62 | 36.33 | 34.28 | 33.86 | 34.62 | 36.33 |
| Gross energy | 355.78 | 356.44 | 356.44 | 356.68 | 355.78 | 356.44 | 356.44 | 356.68 |

[1]Danish fishmeal
[2]soybean meal
[3]corn starch
[4]Black soldier fly larva
[5]Palm oil
[6]Vitamin premix (Rovimix 6288; F.Hoffman La-Roche Ltd, Basel, Switzerland), containing (per kg, dry weight): Vitamin A, 50 million IU; Vitamin D3, 10 million IU; Vitamin E, 130 g; Vitamin B1, 10 g; Vitamin B2, 25 g; Vitamin B6, 16 g; Vitamin B12, 100 mg; biotin,500 mg; panthothenic acid, 56 g; folic acid, 8 g; niacin, 200 g; anti-cake20 g; antioxidant, 200 mg; Vitamin K3, 10 g; and Vitamin c, 35 g
[7]Mineral premix (g/kg)-cobalt carbonate, 100mg; copper sulphate, 780 mg; magnesium sulphate, 137 g;mmanganese oxide, 800 mg; potassium chloride, 50 g; potassium iodide, 150 mg; sodium chloride, 60 g; sodium selenite, 200 mg and zinc oxide, 1.5 g; calcium lactate, 327 g; ferrous sulphate, 25 g; calcium phosphate (monobasic), 397.5 g.
[8]Cellulose
[9]Nitrogen free extract: 100 - (moisture + protein+ lipid +ash+ fibre)

### *Experimental fish and rearing conditions*

Fry of red hybrid tilapia were purchased from a local fish supply in Kedah, Malaysia. Upon their arrival at the Aquatic Research Complex, USM, the fingerlings were acclimatised to the culture conditions for two weeks before the feeding trial began. During this period, the fish were fed with a commercial diet (TP 1 Star Feed) twice daily, at 9:00 a.m. and 5:00 p.m. Following the acclimatisation period, all fish were food-deprived for 24 hours to empty their gastrointestinal tract and reduce stress during stocking. Twenty fish were randomly selected from the holding tanks and stored at -20°C for subsequent determination of the initial whole-body composition.

A total of 10 fish with an average size of 2.29 ± 0.06 g were randomly placed in a rectangular glass aquarium (70 cm (height) × 46.7 (depth) cm × 30.0 cm (width)) filled with 30 L of carbon-filtered water and continuously supplied with air. Each experimental diet was randomly fed to three groups of fish. During the experiment, the fish were manually fed twice daily at 9:00 a.m. and 5:00 p.m. until they were satiated. Daily water exchange was conducted to maintain good culture conditions. The food consumed was measured daily. Every fortnight, the fish from each aquarium were weighed together to monitor their growth. The feeding trial was conducted for eight weeks. The temperature, pH, and dissolved oxygen levels of the water in the aquaria were measured weekly. The concentration of nitrogen ammonia, nitrite, nitrate, and phosphorus were evaluated every two weeks. These parameters were monitored to ensure that the water quality was suitable for tilapia culture.
Prior to final sampling, fish were food-deprived for 24 hours to minimize the stress associated with the sampling process. The fish were euthanized using a tricaine methane sulfonate (MS-222) bath at 150 $mg^{-L}$. Fish were counted individually to determine the survival rate, and they were weighed individually to evaluate growth performance parameters.

### *Tissue collection for body indices assessment*

Three fish were randomly selected from each fish tank and were used for the determination of body indices. The fish were dissected to remove the entire viscera. The viscera were weighed to determine the viscero-somatic index. The liver of each fish was weighed to determine the hepato-somatic index. Carcasses were cleaned and stored at -20 °C for subsequent analysis of the whole-body composition.

### *Blood collection for haematological analysis*

Three fish of each treatment were selected randomly for blood collection. Prior to collecting the blood samples, the fish were euthanized by immersing them in MS-222 bath at 150 $mg^{-L}$. Blood was withdrawn from each fresh euthanized- fish using a sterile 1 cc syringe. Collected blood samples were placed into tubes containing heparin. Standard haematological procedures were used to analyze the haematological parameters described by Kari et al. (2021).

### *Growth performance and body indices*

The collected samples were analyzed to estimate the growth performances using the following formulae:

i. Weight gain (WG)= final weight – initial weight
ii. Feed conversion ratio= total feed intake. weight gain-1 Specific Growth Rate = (ln $Wt_{final}$ – $lnWt_{initial}$). duration-1
iii. Protein efficiency ratio (PER) = weight gain.total protein intake-1 index (HSI %) = (liver weight.body weight-1) x 100
iv. Viscerosomatic Index (VSI %) = (viscera weight.body weight-1) x 100
v. Survival = final number fish/ initial number fish x 100 Hepatosomatic

### *Proximate Analysis*

Proximate analyses of feed ingredients, diets, and carcasses (initial and final) were performed using standard techniques outlined by the Association of Analytical Chemists, AOAC (1997).

### *Statistical analysis*

Data for proximate analysis, growth performance, body indices and haematological parameters were subjected to one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests when analyzing the treatments individually. Two-way ANOVA was used to compare the means of the main effects, followed by Tukey's multiple range test. The level of probability was set at 0.05 %. All analyses were performed using IBM SPS version 27 software.

## RESULTS

### *Growth Performance, body indices and survival*

The growth performance and survival rate of hybrid red tilapia after the feeding experiment are shown in Table 2. Overall, the addition of papaya leaf extract in the experimental diets had a positive effect on the performance of the fish. Fish fed with papaya leaf extract performed significantly better than those fed without papaya leaf extract (P < 0.05). Fish fed with the diet containing BSFL and 1.95% papaya leaf extract had significantly higher final weight than the other feeding treatments, except for the control group fed papaya leaf extract (P < 0.05), followed by the treatments fed Azolla with papaya leaf extract, and the fish fed the combination of Azolla and BSFL with papaya leaf extract in the experimental diet. A similar pattern was observed in weight gain, SGR and feed intake in the diet-treated groups. Feed intake was significantly higher in the treatment group receiving the experimental diet supplemented with papaya leaf extract compared to the other treatment groups, followed by the treatment group receiving a control diet supplemented with papaya leaf extract, except for the treatment group receiving azolla diet supplemented with papaya leaf extract (P < 0.05). Adding 1.95% papaya leaf extract to the experimental diets did not affect FCR and PER (P > 0.05).

The results of body indices (VSI, HSI) showed that the alternative ingredients and papaya leaf extract had not significantly influenced the VSI red hybrid tilapia (P > 0.05). However, a significant interaction between these two factors was observed (p<0.05). The supplementation of papaya leaf extract in the experimental diets had no adverse effects on the hepato-somatic index of red hybrid tilapia, regardless of the protein sources (P > 0.05). High survival amongst the treatment was above 90% and was not significantly impacted by the dietary treatment (P > 0.05).

**Table 2.** Growth performance, body indices and survival of fish fed experimental diets for eight weeks

| Diet | Initial weight (g) | Final weight (g) | Weight gain (g) | SGR (% day$^{-1}$) | FCR | Feed intake | PER | VSI (%) | HSI (%) | Survival (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Control (-) | 2.28 ± 0.06 | 22.95 ± 1.66$^{ab}$ | 20.81 ± 2.13$^{abc}$ | 4.19 ± 0.16$^{ab}$ | 1.36 ± 0.36 | 27.98 ± 1.56$^{ab}$ | 1.88±0.92 | 11.37±0.83 | 2.51±0.23 | 93.33 ± 3.33 |
| Azolla (-) | 2.29 ± 0.06 | 20.81 ± 1.18$^{a}$ | 18.54 ± 0.60$^{a}$ | 4.02 ± 0.03$^{a}$ | 1.34 ± 0.43 | 24.81 ± 1.33$^{a}$ | 1.88±0.07 | 10.19±0.81 | 2.18±0.26 | 96.67 ± 3.33 |
| BSF (-) | 2.29 ± 0.06 | 24.69 ± 1.20$^{ab}$ | 22.41 ± 0.70$^{abc}$ | 4.32 ±0.05$^{abc}$ | 1.28 ± 0.01 | 28.76 ± 1.01$^{bc}$ | 2.00±0.01 | 10.44±0.35 | 2.37±0.19 | 96.67 ± 3.33 |
| Azolla + BSF (-) | 2.30 ± 0.06 | 21.04 ± 1.56$^{a}$ | 18.75 ± 0.60$^{ab}$ | 4.03 ± 0.04$^{a}$ | 1.31 ± 0.03 | 24.59 ± 0.59$^{a}$ | 1.93±0.05 | 11.05±0.52 | 2.88±0.28 | 93.33 ± 3.33 |
| Control (+) | 2.37 ± 0.06 | 30.31 ± 2.36$^{cd}$ | 27.88 ± 2.73$^{de}$ | 4.62 ± 0.19$^{cd}$ | 1.26 ± 0.07 | 34.67 ± 1.37$^{d}$ | 1.95±0.11 | 10.92±0.82 | 2.22±0.17 | 96.67 ± 3.33 |
| Azolla (+) | 2.25 ± 0.07 | 26.39 ± 1.77$^{bc}$ | 24.00 ± 2.05$^{bcd}$ | 4.45 ± 0.16$^{bc}$ | 1.34 ± 0.99 | 31.86 ± 0.65$^{cd}$ | 1.92±0.13 | 12.15±0.87 | 2.51±0.28 | 96.67 ± 3.33 |
| BSF (+) | 2.31 ± 0.06 | 34.14 ± 1.42$^{d}$ | 31.84 ± 1.32$^{e}$ | 4.89 ± 0.07$^{d}$ | 1.23 ± 0.02 | 39.15 ± 1.39$^{e}$ | 1.96±0.03 | 10.94±0.59 | 2.56±0.22 | 100.00 ± 0.00 |
| Azolla + BSF (+) | 2.29 ± 0.06 | 27.42 ± 1.06$^{bc}$ | 25.08 ± 1.53$^{cd}$ | 4.50 ± 0.11$^{bc}$ | 1.23 ± 0.02 | 30.68 ± 1.29$^{bc}$ | 2.07±0.04 | 10.85±0.74 | 2.24±0.12 | 96.67 ± 3.33 |
| **Alternative Ingredients** | | | | | | | | | | |
| Control | 2.22 ± 0.44 | 26.63 ± 1.12$^{ab}$ | 24.34 ± 1.16$^{ab}$ | 4.41 ± 0.83$^{ab}$ | 1.31 ± 0.04 | 31.32 ± 0.85 | 1.92±0.05 | 11.15±0.05 | 2.37±0.17 | 95.00 ± 2.21 |
| Azolla | 2.27 ± 0.44 | 23.60 ± 1.11$^{a}$ | 21.78 ± 1.16$^{a}$ | 4.24 ± 0.83$^{a}$ | 1.34 ± 0.04 | 28.34 ± 0.85 | 1.91±0.05 | 11.17±0.05 | 2.34±0.16 | 96.67 ± 2.21 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| BSF | 2.30 ± 0.44 | 29.41 ± 1.10$^{b}$ | 27.12 ± 1.16$^{b}$ | 4.61 ± 0.83$^{b}$ | 1.26 ± 0.04 | 33.96 ± 0.86 | 1.98±0.05 | 10.69±0.05 | 2.47±0.16 | 98.33 ± 2.21 |
| Azolla +BSF | 2.30 ± 0.44 | 24.23 ± 1.12$^{a}$ | 21.91 ± 1.16$^{a}$ | 4.26 ± 0.83$^{a}$ | 1.27 ± 0.04 | 27.63 ± 0.85 | 2.00±0.05 | 10.95±0.05 | 2.56±0.17 | 95.00 ± 2.21 |
| **Additive** | | | | | | | | | | |
| 0 % PLE | 2.29 ± 0.03 | 22.37 ± 0.79 | 20.13 ± 0.82 | 4.14 ± 0.06 | 1.32 ± 0.03 | 26.53 ± 0.60 | 1.93±0.04 | 10.76±0.04 | 2.49±0.12 | 95.00 ± 1.56 |
| 1.95 % PLE | 2.31 ± 0.03 | 29.57 ± 0.78 | 27.20 ± 0.82 | 4.62 ± 0.06 | 1.26 ± 0.03 | 34.09 ± 0.60 | 1.98±0.04 | 11.22±0.04 | 2.38±0.12 | 97.50 ± 1.56 |
| **Two-way ANOVA** | | | | | | | | | | |
| Protein source | NS | P < 0.05 | P < 0.05 | P < 0.05 | NS | P < 0.05 | NS | NS | NS | NS |
| Additive | NS | P < 0.05 | P < 0.05 | P < 0.05 | NS | P < 0.05 | NS | NS | NS | NS |
| Interaction | NS | NS | NS | NS | NS | NS | NS | P < 0.05 | NS | NS |

Values (means ± standard error for three replicate groups) in each column with different superscripts show significant difference (p < 0.05). NS: not significant.

### *Whole-body Proximate composition*

The whole-body composition of red hybrid tilapia fed diets with different protein sources with or without papaya leaf extract supplementation is presented in Table 3. Dietary treatments had no significant effect on the moisture and protein contents of the fish (P > 0.05). However, dietary treatments had significant effect on the lipid and ash contents, with no clear trend (P < 0.05). Overall, group fed dietary supplementation of papaya leaf extract had increased lipid content and decreased ash content compared with the groups fed diets without the supplementation of the additive in the diets, regardless of the protein sources (P < 0.05).

**Table 3**. Proximate composition of red hybrid tilapia fed the experimental diets for eight weeks.

| | Moisture | Protein | Lipid | Ash |
|---|---|---|---|---|
| **Diet** | | | | |
| Control (-) | 75.36 ± 0.14 | 69.03 ± 1.11 | 9.82 ± 0.29$^{ab}$ | 14.59 ± 0.13$^{ab}$ |
| Azolla (-) | 75.18 ± 0.27 | 69.87 ± 0.94 | 8.70 ± 0.28$^{a}$ | 16.10 ± 0.36$^{b}$ |
| BSF (-) | 76.00 ± 0.16 | 70.67 ± 0.19 | 9.51 ± 0.61$^{ab}$ | 15.49 ± 0.69$^{b}$ |
| Azolla + BSF (-) | 75.88 ± 0.27 | 70.90 ± 0.40 | 8.74 ± 0.33$^{a}$ | 15.60 ± 0.55$^{b}$ |
| Control (+) | 74.73 ± 0.87 | 71.67 ± 1.64 | 10.96 ± 0.39$^{b}$ | 13.71 ± 0.40$^{a}$ |
| Azolla (+) | 75.47 ± 0.45 | 70.40 ± 0.42 | 10.50 ± 1.08$^{ab}$ | 13.42 ± 0.73$^{a}$ |
| BSF (+) | 74.87 ± 0.25 | 70.33 ± 0.38 | 8.85 ± 0.70$^{a}$ | 13.67 ± 0.20$^{a}$ |
| Azolla + BSF (+) | 75.02 ± 0.18 | 70.70 ± 0.31 | 10.86 ± 0.51$^{b}$ | 14.59 ± 0.61$^{ab}$ |
| **Alternative Ingredients** | | | | |
| Control | 75.05 ± 0.28 | 70.35 ± 0.58 | 10.39 ± 0.41 | 14.15 ± 0.36 |
| Azolla | 75.33 ± 0.28 | 70.13 ± 0.58 | 9.60 ± 0.41 | 14.76 ± 0.36 |
| BSF | 75.44 ± 0.28 | 70.50 ± 0.58 | 9.18 ± 0.41 | 14.58 ± 0.36 |
| Azolla +BSF | 75.45 ± 0.28 | 70.80 ± 0.58 | 9.80 ± 0.41 | 15.09 ± 0.36 |
| **Additive** | | | | |
| 0 % PLE | 75.61 ± 0.20 | 70.12 ± 0.41 | 9.19 ± 0.29 | 15.44 ± 0.25 |
| 1.95 % PLE | 75.02 ± 0.20 | 70.78 ± 0.41 | 10.29 ± 0.29 | 13.85 ± 0.25 |
| **P-value** | | | | |
| Protein source | NS | NS | NS | NS |
| Additive | NS | NS | P < 0.05 | P < 0.05 |

| | | | | |
|---|---|---|---|---|
| Protein source × additive | NS | NS | NS | NS |

Values are the means ± standard errors of three replicates. Means with different superscripts in the same column are significantly different (P < 0.05).

***Haematological parameters***

Haematological parameters are presented in Table 4. Dietary treatments had no significant effect on the LYM, MON, GRA, LYM (%), MON (%), GRA (%), MCV, MCH, and MCHC of the fish (P > 0.05). However, dietary treatments significantly affected the fish's WBC, RBC, HGB, and HCT (P < 0.05). Fish provided with BSFL and Azo + BSFL without papaya leaf inclusion in the diet had significantly higher WBC compared with other treatments, except for the control. The highest RBC was apparent in group fed BSFL with papaya leaf inclusion in the diet compared with other treatments, except for fish fed control and azo + BSFL, regardless of papaya leaf inclusion. Additionally, there was no notable variation in RBC between red hybrid tilapia fed diet containing BSFL with papaya leaf supplement and group fed azolla with papaya leaf extract supplement in the diet. A similar trend was found in the HGB and HCT. However, there was no significant differences in HGB between fish fed BSFL added with papaya leaf and fish fed azo+ BSFL added with papaya leaf.

The results of the blood biochemical parameters of red hybrid tilapia fed experimental diets are presented in Table 5. Generally, regardless of the protein sources, there were no significant differences in the blood biochemical parameters of the fish (P>0.05). However, the dietary treatments affected the ALKP and CHOL levels of the fish as fish fed control diets had significantly higher ALKP levels in comparison to the group fed azolla, except for the group fed BSFL and Azo + BSFL (P<0.05). Fish fed control diet and azolla had significantly higher CHOL levels compared to the group fed BSFL and Azo + BSFL. A significant interaction was observed between the protein source and additive for BUN, ALKP and CHOL.

Red hybrid tilapia fed Azolla had significantly higher TP levels in comparison to other dietary treatments, except for the group fed BSFL-, Con+, Azo+, BSFL+, Azo + BSF + (P< 0.05). Similarly, the highest BUN level was apparent in red hybrid tilapia fed Azo in comparison with other dietary treatments, apart from the control group (P <0.05). Significantly higher ALT levels were observed in fish fed azo compared to other dietary treatments, except for those fed control, Azo + BSFL-, Azo + and BSFL+ (P< 0.05). Fish fed the control diet and those fed BSFL+ had the highest ALKP levels in comparison with other dietary treatments (P<0.05). The highest CHOL level was apparent in red hybrid tilapia fed the control diet and Azo- in comparison with other dietary treatments (P< 0.05). Red hybrid tilapia fed Azo + BSFL- had the highest CA level compared to other dietary treatments, except for the group fed control and Azo- diets (P < 0.05).

**Table 4.** Haematological parameters of experimental fish fed experimental diets for eight weeks.

| Diet | WBS | LYM | MON | GRA | LYM% | MON% | GRA% | RBC | HGB | HCT | MCV | MCH | MCHC | RDW | PLT | MPV | PCT | PDW |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control (-) | 108.18±2.16[d] | 91.15±0.66 | 13.42±3.02 | 2.90±0.90 | 84.60±3.17 | 12.68±2.44 | 2.72±0.78 | 1.91±0.18[ab] | 8.23±0.70[ab] | 23.38±3.24[ab] | 127.22±3.16 | 42.45±0.06 | 32.18±0.45 | 10.44±0.72 | 44.50±1.19 | 7.50±0.00 | 0.216±0.00 | 9.40±0.00 |
| Azolla (-) | 72.25±21.14[bc] | 65.53±18.80 | 5.55±2.70 | 1.18±0.36 | 90.53±2.34 | 5.85±2.35 | 3.63±2.58 | 1.29±0.41[a] | 6.10±1.47[a] | 16.08±5.40[a] | 118.50±14.16 | 52.78±7.95 | 46.43±8.45 | 14.40±4.33 | 50.25±13.16 | 7.97±0.77 | 0.042±0.01 | 6.90±0.72 |
| BSFL (-) | 90.25±13.49[cd] | 75.80±9.99 | 11.85±3.49 | 2.55±0.00 | 85.25±3.53 | 12.18±2.68 | 2.57±0.88 | 1.63±0.30[ab] | 7.58±0.88[ab] | 21.23±3.56[ab] | 131.65±4.41 | 48.65±4.71 | 36.78±2.53 | 9.78±0.85 | 33.00±4.93 | 6.85±0.05 | 0.025±0.01 | 9.20±0.10 |
| Azo +BSFL (-) | 90.72±16.50[cd] | 76.90±13.20 | 11.65±2.83 | 2.18±0.68 | 85.63±1.78 | 12.15±1.37 | 2.23±0.46 | 1.92±0.21[ab] | 8.58±0.77[ab] | 25.58±2.62[ab] | 133.45±4.24 | 44.90±1.23 | 33.70±0.88 | 10.03±0.42 | 21.70±1.65 | 7.40±0.20 | 0.016±0.00 | 9.73±1.03 |
| Control (+) | 56.10±6.32[bc] | 50.80±5.80 | 3.83±1.05 | 1.40±0.88 | 90.53±1.77 | 6.53±1.02 | 2.95±2.05 | 1.75±0.17[ab] | 8.32±1.17[ab] | 21.33±4.08[ab] | 119.18±13.60 | 47.18±2.99 | 41.15±5.30 | 16.70±6.17 | 101.00±71.09 | 7.25±0.95 | 0.141±0.12 | 8.55±0.35 |
| Azolla (+) | 45.25±6.02[ab] | 41.65±4.85 | 3.08±1.04 | 0.55±0.22 | 92.60±1.51 | 6.28±1.26 | 1.13±0.27 | 1.89±0.08[ab] | 8.30±0.27[ab] | 25.10±1.29[ab] | 133.03±2.93 | 44.15±1.66 | 33.18±1.04 | 9.13±0.68 | 20.75±1.93 | 7.50±0.21 | 0.015±0.00 | 7.53±1.03 |
| BSFL (+) | 50.77±5.92[b] | 45.45±4.60 | 4.48±1.10 | 0.83±0.25 | 90.00±1.47 | 8.45±1.16 | 1.55±0.23 | 2.18±0.10[b] | 9.55±0.24[b] | 29.05±1.16[b] | 133.65±3.67 | 44.05±1.80 | 33.00±1.37 | 8.98±0.80 | 45.75±9.41 | 7.38±0.18 | 0.034±0.01 | 8.90±0.44 |
| Azo +BSFL (+) | 14.08±7.89[a] | 26.00±0.00 | 2.40±0.00 | 0.60±0.00 | 89.00±0.00 | 8.30±0.00 | 1.90±0.00 | 1.97±0.31[ab] | 9.75±0.86[b] | 26.40±4.62[ab] | 134.98±7.55 | 55.33±13.72 | 40.28±8.32 | 9.03±0.13 | 38.00±9.03 | 7.87±0.48 | 0.034±0.01 | 8.23±0.64 |
| **Alternative Ingredients** | | | | | | | | | | | | | | | | | | |
| Control | 82.14±7.65[b] | 70.98±7.10 | 8.62±1.71 | 2.15±0.49 | 87.56±1.71 | 9.60±1.35 | 2.83±0.86 | 1.83±0.16 | 8.28±0.58 | 22.35±2.36 | 123.20±5.29 | 44.81±4.26 | 36.66±3.34 | 13.57±1.79 | 72.75±18.42 | 7.38±0.47 | 0.179±0.03b | 8.98±0.78 |
| Azolla | 58.75±8.06[ab] | 53.59±7.10 | 4.31±1.87 | 0.86±0.54 | 91.56±1.87 | 6.06±1.48 | 2.38±0.94 | 1.59±0.18 | 7.20±0.64 | 20.59±2.59 | 125.76±5.58 | 48.46±4.26 | 39.80±3.34 | 11.76±1.88 | 35.50±18.42 | 7.33±0.31 | 0.029±0.02a | 7.22±0.62 |
| BSFL | 70.51±8.06[ab] | 60.63±7.10 | 8.16±1.87 | 1.69±0.54 | 87.63±1.87 | 10.31±1.48 | 2.06±0.94 | 1.90±0.18 | 8.56±0.64 | 25.14±2.59 | 132.65±5.58 | 46.35±4.26 | 34.89±3.34 | 9.38±1.88 | 39.38±18.42 | 7.11±0.33 | 0.029±0.02a | 9.05±0.55 |
| Azo +BSFL | 52.40±8.06[a] | 51.45±11.23 | 7.03±2.96 | 1.39±0.85 | 87.71±2.96 | 10.23±2.34 | 2.06±0.94 | 1.94±0.18 | 9.16±0.64 | 25.99±2.59 | 134.21±5.58 | 50.11±4.26 | 36.99±3.34 | 9.53±1.88 | 29.88±18.42 | 7.63±0.31 | 0.025±0.02a | 8.98±0.52 |
| **Additive** | | | | | | | | | | | | | | | | | | |
| 0% PLE | 90.35±5.55 | 77.34±5.02 | 10.62±1.27 | 2.20±0.36 | 86.50±1.27 | 10.72±1.00 | 2.79±0.64 | 1.69±0.12 | 7.62±0.43 | 21.57±1.75 | 127.71±3.85 | 47.19±3.01 | 37.27±2.36 | 11.16±1.30 | 37.38±13.03 | 7.43±0.28 | 0.075±0.02 | 8.81±0.47 |

| | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1.95 % PLE | 41.55±5.66 | 40.98±6.64 | 3.44±1.75 | 0.84±0.50 | 90.73±1.75 | 7.39±1.38 | 1.88±0.88 | 1.94±0.12 | 8.98±0.45 | 25.47±1.83 | 130.21±3.95 | 47.68±3.01 | 36.90±2.36 | 10.96±1.33 | 51.38±13.03 | 7.50±0.23 | 0.056±0.01 | 8.30±0.38 |
| **P-value** | | | | | | | | | | | | | | | | | | |
| Protein sources | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | <0.05 | NS |
| Additive | <0.05 | <0.05 | <0.05 | <0.05 | NS | NS | NS | NS | <0.05 | NS | NS | NS | NS | NS | NS | NS | NS | NS |
| Interaction | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS | NS |

Values (means ± standard error for three replicate groups) in each column with different superscripts show significant difference (p < 0.05). NS: not significant. WBC, white blood cell; LYM, lymphocytes; MON, monocytes; GRA, granulocytes; RBC, red blood cells; HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red cell distribution width; PLT, platelet; MPV, mean platelet volume; PCT, procalcitonin; PDW, platelet distribution width.

**Table 5.** Blood biochemical parameters of red hybrid tilapia fed experimental diets for eight weeks

| | ALB (g/dl) | TP (g/dl) | CREA (mg/dl) | BUN (mg/dl) | ALT (u/l) | ALKP (u/l) | CHOL (mg/dl) | CA (mg/dl) |
|---|---|---|---|---|---|---|---|---|
| **Diet** | | | | | | | | |
| Control (-) | 0.695 ± 0.06 | 1.162±0.18$^{a}$ | 0.613±0.06 | 1.783±0.24$^{ab}$ | 21.379±0.86$^{ab}$ | 9.817±0.82$^{c}$ | 9.433±0.76$^{b}$ | 5.057±0.09$^{bc}$ |
| Azolla (-) | 0.633 ± 0.04 | 1.555±0.06$^{b}$ | 0.633±0.05 | 2.453±0.55$^{b}$ | 23.450±0.73$^{b}$ | 6.500±0.48$^{ab}$ | 10.425±0.88$^{b}$ | 5.018±0.15$^{bc}$ |
| BSF (-) | 0.673±0.05 | 1.403±0.04$^{ab}$ | 0.668±0.03 | 1.418±0.06$^{a}$ | 20.075±0.82$^{a}$ | 5.450±0.46$^{ab}$ | 5.075±0.47$^{a}$ | 4.380±0.30$^{ab}$ |
| Azolla + BSF (-) | 0.745±0.04 | 1.185±0.12$^{a}$ | 0.675±0.08 | 1.350±0.04$^{a}$ | 21.050±0.27$^{ab}$ | 5.025±0.31$^{a}$ | 5.153±0.34$^{a}$ | 5.360±0.38$^{c}$ |
| Control (+) | 0.753±0.03 | 1.360±0.06$^{ab}$ | 0.585±0.11 | 1.343±0.03$^{a}$ | 19.825±0.55$^{a}$ | 5.700±1.20$^{ab}$ | 5.900±0.20$^{a}$ | 3.825±0.29$^{a}$ |
| Azolla (+) | 0.765±0.02 | 1.260±0.06$^{ab}$ | 0.663±0.07 | 1.113±0.05$^{a}$ | 21.625±1.56$^{ab}$ | 5.575±0.50$^{ab}$ | 6.125±0.20$^{a}$ | 4.123±0.27$^{a}$ |
| BSF (+) | 0.675±0.03 | 1.323±0.08$^{ab}$ | 0.653±0.03 | 1.333±0.04$^{a}$ | 22.375±0.38$^{ab}$ | 10.350±0.8$^{c}$ | 4.775±0.24$^{a}$ | 4.500±0.34$^{ab}$ |
| Azolla + BSF (+) | 0.730±0.01 | 1.355±0.04$^{ab}$ | 0.725±0.03 | 1.305±0.03$^{a}$ | 20.525±0.33$^{a}$ | 7.650±0.64$^{b}$ | 5.450±0.23$^{a}$ | 4.425±0.19$^{ab}$ |
| **Alternative Ingredients** | | | | | | | | |
| Control | 0.724±0.03 | 1.261±0.07 | 0.599±0.04 | 1.563±0.15 | 20.597±0.54 | 7.758±0.49$^{b}$ | 7.667±0.36$^{b}$ | 4.441±0.17 |
| Azolla | 0.699±0.03 | 1.408±0.08 | 0.647±0.05 | 1.783±0.17 | 22.538±0.59 | 6.038±0.54$^{a}$ | 8.275±0.40$^{b}$ | 4.570±0.18 |
| BSF | 0.674±0.03 | 1.363±0.08 | 0.660±0.05 | 1.375±0.18 | 21.225±0.59 | 7.900±0.54$^{ab}$ | 4.925±0.40$^{a}$ | 4.440±0.18 |
| Azolla +BSF | 0.738±0.03 | 1.270±0.08 | 0.700±0.05 | 1.328±0.17 | 20.788±0.59 | 6.338±0.54$^{ab}$ | 5.301±0.40$^{a}$ | 4.893±0.18 |
| Additive | | | | | | | | |
| 0 % PLE | 0.686±0.02 | 1.326±0.05 | 0.647±0.03 | 1.751±0.11 | 21.486±0.40 | 6.698±0.36 | 7.521±0.27 | 4.954±0.13 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 1.95 % PLE | 0.731± 0.02 | 1.324±0.06 | 0.656± 0.03 | 1.273±0.12 | 21.088±0.42 | 7.319±0.38 | 5.563±0.28 | 4.218±0.13 |
| **Two-way ANOVA** | | | | | | | | |
| Protein source | NS | NS | NS | NS | NS | P<0.05 | P<0.05 | NS |
| Additive | NS | NS | NS | P<0.05 | NS | NS | P<0.05 | P<0.05 |
| Interaction | NS | NS | NS | P<0.05 | NS | P<0.05 | P<0.05 | NS |

Values (means ± standard error for three replicate groups) in each column with different superscripts show significant difference ($p < 0.05$). NS: not significant. Abbreviation: ALB = Albumin, GLOB = globulin, TP = total protein, BUN = blood urea nitrogen, Crea = creatinine, ALKP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma glutamyltransferase, GLU = glucose, CHOL = cholesterol, TBIL = total bilirubin.

## DISCUSSION

In this study, we seek to confirm the potential use of alternative ingredients, black soldier fly larvae and Azolla along with the inclusion of papaya leaf extract as anadditive to improve the growth performance of red hybrid tilapia. It is confirmed that adding the papaya leaf extract had positive effects on the growth and nutrient utilization of red hybrid tilapia. This finding was consistent with a study conducted (Hamid et al., 2022) in which at 2% papaya leaf extract improved growth performance of red hybrid tilapia. In addition, fish fed diet containing BSFL and papaya leaf supplemented diet had the highest final weight compared with other treatments. Papaya plant (*Carica papaya*) is known to produce a specific proteolytic enzyme called papain. Derived from plants, this enzyme is highly beneficial for health, especially as a protein-dissolving agent (Isa., 2010). Studies showed that papain inclusion in aquafeed promotes growth performance and improved feed utilisation in sterlet (*Acipencer ruthenus*; Wiszniewski et al., 2022), African catfish (*Clarias gariepinus*; Rachmawati et al., 2019), mahseer (*Tor tambra*; Muchlisin et al. 2016) and rohu (*Labeo rohita*; Khati et al., 2015).

This finding aligns with previous studies evaluating the impact of dietary addition of protease on the performance of Nile tilapia, *Oreochromis niloticus* fed plant-based alternative feedstuffs. The authors found that inclusion of protease at 0.5 % and 1.0 % in the diets resulted in significantly increased final weight, weight gain, and SGR and improved feed utilization efficiency (M. S. Hassaan et al., 2020). M. Hassaan et al. (2019) found that supplementation of exogenous protease in diet of Nile tilapia, *Oreochromis niloticus* formulated using cotton seed meal to partially replace fishmeal had significantly increased weight rain, PER and improved FCR. A recent study found that the supplementation of protease in diets of grass carp, *Ctenopharyngodon idella* containing cottonseed meal, rapeseed meal, and soybean meal increased the final weight, weight gain and SGR of the fish significantly (Feng et al., 2023). Shi, Li, Chowdhury, Chen, and Leng (2016) found that addition of protease in low fishmeal diets formulated for gibel carp, *Carassius auratus gibelio* with alternative protein feedstuffs significantly improved growth and feed utilization.

Another study by Li, Chai, Liu, Kabir Chowdhury, and Leng (2016) investigated the effects of dietary inclusion of protease on the growth performance of hybrid tilapia, *Oreochromis niloticus* × *O. aureus* fed diet containing alternative protein feedstuffs (fishmeal, soybean meal, rapeseed meal and cottonseed meal) and found that the inclusion of protease in the diet significantly increased the weight gain and improved the FCR of the fish. Additionally, previous study on the dietary addition of protease in diet of rainbow trout, *Oncorhynchus mykiss,* formulated with pea protein concentrate and canola seed had significantly improved the growth performance (Drew, Racz, Gauthier, & Thiessen, 2005). These studies suggest that dietary inclusion of papaya leaf extract in diets of fish formulated with alternative protein sources can enhance the growth performance. Even though the mechanism behind the growth-promoting effect of papaya leaf extract is not fully understood, it may be associated with the improvement in feed utilization efficiency, gut health, and immunity of fish as it is rich in bioactive compounds such as papain, chymopapain, and carpaine. These bioactive substances have anti-inflammatory, antioxidant, and immune-enhancing properties, which can promote better nutrient absorption and utilization in fish.

Despite the positive impacts of dietary inclusion of exogenous enzyme in these studies, Farhangi and Carter (2007) reported that inclusion of protease in diet of rainbow trout, *Oncorhynchus mykiss* diets formulated with dehulled lupin had no significant impact on the performance of the fish. Bolton et al.

(2021) also demonstrated that the addition of exogenous protease in diet of rainbow trout, *Oncorhynchus mykiss* formulated using BSFL as partial substitute for fishmeal had no significant impact on the growth performance (weight gain, SGR, PER, FCR). The inconsistent results reported in these studies can be attributed to several factors such as species type, age, size, type and level of supplementation of the additive, ingredients for diet formulation and duration of the feeding trial.

In recent years, there has been a growing interest in the utilization of alternative protein sources in fish diets due to the rising cost and limited availability of major conventional protein sources such as fishmeal and soybean meal. However, limitations associated with using alternative protein sources, such as lower nutritional quality and anti-nutritional factors, can limit digestibility, resulting in decreased growth performance, poor digestibility, and poor nutrient utilization in fish. In order to address these limitations, several additives, including exogenous enzymes, phytogenics, probiotics, prebiotics, organic acids and immunostimulants, have been applied in fish diets formulated using alternative protein sources, and the results showed improved growth performance (Gobi et al., 2018; Hamid et al., 2022; Nawaz, Irshad, Hoseinifar, & Xiong, 2018; Tan, Chen, & Hu, 2019). Particularly, exogenous enzymes have been proposed to improve the utilization of alternative protein sources in feeds as they aid in breaking down complex proteins and carbohydrates into more easily digestible molecules. For instance, exogenous proteases are important in improving digestibility by enhancing digestion by hydrolyzing complex proteins into polypeptides and amino acids, thus, facilitating digestion and absorption of nutrients. Also, exogenous proteases can reduce or eliminate protease inhibitors in various alternative feedstuffs.

Although several studies have investigated the impacts of various protein sources on the performance of aquaculture species (Deng et al., 2010; Egerton et al., 2020; Kaushik et al., 1995), a review of related studies revealed that there is paucity of information on the impact of papaya leaf extract supplementation on growth performance of aquaculture species fed diets containing potential sustainable alternative protein sources, BSFL and azolla.

Hepato-somatic index is an indicator of the energy reserve of fish. This study found that dietary treatments had no harmful effects on the HSI of red hybrid tilapia. These findings are consistent with previous studies investigating the addition of inclusion of protease in diet of rainbow trout, *Oncorhynchus mykiss* containing BSFL as partial replacement for fishmeal (Bolton et al., 2021). This suggests that papaya that papaya leaf extract did not reduce organ fat accumulation and did not improve organ function hence, the non-significant effects. Additionally, it can be inferred that the supplementation of papaya leaf extract in diets of red hybrid tilapia formulated using potential sustainable alternative protein sources had no significant impact on the energy reserve of the fish, inferring that the fish had normal metabolic rate when fed dietary treatment diets.

Similarly, adding papaya leaf extract in the experimental diets did not cause any significant changes in the viscero-somatic index of the fish. This findings are in agreement with Bolton et al. (2021) who found that addition of exogenous protease in formulated diet of rainbow trout, *Oncorhynchus mykiss* containing BSFL had no significant impact on the VSI. This indicates that the fish had a normal gastrointestinal condition, irrespective of the specific diets they were fed on. However, it is important to note that the effects of papaya leaf extract may vary depending on the fish species, level of inclusion, duration of the feeding trial, type and level of alternative protein source, and the age and size of the fish.

The results of whole-body composition in this study showed that dietary treatments had significant effect on the lipid and ash content of the fish. However, no significant differences were observed in the moisture and protein contents of the fish among the dietary treatments. Shi et al. (2016) found that inclusion of exogenous protease enzyme in diet of gibel carp, *Carassius auratus gibelio* formulated with low fishmeal using different plant protein sources had no significant influence on the moisture, protein, and ash contents of the fish. However, significantly improved lipid content was apparent in the fish. In another study, dietary protease supplementation had no significant effects on whole-body composition (moisture, protein, lipid, ash) of rainbow trout, *Oncorhynchus mykiss* (Farhangi & Carter, 2007). Similarly, Bolton et al. (2021) demonstrated that supplementation of protease in diets containing BSFL as fishmeal substitute had no significant effect on the protein, lipid and ash of rainbow trout *Oncorhynchus mykiss* but significantly affected the moisture content.

Hematological parameters are important indicators of fish health and are commonly used in aquaculture to evaluate the physiological status of fish. In this study, dietary protein sources and papaya leaf extract supplementation had no deleterious effects on most of the hematological parameters evaluated, indicating that supplementation of the extract in formulated diet with alternative protein feed ingredients had no influence on the normal blood condition of the fish. However, the protein sources and papaya leaf inclusion significantly affected the WBCs, HGB, HCT, and RBCs of red hybrid tilapia, although without a clear trend. Supplementation of protease in diets of Nile tilapia, *Oreochromis niloticus* resulted in improved the HGB, HCT and RBCs, but the WBCs were unaffected (M. S. Hassaan et al., 2020). Similarly, supplementation of protease in diets of Nile tilapia, *Oreochromis niloticus* formulated using cotton seed meal to partially replace fishmeal had significantly increased HGB, HCT and RBC (M. Hassaan et al., 2019). A recent study by Islam, Ferdous, Mamun, Akhter, and Zahangir (2021) reported that supplementation exogenous protease enzyme, trypsin in diet of striped catfish, *Pangasianodon hypophthalmus* significantly increased the hematological parameters (RBC, WBC, HGB, and HCT). However, Adeoye, Jaramillo-Torres, Fox, Merrifield, and Davies (2016) found that supplementation exogenous protease, phytase and xylanase had no significant effect on the hematological parameters of Nile tilapia, *Oreochromis niloticus.*

A recent study indicated that by replacing fishmeal with azolla in diet of Nile tilapia, *Oreochromis niloticus*, the growth and feed utilization of the fish were enhanced. The supplementation of azolla on growth of Rohu, *Labeo rohita* had significantly improved growth even at 40 % level of inclusion compared with the control. In contrast, supplementation of azolla up to 20 % did not affect the growth performance of genetically improved tilapia but reduced the growth at higher levels of inclusion (Magouz, Dawood, Salem, & Mohamed, 2020). High fibre contents and anti-nutritional factors linked to plant-based protein sources can interfere with the utilization of nutrients thereby affecting growth performance in fish.

Additionally, BSFL plays an important role in reducing environmental degradation by recycling organic wastes into valuable protein, and it can be sustainably produced using organic wastes as feedstock. Studies investigating the utilization of BSFL in the diet of rice field eel, *Monopterus albus* reported improved weight gain, condition factor and decreased FCR compared with the control (Hu et al., 2020).

It is crucial to explore other alternative sources that are economically and ecologically feasible. During the past several years, there has been increased interest in using alternative protein sources in aquaculture feed production to substitute fishmeal and soybean meals in the diets of farmed fish. The use of alternative protein sources can reduce the pressure on wild-caught fish stocks, promote sustainable aquaculture practices and reduce the cost of feed production as most of the alternative ingredients are cheaper than fishmeal (Hardy, 2010; Hongmanee, Wongmaneeprateep, Boonyoung, & Yuangsoi, 2022; Klinger & Naylor, 2012; Olsen & Hasan, 2012)..

One of promising sustainable alternative protein source for aquaculture feed production is insect meals. Insects are rich in protein and are currently considered a significant, potentially sustainable alternative protein source. Conversely, the dietary addition of BSFL had no significant improvement in the growth performance of Atlantic salmon, *Salmo salar* (Belghit et al., 2019). According to Zhou, Liu, Ji, and Yu (2018), substituting fishmeal with BSFL did not significantly impact the growth performance, serum biochemical and whole-body composition of Jian carp, *Cyprinus carpio var. Jian*. Tippayadara et al. (2021) also found that the replacement of fishmeal with BSFL in the diets of Nile tilapia, *Oreochromis niloticus* had no significant influence on the growth performance and hematological parameters of the fish. Although studies on the use of black soldier fly larva reported mixed but promising results, the presence of chitin can potentially limit its use in aquaculture feed production.

Although the utilization of alternative protein sources in aquaculture feed production is becoming increasingly popular due to the rising demand for sustainable food production, these alternative protein sources often lack certain essential nutrients, which can limit their effectiveness in fish diets. Additionally, they are of lower nutritional value compared to fishmeal. BSFL and azolla meals are potential alternative protein sources as they are cheap and less competitive in comparison to fishmeal. However, these feedstuffs have lower nutritional profile compared to fishmeal. Studies have shown that a combination of protein sources with diverse nutritional properties is generally a more efficient approach for substituting fishmeal compared to relying on individual protein sources for replacement as they can complement the deficiencies by improving the nutritional value (Oliva-Teles, Enes, & Peres, 2015). However, low palatability, unbalanced essential amino acid profiles and the presence of anti-nutritional factors are often associated with alternative protein sources, with adverse effects on the growth performance and overall health status of fish. Feed additives can help to overcome these limitations by increasing the availability and utilization of nutrients thus, improving the efficiency of feeds formulated using alternative protein feedstuffs.

For the sustainability of aquaculture, it is essential to find novel, cost-effective alternative protein sources and feed additives that can replace fishmeal without negatively impacting the performance of aquaculture species (Soltan, Soaudy, Abdella, & Hassaan, 2023; Ye et al., 2019). Despite the beneficial effects of papaya leaf, a review of related literature shows that there is scarcity of information on its use as additives in aquaculture feed production. Additionally, there is death of information on the dietary supplementation of the extract in practical diets of red hybrid tilapia formulated using BSLFL, azolla and the combination of azolla and BSFL. Therefore, it is necessary to investigate the effect of various alternative protein sources, with or without papaya leaf extract supplementation on the performance and overall health status of red hybrid tilapia, *Oreochromis mossambicus* × *Oreochromis niloticus*, an important model species for freshwater aquaculture.

## CONCLUSION

This study shows papaya leaf extract can be included in practical diets of fish formulated with sustainable protein sources to improve growth performance without any harmful effects. The authors recommend further studies to understand the mode of action between papaya leaf extract and hematological parameters to better understand how the extract impact on the overall health of fish.

### Patents

Not applicable.

### Author Contribution

Conceptualization, N.K.A.H, methodology, P.O. S & Z. A. K ; software, P.O. S.; validation, N.K.A.H., and.; formal analysis, N.K.A.H., investigation, N.K.A.H.; resources, N.K.A.H., H.A.H; data curation, P.O.S, N.K.A.H ; writing—original draft preparation, P.O.S & N. K.A.H ; writing—review and editing,N.K.AH

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