Chapter 3

Materials and Methods

3. Materials and Methods

3.1. Collection of *Lemna minor* and *Ipomoea aquatica*

Two aquatic macrophytes, *Lemna minor* L. and *Ipomoea aquatica* Forssk. Were selected for the evaluation of the plant feed ingredient for possible replacement of the fish meal for the present study. *L. minor* is a small, free-floating aquatic plant widely distributed across temperate and tropical regions (Falaye et al., 2022), while *I. aquatica* is a semi-aquatic plant native to tropical and subtropical regions that thrive in wetlands, ponds, lakes, rivers, and slow-moving streams (Roy et al., 2022). The samples of these two plants were collected from freshwater bodies in the Deborgaon, Kokrajhar region of Assam, India. Immediately after collection, the samples were transported to the Fish Biology Laboratory at the Department of Zoology, Bodoland University, for further processing and identification. Herbarium specimens were prepared following standard protocols to ensure accurate identification. These specimens were then submitted to the Bodoland University Botanical Herbarium (BUBH) in the Department of Botany, Bodoland University, where their identities were confirmed and submitted to BUBH with accession numbers BUBH0000898 for *Ipomoea aquatica* Forssk. and BUBH0000899 for *Lemna minor* L. The images of plants are displayed in Figure 1(a) and (b).

The plants were manually screened for contamination and then washed thoroughly using tap water to remove dirt, debris, and potential contaminants from the plant surfaces. After washing, these were spread out on a clean surface and left to air dry under the shade. Following the initial air drying, the plants were further dried in an oven at 45 °C to ensure they were completely dried. Once dried, the plants were crushed into smaller fragments and ground into a fine powder using a mechanical grinder. The ground powder was then sieved (mesh size, 150 μ) to ensure uniform particle size. The finely powdered plant materials were transferred in airtight containers and stored at -20 °C until further analysis.

Classification of *Lemna minor*:

Kingdom:	Plantae
Class:	Liliopsida
Order:	Arales
Family:	Lemnaceae
Genus:	*Lemna*
Species:	*Lemna minor*

Figure 1(a)

Classification of *Ipomoea aquatica*:

Kingdom:	Plantae
Class:	Magnoliopsida
Order:	Solanales
Family:	Convolvulaceae
Genus:	*Ipomoea*
Species:	*Ipomoea aquatica*

Figure 1(b)

Figure 1. Aquatic macrophytes (a) *Lemna minor* and (b) *Ipomoea aquatica*.

3.2. Experimental fish species

Anabas testudineus (a freshwater fish native to South and Southeast Asia, known to inhabit diverse aquatic habitats such as rivers, lakes, ponds, swamps, and rice paddies) and *Heteropneustes fossilis* (a freshwater fish native to South and Southeast Asia, typically found in freshwater habitats including rivers, ponds, lakes, canals, and seasonally flooded fields) were selected as the test species for our study. The fish were collected from Bijni Fish Farm in Goraimari, Assam. The larvae of the two fish species were obtained by induced breeding technique. The fish larvae of similar size were carefully transported to the wet lab facility of the Department of Zoology, Bodoland University, Kokrajhar, in transparent polythene bags filled with water and oxygen to ensure optimal conditions for the fish.

Upon arrival at the laboratory, the fish were placed in holding tanks and acclimated to laboratory conditions (temperature, 25.2 °C - 27.4 °C; dissolved oxygen, 6.40 - 7.36 mg L^{-1} and pH, 6.97 - 7.09) for 15 days. These fish were fed a 40% protein diet twice daily at 5% body weight during this period. This was intended to allow them to adjust to the controlled environment before the experimental trials commenced, ensuring they were in a stable physiological state. The fish were closely monitored during this time to ensure their health and well-being. The scientific classification of *A. testudineus* and *H. fossilis* is presented in Figures 2(a) and (b).

Classification of *Anabas testudineus*:

Kingdom:	Animalia
Phylum:	Chordata
Class:	Actinopterygii
Order:	Anabantiformes
Family:	Anabantidae
Genus:	*Anabas*
Species:	*Anabas testudineus*

Figure 2 (a)

Classification of *Heteropneustes fossilis*:

Kingdom:	Animalia
Phylum:	Chordata
Class:	Actinopterygii
Order:	Siluriformes
Family:	Heteropneustidae
Genus:	*Heteropneustes*
Species:	*Heteropneustes fossilis*

Figure 2 (b)

Figure 2. Experimental fish (a) *Anabas testudineus* and (b) *Heteropneustes fossilis* used for the study.

3.3. Fish culture unit and experimental design

Fish were housed in individual glass tanks (50 L), each with dimensions of 60 cm in length, 30 cm in width, and 30 cm in height. For each treatment, three tanks were used to stock the fish larvae for the feeding trial. Water in each tank was replaced by 50% daily with fresh water by removing an equal volume of the existing water. The water quality parameters (dissolved oxygen, pH and temperature) were checked (APHA, 2017) and maintained at optimal levels during the experimental period.

Each tank was also connected to an aeration system, which provided continuous aeration throughout the experimental period. This aeration system was essential to ensure adequate oxygen levels in the water, supporting the fish's respiratory needs. The tanks were organised systematically, with sufficient spacing for easy access and maintenance. An individual tank and a section of the feeding trial set up is shown in Figures 3 (a) and (b).

Figure 3 (a)

Figure 3 (b)

Figure 3. The fish culture unit facility (a) an individual tank and (b) a section of the feeding trial set up.

3.4. Proximate composition analysis

Proximate composition analysis of dried plants (*L. minor* and *I. aquatica*), different feed prepared and fish (*A. testudineus* and *H. fossilis*) muscle tissue were conducted following AOAC (2000) procedures, with all analyses performed in triplicate. Total nitrogen content was measured using the Micro Kjeldahl method. Samples were digested with concentrated H_2SO_4 (SRL, India) and the resulting ammonia was distilled, trapped in boric acid, and titrated with HCl (Loba Chemie, India). Crude protein was calculated using a 6.25 conversion factor. Moisture content was calculated by drying the sample at 105 °C until a constant weight was achieved, and ash content was obtained by burning the sample at 550 °C and weighing the residual ash. Fat content was measured using Soxhlet extraction with petroleum ether, and the fat residue was weighed. Crude fibre was determined by treating the sample with dilute acid and alkali, leaving indigestible fibre dried and weighed to calculate crude fibre content.

3.5. Antinutritional factors

Antinutritional factors, including oxalate, phytic acid, alkaloids, tannins, and saponins, were quantitatively analysed using standardised protocols. The methods used for each specific factor are described below, with references to established procedures.

3.5.1. Oxalate

The oxalate content was analysed using a titrimetric method (Unuofin et al., 2017) with some modifications. Five grams of the powdered sample were extracted with 75 mL of 3 M H_2SO_4 (Loba Chemie, India) for 1 hour and then filtered. From the filtrate, 5 mL aliquots were titrated with 0.05 M $KMnO_4$ (SRL, India) until a reddish-brown endpoint was reached. Oxalate content was calculated using the conversion factor (2.2 mg oxalate = 1 mL of 0.05 M $KMnO_4$) and expressed as a percentage (%) based on triplicate determinations.

3.5.2. Phytic acid

A titrimetric method (Aina et al., 2012) was used to determine the phytic acid content, with slight modifications. Five grams of powdered sample were extracted with 100 mL of 2% HCl (Loba Chemie, India) for 3 hours. The solution was filtered, and a 25 mL aliquot of the filtrate was mixed with 5 mL of 0.3% ammonium thiocyanate (SRL, India). The mixture was titrated with standard $FeCl_3$ (SRL, India) solution until a brownish-yellow endpoint appeared. Phytic acid content was calculated using the formula:

$$\text{Phytic acid (\%)} = \text{titre value} \times 0.00195 \times 1.19 \times 100.$$

3.5.3. Alkaloid

The alkaloid content was determined according to Harborne (1998). Five grams of powdered sample were extracted with 200 mL of 10% acetic acid in ethanol for 4 hours. The extract was filtered, concentrated to one-quarter of its original volume, and treated with concentrated ammonium hydroxide to precipitate the alkaloids. After allowing the precipitate to settle for 3 hours, it was washed with

0.1M ammonium hydroxide (SRL, India), filtered, dried, and weighed. The alkaloid content percentage was then calculated.

$$\text{Alkaloids (\%)} = \frac{\text{Weight of alkaloid}}{\text{Weight of sample}} \times 100$$

3.5.4. Tannic acid

Tannic acid content was determined using the method of Ogunjobi et al. (2020) and Panchal & Jha (2021), with slight modifications. Five grams of powdered sample were boiled in 500 mL of distilled water for 1 hour. The mixture was filtered, and the filtrate was diluted with 50 mL of water. From the diluted filtrate, 1 mL was mixed with 0.5 mL Folin-Denis reagent (SRL, India), 1 mL sodium carbonate solution, and 8 mL distilled water. The mixture was vortexed and left for 30 minutes at 25 °C for colour development. Absorbance was measured at 725 nm (UV-Visible Double Beam Spectrophotometer 2203, Systronics, India). Tannic acid content was determined using a standard curve prepared with known concentrations of tannic acid and expressed as a percentage (%) based on triplicate determinations.

3.5.5. Saponin

Saponin determination was performed using the methods described by Ejikeme et al. (2014) and Obadoni and Ochuko (2002). Five grams of powdered sample were mixed with 100 mL of 20% aqueous ethanol in a 250 mL conical flask and heated at 55 °C for 4 hours with continuous stirring. The residue was filtered and extracted again with another 100 mL of 20% aqueous ethanol. The extracts were concentrated by boiling at 90 °C for 40 minutes. The mixture was transferred to a separating funnel, shaken with 20 mL of diethyl ether, and after 10-20 minutes, the ether layer was discarded. This process was repeated with 60 mL of butanol (SRL, India), and the butanol extract was washed with 5% NaCl (SRL, India). After discarding the NaCl layer, the solution was heated for 30 minutes in a water bath, dried in an oven, and weighed. The saponin content was then calculated:

$$\text{Saponin (\%)} = \frac{\text{Weight of saponin}}{\text{Weight of sample}} \times 100$$

3.6. Amino acid analysis

Amino acid profiling of plant feed ingredients and fish muscle tissue was conducted using LC-MS with TQD MS/MS (Nimbalkar et al., 2012). The sample was homogenised with 0.1% formic acid in 20% methanol, centrifuged, and diluted. After filtering through a 0.2 µm membrane, the solution was injected into the UPLC-MS/MS system. For hydrolysis, the sample was mixed with 6 M HCl (SRL, India), heated under vacuum at 110 °C for 6 hours, then cooled and diluted. The supernatant was reconstituted in 0.1% formic acid in 20% methanol, filtered, and injected into the system.

Chromatographic separation was performed using a Waters UPLC H-Class system (Waters Corporation, Milford, USA) with a BEH C18 column. A gradient elution method was applied, with phases varying from 95% aqueous to 40% organic over 19 minutes at a flow rate of 0.1 mL min^{-1}. Amino acids were monitored using a PDA detector, and the UPLC effluent was introduced directly into the TQD-MS/MS system for accurate identification and quantification.

3.7. Fatty acid analysis

The fatty acid profile of the plant feed ingredient and fish muscle tissue was analysed using gas chromatography-mass spectrometry (GC-MS) with a Clarus 680 GC and a Clarus 600C MS (PerkinElmer, USA). Following the protocol of Folch et al. (1957), crude lipid was extracted from the sample using a chloroform/methanol solution (2:1, v/v), with three replicates for each dietary group. Fatty acid methyl esters (FAME) were prepared from the crude lipid extracts by acid transesterification, where the lipid was treated with 1% H_2SO_4 (SRL, India) in methanol (Loba Chemie, India) at 50 °C for 16 hours (Christie, 2003). Following purification and extraction, helium gas (99.99% purity) was used as the carrier gas at 1 mL min^{-1} for the fatty acid analysis. A 1 µL sample was injected in splitless mode, with the injector set at 280 °C and the ion source temperature at 180 °C. The oven temperature was programmed to start at 40 °C for 2 minutes, rise to 140 °C at a rate of 7 °C min^{-1} (held for 2 minutes), then increase to 300 °C at 10 °C min^{-1} (held for 5 minutes), completing the analysis

in 41.86 minutes. A 5-minute solvent delay was set. Mass spectra were recorded in Electron Impact positive (EI+) mode at 70 eV, with an m/z range of 50-600 amu. Peaks in the chromatogram were identified by comparing mass spectra to the National Institute Standard and Technology- 2014 (NIST-2014) library, reporting the names, molecular weights, and empirical formulas of the fatty acids.

3.8. Experimental feed formulation

3.8.1. *Lemna minor* supplemented feed formulation

Five isonitrogenous experimental diets containing 40% crude protein were formulated using WinFeed 2.8 software (Cambridge, United Kingdom). These diets were designed to include increasing levels of *L. minor* replacing fish meal, with the following designations: LM0 (0% *L. minor*), LM5 (5% *L. minor*), LM10 (10% *L. minor*), LM15 (15% *L. minor*), and LM20 (20% *L. minor*). The detailed formulation of each diet is presented in Table 2, showing the percentage composition of dry matter ingredients. Dry fish powder was procured from a local fish market at Kokrajhar, Assam, India.

The experimental diets were prepared by weighing and thoroughly mixing all ingredients to achieve uniformity. Water was gradually incorporated to create a dough-like consistency, which was then shaped into 2 mm pellets using a manual pelletiser. The pellets were dried in an oven at 50 °C to ensure proper moisture removal and were stored in airtight containers until the feeding trial.

3.8.2. *Ipomoea aquatica* incorporated diet formulation

Five isonitrogenous experimental diets containing 40% crude protein were formulated using WinFeed 2.8 software (Cambridge, United Kingdom). The diets were designed with increasing levels of *I. aquatica* replacing fish meal and were designated as follows: IA0 (0% *I. aquatica*), IA5 (5% *I. aquatica*), IA10 (10% *I. aquatica*), IA15 (15% *I. aquatica*), and IA20 (20% *I. aquatica*). The detailed formulation of each diet is presented in Table 3, outlining the

percentage composition of dry matter ingredients. Dry fish powder was procured from a local fish market at Kokrajhar, Assam, India.

The diet preparation involved accurately weighing and thoroughly mixing all ingredients to achieve a homogenous blend. Water was slowly added to the dry mixture until it reached a dough-like consistency. The dough was then processed through a manual pelletiser to form 2 mm diameter pellets. The pellets were oven-dried at 50 °C to reduce moisture and preserve them. After drying, the pellets were stored in airtight containers to maintain their quality and freshness until the feeding trial began.

Table 2. The feed composition and proximate analysis of the *Lemna minor* incorporated experimental diets (% dry matter basis).

Ingredients (%)	**LM0**	**LM5**	**LM10**	**LM15**	**LM20**
Dry fish powder*	47.27	46.49	45.7	44.92	44.14
Lemna minor	0	5	10	15	20
Wheat flour	51.33	47.11	42.9	38.68	34.46
Vitamin & mineral premix$^{\phi}$	0.4	0.4	0.4	0.4	0.4
Cod liver oil$^{\Psi}$	1	1	1	1	1
Proximate analysis (%)					
Protein	38.43	38.93	37.47	38.99	39.76
Moisture	5.26	5.21	5.42	5.50	5.75
Ash	6.79	7.38	8.00	8.96	9.46
Fibre	1.94	1.77	2.43	3.58	3.73
Lipid	4.90	4.48	3.95	5.06	4.99
Carbohydrate	42.68	42.23	42.73	37.91	36.31

*Local Market, Kokrajhar, Assam.

$^{\phi}$Vitamins: 5000IU Vitamin A, 500mcg Methylcobalamin, 400IU Vitamin D3, 150mcg D – Biotin USP, 75mg Ascorbic acid, 50mg Vitamin B3, 25mg Tocopheryl Acetate, 10mg Calcium D-Pantothenate, 5mg Vitamin B2, 5mg Vitamin B1, 1.5mg Folic Acid, 1.5mg Vitamin B6. Trace Elements: 2mg Copper Sulphate, 250mcg Chromium Picolinate, 70mcg Selenium, 25mcg Sodium Molybdate, 5mg Manganese Sulphate Monohydrate. Amino acid: 50mg L- Glutamic acid

$^{\Psi}$SEACOD, Cod Liver Oil (Type B) BP Universal Medicare, Mumbai, India.

Table 3. The feed composition and proximate analysis of the *Ipomoea aquatica* incorporated experimental diets (% dry matter basis).

Ingredients (%)	**IA0**	**IA5**	**IA10**	**IA15**	**IA20**
Dry fish powder*	47.27	46.33	45.38	44.44	43.5
Ipomoea aquatica	0	5	10	15	20
Wheat flour	51.33	47.27	43.22	39.16	35.1
Vitamin & mineral premix$^{\phi}$	0.4	0.4	0.4	0.4	0.4
Cod liver oil$^{\Psi}$	1	1	1	1	1
Proximate analysis (%)					
Protein	39.94	39.98	39.57	39.85	39.95
Moisture	5.60	5.49	5.43	5.55	5.61
Ash	7.34	7.37	7.36	7.38	7.36
Fibre	1.86	1.85	1.91	1.95	1.94
Lipid	4.93	4.96	5.04	5.04	5.13
Carbohydrate	40.33	40.35	40.69	40.23	40.01

*Local Market, Kokrajhar, Assam.

$^{\phi}$Vitamins: 5000IU Vitamin A, 500mcg Methylcobalamin, 400IU Vitamin D3, 150mcg D – Biotin USP, 75mg Ascorbic acid, 50mg Vitamin B3, 25mg Tocopheryl Acetate, 10mg Calcium D-Pantothenate, 5mg Vitamin B2, 5mg Vitamin B1, 1.5mg Folic Acid, 1.5mg Vitamin B6. Trace Elements: 2mg Copper Sulphate, 250mcg Chromium Picolinate, 70mcg Selenium, 25mcg Sodium Molybdate, 5mg Manganese Sulphate Monohydrate. Amino acid: 50mg L- Glutamic acid

$^{\Psi}$SEACOD, Cod Liver Oil (Type B) BP Universal Medicare, Mumbai, India.

3.9. Feeding trial and sampling

The feeding trial was conducted for 60 days. Fish were housed in 15 aquaria, with each diet randomly assigned to three replicate aquaria (n = 3 per diet). Each aquarium contained a set number of fish (n = 30 for *A. testudineus*, n = 50 for *H. fossilis*), and the fish were fed one of the five experimental diets. Feeding was conducted twice daily, at 9:30 AM and 4:30 PM, at a feeding rate of 5% of the fish's body weight. The body weight of the fish was recorded weekly, and the feeding rate was adjusted accordingly to maintain the 5% feeding rate. Uneaten feed was collected one hour after each feeding session to monitor feed

consumption and calculate feed utilisation parameters. The feed was carefully siphoned from the bottom of the aquarium, dried, and weighed. This data was used to calculate feed consumption, which allowed for the calculation of feed conversion ratio (FCR) and other growth-related parameters. The flow charts for the feeding trials using *L. minor* and *I. aquatica* are shown in Figures 4 and 5, respectively.

3.10. Growth performance of fish

3.10.1. Average weight

The initial and final weights of the fish in each tank were measured using an electronic balance (AT-R series, Shimadzu, Japan) with an accuracy of 0.0001 g. The fish (n = 30) in each group were individually weighed regularly up to the end of the trial. The mean weight of all fish in each tank was calculated, and the average weight for that group was considered, expressed in grams (g). Regular weight monitoring was done to assess growth performance throughout the experimental period.

Several parameters related to growth performance were evaluated, such as body weight gain (BWG), specific growth rate (SGR), protein efficiency ratio (PER), feed utilisation efficiency (FE), feed conversion ratio (FCR) and survival rate (SR) following standard protocol (Castell & Tiews, 1980).

3.10.2. Survival rate (SR)

The SR of the fish during the trial was determined to evaluate the percentage of fish that survived until the end of the study. This was calculated using the formula:

$$\text{SR (\%)} = \frac{\text{Final number of fish}}{\text{Initial number of fish}} \times 100$$

The initial number of fish was recorded at the start of the experiment, and the final number was counted at the end. Any mortalities during the study were recorded and subtracted from the initial count to determine the final number.

3.10.3. Body weight gain (BWG)

The BWG of the fish was calculated to determine the percentage increase in weight over the study period. This was expressed as:

$$\text{BWG (\%)} = \frac{\text{Final body weight in g} - \text{Initial body weight in g}}{\text{Initial body mass in g}} \times 100$$

The difference between the fish's final and initial body weight was used to assess growth performance, and the result was expressed as a percentage.

3.10.4. Specific growth rate (SGR)

The specific growth rate (SGR) was calculated to assess the daily growth of the fish over the experimental period. The SGR was determined using the following formula:

$$\text{SGR (\% day}^{-1}\text{)} = \frac{\ln(\text{Final body weight in g}) - \ln(\text{Initial body weight in g})}{\text{Number of trial days}} \times 100$$

The natural logarithms (ln) of the final and initial body weight were calculated to reflect the exponential growth of the fish over time.

3.10.5. Feed conversion ratio (FCR)

The feed conversion ratio (FCR) was calculated to evaluate the efficiency of fish feed utilisation. FCR was expressed as:

$$\text{FCR} = \frac{\text{Dry feed fed (g)}}{\text{Weight gain (g)}}$$

This ratio reflects the amount of dry feed consumed by the fish to achieve a unit gain in body weight.

3.10.6. Feed efficiency (FE)

Feed efficiency (FE) was calculated to determine the effectiveness of the feed in promoting weight gain. The formula used was:

$$\text{FE (\%)} = \frac{\text{Total final body weight in g} - \text{Total initial body weight in g}}{\text{Total dry feed}} \times 100$$

This value indicates the percentage of feed that was converted into body weight.

3.10.7. Protein efficiency ratio (PER)

The protein efficiency ratio (PER) was calculated to assess the efficiency of dietary protein in promoting growth. PER was expressed as:

$$\text{PER} = \frac{\text{Weight gain (g)}}{\text{Protein intake (g)}}$$

This ratio provides insight into how well the fish used dietary protein for growth.

3.11. Digestive enzyme activity

After the 60-day trial period, entire digestive tracts were collected from fish after being subjected to a 24-hour fast and anaesthetisation. Fifteen fish per treatment were included by randomly selecting five fish from each of the three replicates. Dissections were performed in a chilled environment, and samples were homogenised (1:10 w/v, tissue: distilled water) using a mechanical tissue homogeniser. Following 10,000 × g centrifugation (Eppendorf 5425R, Germany), the supernatants were collected and stored separately for further studies at -20 °C.

3.11.1. Amylase

The amylase activity was measured using Bernfeld's method (1955). A substrate mixture of 200 µL 1% starch (SRL, India) solution in 200 µL of 0.1 M phosphate buffer (pH 7.0) with 200 µL of 1% NaCl (SRL, India) was prepared. Then, 200 µL of crude enzyme extract was added to the test solution. The solutions were incubated at 37 °C for 1 hour. After incubation, the reaction was stopped by adding 3,5-dinitrosalicylic acid (SRL, India). The mixture was then placed in hot water for 10 minutes and allowed to cool, and the optical density (OD) was measured at 540 nm using a spectrophotometer (UV-1900i, Shimadzu, Japan). Amylase activity was expressed as units (U) per milligram of protein (U

mg^{-1}), where one unit of amylase activity was defined as the amount of enzyme required to release one µmol of maltose per milligram of protein per hour at 37 °C.

3.11.2. Lipase

Lipase activity was measured using p-nitrophenyl palmitate (Merck, Germany) as the substrate, following the method of Winkler and Stuckmann (1979). To prepare the substrate, 30 mg of p-nitrophenyl palmitate was dissolved in 10 mL of isopropanol (Merck, Germany) and then mixed with 90 mL of 0.05 M Sorensen phosphate buffer (pH 8.0). A freshly prepared solution of this substrate (2.4 mL) was prewarmed at 37 °C and then mixed with 100 µL of enzyme extract. After a 15-minute incubation at 37 °C, the OD was measured at 410 nm using a spectrophotometer (UV-1900i, Shimadzu, Japan). One enzyme unit (U) is defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per minute per millilitre of the substrate (U mL^{-1}). The extinction coefficient of p-nitrophenol is 15,000 cm^2 mg^{-1}.

3.11.3. Pepsin

Pepsin activity was assessed using the method described by Anson (1938), with haemoglobin (SRL, India) serving as the substrate. A reaction mixture was prepared by combining 100 µL of crude enzyme extract with 500 µL of a 2% haemoglobin solution, which was then incubated at 37 °C for 10 minutes. The reaction was halted by adding 1000 µL of 5% trichloroacetic acid (TCA), causing precipitation of the unhydrolysed haemoglobin. The mixture was subsequently centrifuged (Eppendorf 5425R, Germany) at 12,000 ×g for 5 minutes, and the OD of the supernatant was measured at 280 nm using a spectrophotometer (UV-1900i, Shimadzu, Japan). Pepsin activity was reported in units per milligram of protein (U mg^{-1}), with one unit (U) defined as the amount of enzyme needed to hydrolyse 1 µmol of haemoglobin per minute at 37 °C under the assay conditions.

3.11.4. Total protease

The total protease activity was assessed using azocasein (Merck, Germany) as the substrate, following the protocol described by Garcia-Carreno (1992). A reaction mixture containing 500 µL of 1% azocasein (in 50 mM Tris-HCl buffer, pH 7.5) and 500 µL of 50 mM Tris-HCl buffer (pH 7.5) was prepared. Then, a 10 µL sample of crude enzyme extract was added to the mixture. The reaction solution was incubated at 25 °C for 10 minutes. After incubation, the reaction was stopped by adding 500 µL of 20% TCA (SRL, India). The mixture was centrifuged at 12,500 x g for 5 minutes, and the OD of the supernatant was measured at 366 nm using a spectrophotometer (UV-1900i, Shimadzu, Japan). Total protease activity was expressed as units (U) per milligram of protein (U mg^{-1}), where one unit (U) represents the enzyme activity required to produce an absorbance change of 1 unit at 366 nm per minute.

3.11.5. Chymotrypsin

Chymotrypsin activity was measured using N-Benzoyl-L-tyrosine ethyl ester (BTEE, Merck, Germany) as a substrate, following the protocol outlined by Bergmeyer (1974). The reaction mixture consisted of 1.42 mL of 80 mM Tris buffer (pH 7.8, 25 °C), 1.40 mL of 1.18 mM BTEE, and 0.08 mL of 2 M $CaCl_2$ (Merck, Germany), resulting in a total reaction volume of 3.0 mL. To initiate the reaction, 0.05 mL of crude enzyme extract was added, and absorbance was recorded at 256 nm over 5 minutes. The enzyme activity was first calculated in units per millilitre (U mL^{-1}) using the following formula:

$$\text{Units mL}^{-1} = \frac{(\Delta A \text{ test} - \Delta A \text{ blank}) \times TV \times df}{0.964 \times V}$$

Where ΔA = Change in absorbance per minute, TV = total volume (mL) of the reaction mixture in a cuvette, df = Dilution factor of the crude extract solution, 0.964 = millimolar extinction coefficient of BTEE at 256 nm, and V = volume (mL) of crude extract used in the assay. After obtaining the enzyme activity in U mL^{-1}, the result was normalised by dividing by the protein concentration of the crude extract to express the chymotrypsin activity as units

per milligram of protein (U mg^{-1}). Thus, chymotrypsin activity is reported as U mg^{-1}, representing the amount of enzyme required to hydrolyse 1 µmol of BTEE per minute per milligram of protein at pH 7.8 and 25 °C.

3.11.6. Trypsin

Trypsin activity was measured using a spectrophotometric method with N-Benzoyl-L-arginine ethyl ester (Merck, Germany) as the substrate, following Bergmeyer (1974) method. The reaction mixture consisted of 3 mL substrate solution (0.25 mM BAEE prepared in 67 mM sodium phosphate buffer, pH 7.6, 25 °C), 0.125 mL of 1 mM HCl and 0.075 mL of crude extract solution in a total volume of 3.20 mL. Absorbance at 253 nm was recorded for 5 minutes, and the change in absorbance per minute (ΔA) was calculated. The trypsin activity was initially calculated in units per millilitre (U mL^{-1}) using the following formula:

$$\text{Units mL}^{-1} = \frac{(\Delta A \text{ test} - \Delta A \text{ blank}) \times df}{0.001 \times 0.075}$$

Where ΔA= change in absorbance per minute, df = dilution factor of the crude extract solution, 0.001 = the change in absorbance per minute corresponding to one BAEE unit of trypsin activity, 0.075 = volume (mL) of crude extract solution added to the reaction mixture. After obtaining trypsin activity in U mL^{-1}, the result was normalised by dividing by the protein concentration of the crude extract, expressing the activity as units per milligram of protein (U mg^{-1}). Thus, trypsin activity is reported as U mg^{-1}, representing the amount of enzyme necessary to produce a change in absorbance of 0.001 per minute per milligram of protein at pH 7.6 and 25 °C.

3.11.7. Total protein

The total soluble protein content in the enzyme extract was determined using the Bradford method (1976). A standard curve was prepared using bovine serum albumin (SRL, India) at a concentration of 1 mg mL^{-1} as the reference protein. The absorbance of the enzyme extract was measured at 595 nm using a

spectrophotometer (UV-1900i, Shimadzu, Japan), and the total protein was calculated based on the standard curve.

3.12. Biochemical parameters

For the analysis of the biochemical parameters, mucus and wet muscle tissue samples were collected in triplicates from each dietary group at the end of the feeding trial. Mucus collection followed a modified method from Ross et al. (2000), in which fish were starved for 24 hours, anesthetised with phenoxyethanol (0.5 mL L^{-1}), and mucus was collected by placing fish in a polyethylene bag with 10 mL of 50 mM NaCl (Merck, Germany) by shaking gently. The samples were then centrifuged (Eppendorf 5425R, Germany) at 1500 × g (10 minutes, 4 °C), and the supernatant was stored at -20 °C. Muscle tissue extract was prepared separately for each parameter, following the standard method. Total immunoglobulin (TIg), alkaline phosphatase (ALP) and lysozyme (LYZ) activity were measured in the mucus samples, while catalase (CAT), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) activities were assessed in muscle tissue samples.

3.12.1. Total immunoglobulin (TIg)

The TIg concentration in the mucus was measured following the method described by Siwicki and Anderson (1993), with slight modifications. Mucus samples (100 µL) were mixed with 100 µL of 12% polyethylene glycol (SRL, India) solution to precipitate immunoglobulins. The mixture was thoroughly agitated to ensure the immunoglobulins were fully precipitated and then centrifuged (Eppendorf 5425R, Germany) at 10,000 × g for 10 minutes at 4 °C. The supernatant was collected, and the total protein concentration in both the original mucus sample and the supernatant was determined using the Bradford (1976) method, as described above. The total immunoglobulin concentration was calculated using the formula: Total immunoglobulin (mg mL^{-1}) = total protein in mucus sample - total protein in the supernatant.

3.12.2. Lysozyme (LYZ)

LYZ activity was determined using a turbidometric assay (Ross et al., 2000) with slight modifications. 50 µL of mucus sample and 50 µL of 40 mM Na_3PO_4 (Merck, Germany) buffer (pH 6.5) were incubated at 30 °C for 15 minutes. Lyophilised *Micrococcus lysodeikticus* cells (0.3 mg mL^{-1}, Merck, Germany) were used as the substrate, and the reaction was initiated by adding the sample to the bacterial suspension. The absorbance at 450 nm was monitored in a spectrophotometer (UV-1900i, Shimadzu, Japan). LYZ activity was normalised to protein concentration in the sample and is expressed as units per milligram of protein (U mg^{-1}). One unit of LYZ activity was defined as the amount of enzyme causing a 0.001 unit decrease in absorbance per minute.

3.12.3. Alkaline phosphatase (ALP)

ALP activity was determined using the method described by Ross et al. (2000) with modifications. A 50 µL mucus sample was reconstituted in 100 mM NH_4HCO_3 (Merck, Germany) buffer (pH 7.8) containing 1 mM $MgCl_2$ (Merck, Germany) and incubated at 30 °C for 15 minutes. The reaction was initiated by adding the sample to 50 µL of 4mM p-nitrophenyl phosphate (Merck, Germany) substrate. The absorbance was measured at 405 nm over 30 minutes at 30 °C. ALP activity was normalised to protein concentration in the sample and is expressed as units per milligram of protein (U mg^{-1}). One unit of ALP activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol per minute.

3.12.4. Catalase (CAT)

CAT activity was determined using the method described by Aebi (1983), Li & Schellhorn (2007), and Vinagre et al. (2012) with adjustments. 5% muscle homogenate was prepared in cold phosphate buffer (pH 7.4) and then centrifuged (Eppendorf 5425R, Germany) at 16,000 × g for 5 minutes. The 80 µL of sample was added to a 2920 µL of H_2O_2 (0.072% v/v) solution in 50 mM (pH 7.0) potassium phosphate buffer with 1 mM ethylene diamine tetraacetic

acid (EDTA), and absorbance at 240 nm was measured every 15 seconds. Catalase activity was expressed as μmol of H_2O_2 reduced per minute per milligram of protein (U mg^{-1}).

3.12.5. Aspartate aminotransferase (AST)

AST activity was determined using buffered aspartate-α-ketoglutarate substrates following Reitman & Frankel (1957). A 5% muscle homogenate was prepared in phosphate buffer (0.1 M, pH 7.4) at 0 °C. A phosphate buffer (pH 7.4) was prepared by mixing 420 mL of 0.1 M sodium phosphate solution and 80 mL of 0.1 M potassium dihydrogen phosphate solution. A standard curve was prepared using a buffered aspartate substrate and a 2 mM pyruvate standard solution. A pyruvate solution was created for the standard curve, with optical density (OD) values measured at 505 nm.

The AST substrate solution was prepared by dissolving α-ketoglutaric acid (SRL, India) and DL-aspartic acid (SRL, India), adjusted to pH 7.4. For the assay, 500 μL of the buffered aspartate-α-ketoglutarate substrate was initially incubated in test tubes at 37 °C for 5 minutes. Then, 100 μL of tissue extract was added, mixed well, and incubated at 37 °C for 60 minutes. After the incubation, 500 μL of 2,4-dinitrophenylhydrazine (SRL, India) colour reagent was added, mixed well, and allowed to stand at room temperature for 20 minutes. Next, 5 mL of 0.4 N NaOH was added, mixed well, and allowed to stand at room temperature for another 10 minutes. The OD was measured at 505 nm. AST activity was calculated using the standard curve and expressed as units per milligram of protein (U mg^{-1}).

3.12.6. Alanine aminotransferase (ALT)

The ALT activity was determined using buffered alanine-α-ketoglutarate substrates following the method of Reitman and Frankel (1957). A 5% muscle homogenate was prepared in phosphate buffer (0.1 M, pH 7.4) at 0 °C. The phosphate buffer (pH 7.4) was prepared by mixing 420 mL of 0.1 M sodium phosphate solution with 80 mL of 0.1 M potassium dihydrogen phosphate

solution. A standard curve was prepared using a buffered alanine substrate and a 2 mM pyruvate standard solution. The pyruvate solution for the standard curve was prepared, and OD values were measured at 505 nm. The ALT substrate solution was prepared by dissolving α-ketoglutaric acid (SRL, India) and DL-alanine (SRL, India), adjusting the pH to 7.4, and using it in the enzymatic reaction. For the assay, 500 µL of the buffered alanine-α-ketoglutarate substrate was initially incubated in test tubes at 37 °C for 5 minutes. Then, 100 µL of tissue extract was added, mixed well, and incubated at 37 °C for 60 minutes. After the incubation, 500 µL of 2,4-dinitrophenylhydrazine (SRL, India) colour reagent was added, mixed well, and allowed to stand at room temperature for 20 minutes. Next, 5 mL of 0.4 N NaOH was added, mixed well, and allowed to stand at room temperature for another 10 minutes. The OD was measured at 505 nm. ALT activity was calculated using the standard curve and expressed as units per milligram of protein (U mg^{-1}).

3.12.7. Superoxide dismutase (SOD)

SOD activity was determined following the method of Roy et al. (2020), based on the inhibition of superoxide radicals generated by the xanthine oxidase reaction. The reaction mixture consisted of 75 mM phosphate buffer (pH 7.0), 150 mM xanthine (SRL, India), and 1.5 mM nitroblue tetrazolium (SRL, India). The reaction was equilibrated at 25 °C for 5 minutes, after which 0.4 U mL^{-1} xanthine oxidase (SRL, India) was added to initiate superoxide production. OD values at 560 nm were recorded every second for 3 minutes. Following this, 50 µL of the sample extract was added, and the decrease in OD was measured over the next 7 minutes, reflecting SOD activity. The specific activity (U mg^{-1}) was calculated as units per milligram of protein based on the inhibition of superoxide radicals.

3.12.8. Thiobarbituric acid reactive substances (TBARS)

TBARS activity was determined following Ohkawa et al. (1979) to measure lipid peroxidation by detecting malondialdehyde (MDA). Tissue homogenates

(10% w/v in 1.15% KCl) were prepared. To start the reaction, 200 µL sodium dodecyl sulfate (Loba Chemie, India), 1.5 mL acetic acid (pH 3.5, Loba Chemie, India), and 1.5 mL thiobarbituric acid (TBA, SRL, India) were added to 200 µL tissue homogenate, and the volume was adjusted to 4 mL with water. The mixture was heated at 95 °C for 60 minutes to form the MDA-TBA adduct, then cooled. A butanol-pyridine mixture was added, and the mixture was centrifuged. The absorbance was measured at 532 nm. MDA content was calculated using a standard curve with 1,1,3,3-tetramethoxy propane (Himedia, India). The TBARS activity was expressed as units per milligram of protein (U mg^{-1}).

3.13. Statistical analysis

All statistical analyses were performed using SPSS version 23 (IBM Corp., Armonk, NY, USA). The normality of the data was assessed using the Shapiro-Wilk test, where a $P < 0.05$ indicated non-normality. The homogeneity of variances across the groups was tested using Levene's test, with a $P > 0.05$ confirming equal variances, for comparisons between two independent groups, a sample t-test was used. This test compared the means of the two groups, and statistical significance was set at $P < 0.05$.

For comparisons involving more than two groups, a one-way analysis of variance (ANOVA) was conducted to determine whether there were significant differences among group means. When ANOVA results indicated significant differences ($P < 0.05$), Tukey's post hoc test was applied to identify specific pairwise differences between group means.

Regression analysis using a quadratic polynomial model was employed to analyse the relationship between the levels of *I. aquatica* and *L. minor* in the diet and performance parameters such as SGR and FCR. This analysis aimed to identify the optimal inclusion level of *I. aquatica* and *L. minor* that maximises SGR and FCR. All data were expressed as mean ± standard deviation (SD) to indicate variability within groups. Statistical significance was considered at $P < 0.05$ for all tests.

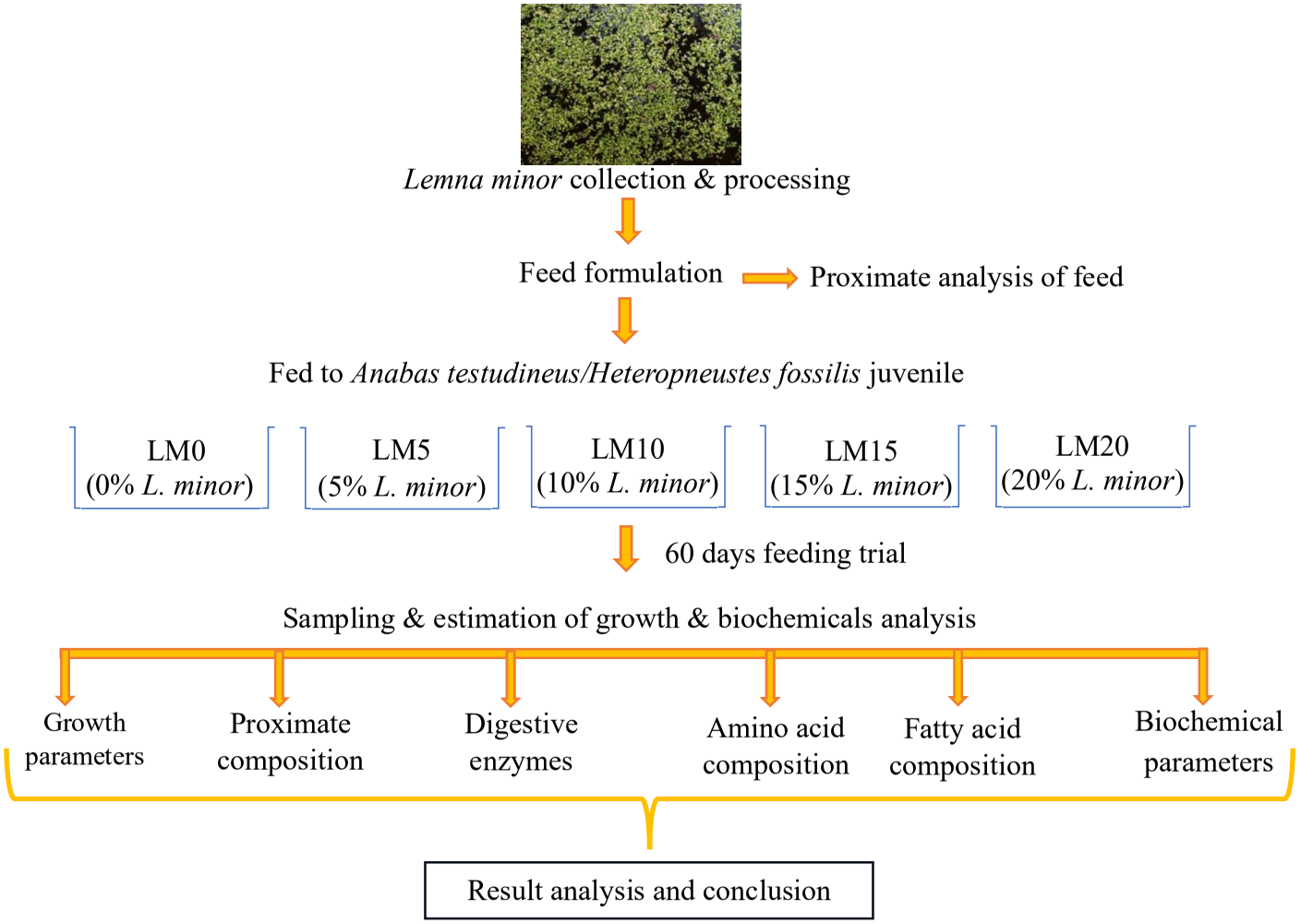

Figure 4. Flow chart showing feeding trial in *Anabas testudineus* and *Heteropneustes fossilis* using *Lemna minor* supplemented feed at different level of supplementation.

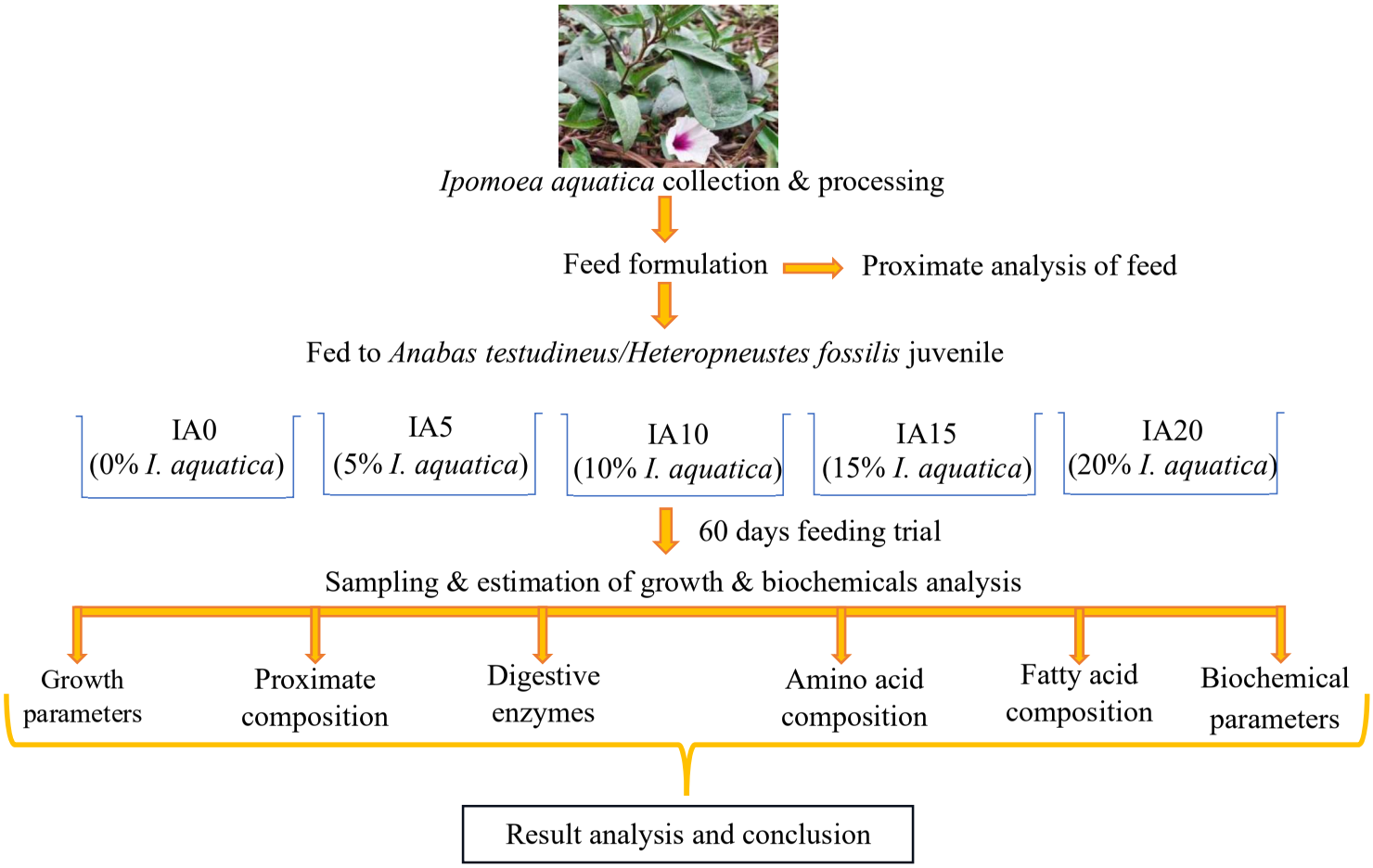

Figure 5. Flow chart showing feeding trial in *Anabas testudineus* and *Heteropneustes fossilis* using *Ipomoea aquatica* supplemented feed at different level of supplementation.