

Can *Azolla filiculoides* be a complementary feed resource for ecological intensification in small-scale fish farming? Biological effects on giant gourami (*Osphronemus goramy*)

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Abstract

Juveniles of giant gourami *Osphronemus goramy* Lacépède (1801) were fed for six weeks with different proportions of fresh *Azolla* (A100% B56% C26% and D0% of replacement). The condition factor (K_{Fulton}) somatic and immunological indicators were measured. Effects of *Azolla* on transport stress and bacterial infection with *Aeromonas hydrophila* were also assessed. Results showed that K_{Fulton} decreased with increasing proportions of *Azolla* in the diet ($p < 0.001$). Total protein, albumin and globulin in fish fed exclusively with *Azolla* (treatment A) were significantly lower than in the other treatments. A significant decrease in lymphocytes was observed in treatments A and B ($p < 0.001$) and fish from these treatments had significantly higher levels of monocytes ($p < 0.001$). Neutrophils were significantly higher in treatment A only ($p = 0.012$). Plasma lysozyme levels and serum bactericidal activity increased significantly with *Azolla* in the diet (both $p < 0.001$). Before transport stress, blood glycaemia was significantly lower in fish from treatment A ($p < 0.001$), after transport, glycaemia increased significantly only in treatments B, C and D ($p < 0.001$) and fifteen days after, survival was significantly higher in fish from treatment A. One week after experimental infection the survival of fish from treatments A and B was significantly higher than for fish from treatments C and D ($p < 0.001$). Results suggest that *A. filiculoides* has positive effects on fish immunological indicators, and resistance to stress and disease but decreases growth performance. We recommend that the replacement of commercial pellets with *A. filiculoides* should not exceed 30%.

1. Introduction

Producing more than 3.4 million tons of farmed freshwater fish in 2020, Indonesia is the third-largest continental fish aquaculture producer after China and India (FAO 2022). As in other Asian countries, farmed fish in Indonesia has become a cash crop (Bostock et al 2010), leading to the increasing use of commercial feeds. Accordingly, the share of unfed species in Asian aquaculture production strongly decreased from 2000 to 2016 (22% vs. 8%; FAO 2018). Although the use of commercial feed is the main driver of the development of aquaculture, this practice causes environmental damage (Edwards 2015; Aubin et al 2019). As an alternative to conventional intensification using commercial feed, “ecological intensification” promotes the use of ecological processes and functions to increase productivity, strengthen ecosystem services and decrease disservices (Aubin et al 2019). Floating macrophytes can provide several ecological services: they can contribute to the diet of many farmed fish species as an alternative to commercial feeds (Fiogbé et al 2004; Yilmaz et al 2004; Velásquez 2016) and may play a role in the remediation of aquaculture effluents (Sipaúba-Tavares et al 2002; Henry-Silva et al 2006; Carlozzi and Padovani 2016). Floating macrophytes are thus increasingly considered for ecological intensification in small-scale aquaculture (Slembrouck et al 2018).

Among floating macrophytes, *Azolla* spp. water ferns are widespread and occur naturally in tropical areas. Beyond its potentially high productivity with doubling times ranging from 1 to 5 days (Wagner 1997; Sadeghi et al 2013; Pouil et al 2020), *Azolla* has interesting nutritional properties, such as a high protein content (15–40% of its dry weight; Brouwer et al 2018; Feedipedia 2018; Slembrouck et al 2018). Thus, *Azolla* is an alternative to costly fish meal and soybeans, especially for the aquaculture of

omnivorous and vegetarian fish (Datta 2011; Gangadhar et al 2015; Das et al 2018). Recently, a multi-criterion study taking six eco-services parameters into account identified *Azolla filiculoides* among five floating macrophytes species as the best candidate to replace — at least partially — commercial feed in freshwater fish aquaculture (Slembrouck et al 2018). Nevertheless, to promote the inclusion of *Azolla* spp. in fish diets, the ecological intensification of fish farming systems requires a thorough investigation of its biological effects on fish, not only on their growth, but also on other aspects, including immunity and/or physiological status.

The effects of dietary *Azolla* on fish physiology have been little investigated. In one rare example, the grass carp *Ctenopharyngodon idella* fed with fresh *A. filiculoides* and formulated pellets show no differences in the concentrations of blood factors including haemoglobin, white blood cells, glucose, cholesterol and cortisol (Nekoubin and Sudagar 2013). In the goldfish *Carassius auratus*, the number of lymphocytes and monocytes is higher in fish consuming 50 g *Azolla* kg⁻¹ diet, declining with increased doses (Vasudhevan et al 2013). Furthermore, *A. microphylla* extracts have hepatoprotective and antioxidant effects on the common carp *Cyprinus carpio* (Kunjiappan et al 2015) and another study has demonstrated that *Azolla* meal does not negatively influence the enzymatic digestive functions or immunological activities of Nile tilapia *Oreochromis niloticus* (Magouz et al 2020). Although divergent, these results suggest that the inclusion of *Azolla* in fish diets can lead to significant physiological changes potentially affecting the sensitivity of fish to disease and stress.

It is also important to underline the challenge in moving from research results to application in the field. For example, nutrition studies have shown that inclusion rates of *Azolla* in feeds of ~ 25–30% do not impair growth in tilapias and Cyprinidae species (e.g. Das et al 2018; Datta, 2011; Gangadhar et al 2015; El-Sayed 1992). Nevertheless, in such investigations, *Azolla* is usually used dried and powdered to replace fish meal in experimental feeds whose formulation has been selected for making isoproteinic, isolipidic and isoenergetic pellets. Although this approach can help to understand the effects of *Azolla* on growth and survival, using dried and powdered *Azolla* requires prior transformation and incurs additional costs and labour that can discourage small-scale fish farmers (~ 98% of small-scale fish farmers are in developing countries; Bhujel 2012). Using *Azolla* in small-scale aquaculture requires the investigation of the potential effects of fresh plant material — partially replacing formulated pellets in the fish diet — on fish growth, physiological state and responses to disease and stress. Instead of considering *Azolla* as an ingredient in formulated pellets, the underlying idea is to include it fresh in fish feeding management, an approach that is easier and directly applicable for fish farmers.

The main objective of this study was thus to assess the biological effects of fresh *A. filiculoides* when used in total or partial replacement of commercial fish feed in giant gourami *Osphronemus goramy*, one of the main freshwater commodities of economic and patrimonial importance in Indonesia (FAO 2019). To assess the wide range of possible impacts of a diet based on *A. filiculoides* on fish growth and health, we performed a series of tests as follows:

1) A six-week feeding trial was conducted to measure growth and evaluate the physiological and immunological state of fish fed with increasing quantities of fresh *Azolla* in replacement of commercial pellets.

2) Then, fish from the previous experiment were divided into two groups and two tests were performed to quantify (1) the sensitivity of fish to transport stress and (2) their sensitivity to bacterial infection (*Aeromonas hydrophila*).

2. Materials And Methods

2.1. Fish and plants: Origin and maintenance

2.1.1. Giant gourami rearing

The experiment was conducted at the experimental facilities of the Research Station for Freshwater Aquaculture (RIFAFE, Cijeruk, West Java, Indonesia). The juveniles of the giant gourami *O. goramy* belonging to the Indonesian “Galunggung” strain (Arifin et al 2018) used in this study came from a single spawn. After one month of rearing the larvae in a recirculating facility, juvenile fish were transferred into net cages set up in an outdoor 400 m² pond until experiments started, approximately 9 months later. Juveniles were pooled, sorted, then individually weighed (body weight, BW: 32.3 ± 4.5 g), measured (total length, TL): 12.4 ± 0.6 cm) and arbitrarily distributed (n = 480) into 12 net cages (surface: 4 m², height: 1 m) placed in the same outdoor pond at a density of 40 individuals cage⁻¹. Fish were acclimated to the experimental conditions for one week before feeding with 1 mm diameter floating commercial pellets and fresh *Azolla* (see Table 1 for proximate composition). After one month and at the end of the experiment, all fish in each net cage were individually measured (BW and TL) to determine growth and to adjust the commercial feed ration.

Table 1

Proximate composition of feeds (commercial pellets and *Azolla*) used to feed giant gourami juveniles. Except for water content, data are expressed as a percentage of dry matter. Data are means $\pm$ SD.

Parameter	Commercial pellets (n = 3)	<i>Azolla</i> (n = 1)
Water content (%)	8.95 $\pm$ 0.21	89.69
Crude protein (%)	38.06 $\pm$ 0.74	28.28
Crude lipid (%)	4.67 $\pm$ 0.47	1.52
Ash (%)	8.78 $\pm$ 0.39	12.17
Crude fibre (%)	3.20 $\pm$ 0.19	10.84
¹ NFE (%)	45.30 $\pm$ 0.28	47.17
Gross energy (MJ kg ⁻¹)	19.23 $\pm$ 0.03	17.30
¹ NFE = Nitrogen-free extract represents carbohydrates.		

Water temperatures were monitored twice a day at 08:00 AM and 04:00 PM and varied between 25.1°C and 26.9°C. Water pH, dissolved oxygen, conductivity and turbidity were measured *in situ* once a week between 07:00 and 08:00 AM using a multi-parameter probe (Hanna HI 9829); their ranges were as follows pH 7.0–8.0, 4.3–7.2 mg L⁻¹ O₂, 102–225 μ S cm⁻¹ conductivity and 20–44 nephelometric turbidity units (NTU). At the same time, water samples were collected and concentrations in ammonia (NH₃), nitrite (NO₂⁻) and nitrate (NO₃⁻) were analysed at the laboratory less than 1 h after sampling using spectrophotometry analysis (Hanna HI83399); the corresponding value ranges were 0.1–0.3 mg NH₃ L⁻¹, 0.00–0.06 mg NO₂⁻ L⁻¹ and 0.6–2.3 mg NO₃⁻ L⁻¹. All experiments involving fish complied with animal welfare regulations (Ethical Approval No. 181–2020 IPB) and were carried out under the Indonesian accreditation SNI 01–6485.2–2000.

2.1.2. *Azolla* production

Three weeks before the experiment, two concrete ponds (7.5 $\times$ 5.5 $\times$ 0.7 m) were emptied, cleaned and filled with water and then each pond was fertilised with chicken manure mixed with rice bran (1:1 ratio, 60 kg). One week later, *A. filiculoides* was seeded (400 g m⁻²) and kept in the same conditions until the experiment began. The observed daily air ranged from 20 to 30°C while water temperatures ranged from 25 to 27°C. Light intensity reached peaks of > 270 μ mol m⁻² s⁻¹. In such culture conditions, *Azolla* yield can reach ~ 4 g DW m⁻² d⁻¹, and the risk of non-production remains < 20% (Slembrouck et al 2018; Pouil et al 2020).

2.2. Experimental procedures

The course of the experiment as well as the different procedures performed are detailed in Fig. 1.

2.2.1. Feeding trial

After acclimation, triplicate net cages containing fish were assigned to experimental fish diet treatments using four levels of fresh *Azolla* in total or partial replacement of floating commercial pellets (100%, 56% and 26% of the feeding events for treatments A, B and C, respectively) and the fish in the three remaining cages (treatment D, i.e. control) were fed exclusively with floating commercial pellets (see Table 2). The feeding trial lasted six weeks as follows: fish from treatment A were fed *Azolla* 7 days week⁻¹, and fish from the two other experimental treatments were fed alternately with fresh *Azolla* and commercial pellets (i.e. 4 days *Azolla*/3 days pellets and 2 days *Azolla*/5 days pellets for treatments B and C, respectively). Fish from treatment D were fed commercial pellets 7 days week⁻¹ (Table 2). Feedings took place at the same time for all the treatments.

Table 2
Experimental design for the different feeding treatments.

Treatments	Number of feedings		Ingested feeds (kg, wet weight) ¹		Ingested proteins (g)	
	Commercial pellets	<i>Azolla</i>	Commercial pellets	<i>Azolla</i>	Commercial pellets	<i>Azolla</i>
A 100% <i>Azolla</i>	-	43	-	10.08 ± 0.40	-	294 ± 12
B 56% <i>Azolla</i>	19	24	1.40 ± 0.05	5.16 ± 0.45	486 ± 18	150 ± 13
C 26% <i>Azolla</i>	32	11	2.68 ± 0.13	1.94 ± 0.07	928 ± 46	57 ± 2
D (control) 0% <i>Azolla</i>	43	-	3.67 ± 0.04	-	1271 ± 12	-
¹ Estimated as described in Section 2.2.1.						

Throughout the experiment, the commercial pellet ration was set to 5% of biomass d⁻¹; *Azolla* rations were the same for treatments A, B and C and constant throughout the experiment (300 g per distribution). Commercial pellets and fresh *Azolla* were placed in round floating frames (diam. 0.4 m) placed on the surface of each cage to prevent the feed from floating out. Before distribution at 08:00 AM, *Azolla* was

carefully wrung out and weighed. At 05:00 PM (i.e. after a 9 h feeding period) the remaining quantities of *Azolla* in the cages were recovered with a dip net, drained and weighed to calculate the proportions of *Azolla* ingested by fish for all the experimental treatments (A, B and C). At the end of the feeding trial, the fish remaining in each net cage were individually measured and counted to assess survival and growth.

The proximate composition of commercial feed, fish and *Azolla* were determined following AOAC methods (AOAC 1999). For *Azolla*, samples were taken each week, then pooled before analysis with equal quantities of mixed *Azolla* (n = 1) and commercial feed (n = 3); fish flesh samples were collected at the beginning (n = 3) and then at the end of the experiment for each feeding treatment (n = 3 per treatment). Gross energy (GE) content was calculated using the following equation $GE = 5.7 \times \text{g protein} + 9.4 \times \text{g crude lipid} + 4.1 \times (\text{g nitrogen-free extract (NFE)} + \text{g fibre})$ (Hall et al 2013).

2.2.2. Transport stress test and disease challenge

A total of 30 fish from the four treatments were used to test resistance to transport stress according to (Urbinati et al 2004). Briefly, fish were placed in double plastic bags (10 fish bag⁻¹) containing approximately 10 L of pond water saturated with oxygen and then transported by car for 6 h. After the transport period, a drop of blood was sampled from each fish (n = 7) from each experimental group to check glycaemia. The fish were released in net cages placed in the concrete pond previously used and survival was checked daily for 14 days. During this time, fish from each feeding treatment were fed as described above.

For the disease challenge, fish from each treatment were transferred to the Depok Research Station for Fish Disease and Control and then assigned to duplicate round 200 L plastic tanks (n = 30 fish per tank). The day after transfer, fish were contaminated by intramuscular injection 0.1 ml 1×10^7 CFU mL⁻¹ of *A. hydrophila*, isolated from diseased African catfish *Clarias gariepinus*. The infecting dose was determined using previous lethal dose 50 (LD₅₀) tests involving giant gourami of similar size. An additional group of 12 fish (n = 3 fish from each feeding treatment) maintained in the same experimental conditions was injected with 1 mL of sterile phosphate buffer saline (PBS) and used as a negative control.

2.3. Measurements and metrics

All the samplings conducted throughout the experiment are summarised in Fig. 1.

2.3.1. Condition factor and somatic indicators

The condition factor (K_{Fulton}) was calculated according to the relationship $K = BW \text{ (g)} / TL \text{ (cm)}^3 \times 100$ (Froese, 2006) on all fish sampled. At the end of the six-week feeding period, 24 fish per treatment were euthanised using Eugenol oil at a concentration of 1 mL L⁻¹, and then individually weighed. To determine the hepatosomatic index (HSI) and spleen somatic index (SSI), the liver and spleen were extracted and weighed to an accuracy of 0.1 mg.

2.3.2. Blood tests and non-specific immune response

Blood was sampled from anaesthetised fish (Eugenol oil, 0.04 mL L^{-1}) at the end of the six-week feeding experiment. Blood taken from the caudal aorta was aliquoted into two microtubes and, for one of them, serum was obtained by allowing blood to clot (without heparin) at 4°C ; plasma was obtained from heparinised blood after centrifugation at 3000 g , for 5 min at 4°C . Serum and plasma were transferred into a sterile Eppendorf tube and then frozen at -20°C until analysis.

Serum total protein, albumin globulin and glutamic pyruvic transaminase (SGPT) were determined on fish ($n = 9$ per treatment) using a benchtop chemistry system Cobas® Mira Plus (Roche Diagnostic Systems). Blood glucose was measured with a portative device (Freestyle® Abbott) for diabetes self-management ($n = 24$ and $n = 7$ per treatment before and after stress tests, respectively). To determine the leucocyte count after the six-week feeding trial, a drop of heparinised blood from nine fish from each experimental treatment was smeared on a glass slide, dried to room temperature and then stained according to the manufacturer's instructions (RAL KIT 555). Differentiation of leucocytes was carried out according to Conroy et al. (2006), dividing white cells into three categories: lymphocytes, monocytes and neutrophil granulocytes; counts were expressed in percentages of 100 counted leucocytes.

Plasma lysozyme measurements on fish at the end of the feeding trial ($n = 15$ per treatment) were performed following the procedure for using an ELISA reader on plasma samples. Aliquots of $30 \mu\text{L}$ of plasma from each sampled fish were added to $170 \mu\text{L}$ of an aqueous suspension of *Micrococcus lysodeikticus* (Sigma, 200 mg L^{-1} in $0.05 \text{ M NaH}_2\text{PO}_4$ at 25°C and $\text{pH } 6.2$). Two adsorption readings were taken at 530 nm using a spectrophotometer after 30 s and then 4.5 min of mixing plasma with the aqueous solution. The unit of lysozyme activity (LU) was defined as the amount of enzyme causing a decrease in absorbance of 0.001 min^{-1} . Bactericidal activity of plasma was determined according to Leano et al., 2003 on the same plasma samples ($n = 15$ per treatment). Equal volumes ($50 \mu\text{L}$) of plasma and bacterial suspension (*A. hydrophila*; 10^4 CFU mL^{-1}) were mixed and incubated for 1 h at 32°C . The mixture was diluted with sterile PBS at a ratio of $1:10$ and $100 \mu\text{L}$ of the dilution was plated onto a nutrient agar plate and incubated at 32°C for $24\text{--}36 \text{ h}$. Colonies were counted (CFU mL^{-1}) and percent killing activity was computed using the following formula:

$$\% \text{ killing} = 1 - (\text{CFU with plasma} / \text{CFU without plasma}) \times 100.$$

A blank control was also run by replacing blood plasma with the same amount of sterile PBS.

2.4. Statistical analysis

Data were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Brown-Forsythe test) and, when necessary, data were $\ln$ -transformed. When normality and homogeneity of variance assumptions were met, one-way (or two-way for glucose data) ANOVAs were used for comparisons of means between treatments, followed by the Holm-Sidak test for pairwise differences; otherwise, when assumptions of normality and homogeneity of variances were not met even after transformation, non-parametric Kruskal-Wallis tests were used followed by Dunn's test. The chi-square test was used to determine significant differences between categories and the Kaplan-Meier survival analysis was used to

analyse the survival curves between treatments following transport and disease challenge. All statistical analyses were performed using SigmaStat software. The level of significance for statistical analyses was always set to $\alpha = 0.05$.

3. Results

3.1. Condition factor and somatic indicators

The final condition factor decreased with increasing proportions of *Azolla* in the diet with significant differences observed between all the treatments ($p < 0.001$). At the end of the experiment, K_{Fulton} values ranged from $1.45 \pm 0.16\%$ in treatment A (100% *Azolla*) to $1.68 \pm 0.09\%$ in treatment D (control, 0% *Azolla*) (Table 3).

Table 3

Growth and survival of giant gourami after 6 weeks of feeding with four levels of fresh *Azolla* in lieu of commercial pellets. Data are means $\pm$ SD. For each parameter, different letters indicate significant differences between treatments.

Parameter	A 100% <i>Azolla</i>	B 56% <i>Azolla</i>	C 26% <i>Azolla</i>	D 0% <i>Azolla</i>	<i>p</i> -value
Initial body weight (BW _i , g)	37.5 $\pm$ 7.0	36.2 $\pm$ 6.7	36.2 $\pm$ 5.5	36.8 $\pm$ 4.8	NS ¹
Final body weight (BW _f , g)	36.8 $\pm$ 6.8 ^a	54.5 $\pm$ 9.6 ^b	61.2 $\pm$ 10.3 ^c	68.4 $\pm$ 10.0 ^d	< 0.001
SGR ² (% d ⁻¹)	-0.04 $\pm$ 0.03 ^a	0.97 $\pm$ 0.09 ^b	1.25 $\pm$ 0.09 ^c	1.48 $\pm$ 0.12 ^d	< 0.001
K_{Fulton}	1.68 $\pm$ 0.09 ^a	1.64 $\pm$ 0.07 ^b	1.56 $\pm$ 0.09 ^c	1.45 $\pm$ 0.16 ^d	< 0.001
Survival (%)	96.7 $\pm$ 2.9	95.8 $\pm$ 2.9	98.3 $\pm$ 2.9	96.7 $\pm$ 3.8	NS ¹
¹ NS = not significant; ² SGR = specific growth rate					

HSI was significantly lower ($p = 0.001$) in giant gourami fed exclusively with *Azolla* (treatment A), but HSI remained similar between the other treatments with a maximum of $1.56 \pm 0.43\%$ in fish from treatment D (0% *Azolla*, Fig. 2A). A similar trend was observed for SSI, with significant differences ($p < 0.001$) between the four experimental treatments and the lowest values observed in fish from treatment A ($0.16 \pm 0.08\%$, Fig. 2B).

3.2. Body composition

Except for crude fibre, the chemical composition of fish was significantly influenced by the inclusion of *Azolla* in the diet (Table 4). The most important changes in proximate composition in giant gourami were observed for crude proteins, crude lipids and NFE. For instance, crude proteins in giant gourami increased significantly ($p < 0.001$) with increased *Azolla*, with the lowest values measured in fish from treatment D ($58.08 \pm 0.37\%$) and the highest values in fish from treatment A ($67.14 \pm 0.66\%$; Table 4). Crude lipids remained similar in fish from treatments B, C and D, but they were 46% lower in fish fed exclusively with *Azolla*. The opposite trend was observed for NFE, for which values were similar in the fish fed (at least partially) with *Azolla*, and values were 87% higher in giant gourami fed exclusively with commercial pellets.

Table 4

Proximate composition of giant gourami fed with experimental feeding regimes using four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D; respectively) after six weeks. Except for water content, data are expressed as a percentage of dry matter. Data are the means $\pm$ SD ($n = 3$ per treatment) and different letters indicate significant differences between treatments.

Parameter	A 100% <i>Azolla</i>	B 56% <i>Azolla</i>	C 26% <i>Azolla</i>	D 0% <i>Azolla</i>	p-values
Water (%)	73.2 ± 0.45^{ab}	70.7 ± 0.67^{abc}	69.1 ± 1.72^c	68.4 ± 0.68^c	0.002
Crude protein (%)	67.1 ± 0.66^a	61.7 ± 2.21^b	61.2 ± 0.96^b	58.1 ± 0.37^c	< 0.001
Crude lipid (%)	9.3 ± 0.77^a	18 ± 0.96^b	17.1 ± 0.86^b	16.9 ± 1.43^b	< 0.001
Ash (%)	17.5 ± 1.13^a	14.5 ± 0.74^{ab}	14.6 ± 1.41^{ab}	13.9 ± 1.15^b	0.018
Crude fibre (%)	0.76 ± 0.05	0.77 ± 0.07	0.75 ± 0.02	0.72 ± 0.03	NS ²
NFE ¹ (%)	5.2 ± 0.33^a	5.1 ± 1.00^a	6.3 ± 1.79^a	10.4 ± 0.27^b	< 0.001
¹ NFE = Nitrogen-free extract					
² NS = non-significant					

3.3. Biochemical and immunological parameters

The glucose concentration in giant gourami measured before transport changed upon inclusion of *Azolla* in their diet, with values ranging from 41 ± 16 to 68 ± 35 to mg dL^{-1} in treatments A and D, respectively. Experimental treatment and transport both affected glucose concentrations, with significant interactions between these two variables ($p < 0.001$), meaning that transport did not affect fish from the different feeding treatments in the same way. Overall, glucose concentrations increased in fish from treatments B, C and D, but remained similar in fish from treatment A, which fed exclusively on *Azolla*. Total protein,

albumin and globulin content in the serum were significantly lower ($p < 0.001$) in fish from treatment A with respect to fish from the other treatments (i.e. B, C and D; Fig. 3B).

SGPT was not modified across feeding regimes and the average values within the groups ranged from 17 to 23 IU L⁻¹ ($p = 0.760$; Fig. 4).

Bactericidal activity of serum increased significantly with increasing proportions of *Azolla* in the diet ($p < 0.001$) and fish from treatment D, fed exclusively with commercial pellets showed the lowest killing activity ($16.0 \pm 7.6\%$, Fig. 5A). For plasma lysozyme, significant differences ($p < 0.001$) were observed between the four experimental treatments, with a clear increase in plasma lysozyme with increasing *Azolla* proportions in the fish diet (from 62.3 ± 15.7 to 135.3 ± 8.8 IU mL⁻¹, Fig. 5B).

Leucocyte count (Fig. 5C) was strongly influenced by the feeding regime.

A significant reduction in lymphocytes was observed in giant gourami fed the highest proportions of *Azolla* (i.e. 56% and 100% for treatments A and B, respectively) compared with the fish at 0% *Azolla* (treatment D) or at low proportions (i.e. 26%, treatment C) with values ranging from $70 \pm 4\%$ to $86 \pm 1\%$ of the leucocytes. Giant gourami from treatments A and B showed significantly higher ($p < 0.001$) levels of monocytes ($17 \pm 4\%$ of the leucocytes) compared with the other treatments ($9 \pm 2\%$ of the leucocytes). Neutrophils were significantly higher in fish from treatment A ($p = 0.012$) with a value of $10 \pm 3\%$ of the leucocytes.

3.4. Transport stress test and disease challenge

Statistical comparisons between fish before and after transport revealed a significant increase in glucose concentrations ($p = 0.001$) for fish from treatments B, C and D, but glucose concentrations remained similar before and after transport in fish from treatment A (Fig. 6A). Fifteen days after the six-hour transport stress test, cumulative mortality differed significantly between the experimental treatments ($\chi^2 = 16.58$, $df = 3$, $p < 0.001$) and survival was significantly higher (97%) in giant gourami fed exclusively with *Azolla* (treatment A), compared with the other treatments (53–77%). There were no significant differences in the survival of fish from the other treatments (B, C and D) (Kaplan-Meier survival analysis, $p < 0.001$; Fig. 6A).

High mortality (91–100%) was observed in the four experimental treatments 144 h after *A. hydrophila* injection. Significant differences in cumulative mortality ($\chi^2 = 11.66$, $df = 3$, $p = 0.009$) were observed between the experimental treatments (Fig. 6B). Overall, the survival rate of giant gourami from treatments A and B was significantly ($\chi^2 = 53.56$, $df = 3$, $p < 0.001$) higher than the survival rate in the two other experimental treatments (8–9% vs. 0–3% for treatments C and D, Fig. 5B). However, after 14 days post-injection, the survival rates were 1.8 and 3.5% for giant gourami from treatments A and B, whereas all the fish died after 5 and 10 d in treatments C and B, respectively (Fig. 6B). No fish died in the negative control.

4. Discussion

Commercial feeds not only leave a non-negligible environmental footprint in many aquaculture farming systems, but also represent a major expense for fish farmers (Henriksson et al 2015). In small-scale fish farming, the use of alternatives to commercial feeds based on local agrosystem resources may offer fish farmers the possibility to better integrate their farm into the ecosystem (Phong et al 2010). Floating macrophytes such as proposed in the present study may be affordable alternatives for the partial or total replacement of commercial feed. When these plants are coproduced on part of the rearing surface of a fish pond, they may substantially contribute to the reduction of the use of commercial feeds and also improve water quality without competition for land (Hasan and Chakrabarti 2009).

The lower growth performance of fish fed with high proportions of fresh *A. filiculoides* is essentially due to the low energy content ingested by fish during feeding treatments with *Azolla*, although the poor digestibility of raw plant ingredients (Das et al 2018) may also play a role. The condition factor decreased with the increasing levels of *Azolla* in the diet. This result suggests that giant gourami fed with diets containing *Azolla* may be less vigorous than fish fed the control diet, and contrast with the findings on rohu *Labeo rohita* (Datta 2011). Such results can be reasonably attributed to our protocol, which included fresh *Azolla* in the diet of giant gourami instead of using it as an ingredient (in dried powdered form) in manufactured pellets.

HSI decreased with increasing *Azolla* in the diet. A drastic decrease in HSI can also be observed during fasting, linked to the depletion of glycogen in the liver; in fact, this polysaccharide is highly present in fish liver and rapidly used for energy by fish during stress conditions or fasting (Barcellos et al., 2010; Nebo et al., 2018). Given the possible suspended or reduced storage of lipids and glycogen in the liver of giant gourami, it is likely that the reduction in HSI was also linked to the active mobilisation and high metabolic rates in the liver induced by the lower energy content of the *Azolla*-based diet. In Nile tilapia fed a low-fat diet, the liver is the primary organ that responds, resulting in elevated glycolysis and accelerated biosynthesis of fatty acids (He et al 2015). SGPT is the most significant indicator of liver inflammation and damage (Kulkarni and Pruthviraj, 2016). Here, there were no differences in the SGPT levels in fish from the four treatments, suggesting that no hepatic damage was observed in giant gourami at any level of pellet replacement with *Azolla*. In an *in vitro* experiment, Kunjiappan et al (2015) even found hepatoprotective effects of *A. microphylla* extract on common carp hepatocytes. *Azolla* supplementation can reduce oxidative stress in fish liver, as demonstrated by higher antioxidant enzyme activities such as SOD, GPx, and CAT, along with reduction of lipid peroxidation products (Attia et al 2021). Such hepatoprotective and antioxidant activity may be linked to the antioxidant ability of *Azolla* species, reducing the accumulation of free radicals (Nayak et al., 2015): *Azolla*'s high polyphenol content presumably helps to scavenge free radicals and increase the expression of antioxidant enzymes (Attia et al 2021).

The effects of *Azolla* on serum proteins in fish have been poorly investigated in the literature. Nevertheless, in the grass carp, there are no significant differences in the levels of albumin, globulin and total proteins between fish fed with *A. filiculoides* and fish fed with commercial pellets for 90 days (Nekoubin and Sudagar 2013). Similar findings have been reported in Nile tilapia by Magouz et al (2013)

at *Azolla* inclusion rates of up to 30%. Our results showed a significant decrease in albumin, globulin and total protein levels in fish fed exclusively with *Azolla* (treatment A) compared with the other treatments with no or lower proportions of *Azolla*. Again, starvation may also reduce serum protein levels, as observed in the gilthead seabream *Sparus aurata* and the common carp (Sala-Rabanal et al 2003; Varga et al 2016). In the European seabass *Dicentrarchus labrax*, tannins reduce the apparent digestibility coefficient (ADC) of crude protein, protein retention (PR) and the protein efficiency ratio (PER) (Omnes et al 2017). Thus, the hypoproteinaemia observed here, may also be an indirect effect of the reduced protein absorption due to tannins contained in *Azolla*. However, this generalised hypoproteinaemia contrasts with the high protein content in the body composition of giant gourami exclusively fed with *Azolla*. In literature, there are contrasting observations on protein content in the body composition of tropical fish fed with fresh or dried *Azolla* (Antoine et al., 1986; Micha et al 1988; El-Sayed 1992; Datta 2011). Ultimately, results observed in giant gourami fed exclusively with *Azolla* and to a lesser extent fed at high replacement rates were very close to those observed in common carp during starvation. The reduction in HSI, the condition factor and lipids in carcasses with an increase in protein content has been reported in this species of fish (Varga et al 2014). In the grass carp, long-term food restriction reduces the accumulation of lipids, with down-regulation of lipogenesis genes and up-regulation of lipolysis genes (Gong et al 2016).

There is a strong relationship between the diet and the immune defences of aquatic organisms (Lumsangkul et al 2022). Intestinal immunity is the first line of defence affected by feed quality (Dawood 2021), whereas skin mucus is more associated with the surrounding environmental conditions (Pérez-Sánchez et al 2017). Lysozymes play an important role in fish immune defences, inducing antibacterial activity in the presence of the complement system (Saurabh and Sahoo 2008; Harikrishnan et al 2012). We observed significantly higher plasma lysozyme levels with increasing levels of *Azolla* in the diet, leading to higher bactericidal activity in fish fed with *Azolla*. Increases in lysozyme levels may be attributed to neutrophilia observed in fish, because lysozymes are mainly produced by these cells (Ellis 2001). The leucocyte counts also revealed influences of an *Azolla*-based diet with an increase in monocytes and neutrophils in fish fed exclusively with *Azolla*, whereas lymphocytes decreased in fish from the same treatment. Similar findings were highlighted in goldfish fed with *Azolla* (Vasudhevan et al., 2013). The high content of carotene, vitamins (vitamin A, vitamin B12, beta carotenes) and phenolic and flavonoid components in *Azolla* is often suggested to explain its positive effects on immunity (e.g., Nayak and Padhy 2017, Noor Nawaz et al 2014). Additionally, dietary inclusion of *Azolla* may increase fatty acid production (e.g., propionate, acetate, and butyrate) (Brouwer et al 2019), which in turn enhances the immune response (Kim 2021; Lumsangkul et al 2022).

Interestingly, one of the rare quantitative studies on this topic isolated immune-stimulating substances from *Azolla* species (Shemami et al 2018), which may explain the higher non-specific immune response in giant gourami fed with *Azolla*. Furthermore, the *Azolla* symbiont *Anabaena azollae* may also play a role in the response to bacterial infection. For instance, Barakat et al (2015) found that *A. azollae* has an antibacterial effect against the bacterium *A. hydrophila*. Similar results have been shown in 10 cyanobacterial species (*Anabaena* sp. and *Oscillatoria* sp.) with antibacterial effects against four species

of the genus *Aeromonas* including *A. hydrophila* (Abdel-Raouf and Ibraheem, 2008). Such findings may explain the reduced mortality in experimental *A. hydrophila* infections we observed in fish fed with high levels of *Azolla*.

Overall, the mortalities observed in the disease challenge confirm that giant gourami is a fish very sensitive to opportunistic diseases typical of intensified aquaculture systems, as well as to stressful conditions simulated here by 6 h of transport by car in oxygen-saturated plastic bags. Interestingly, we found higher survival after transport in fish fed with high levels of *Azolla*. We also showed that the glucose concentration, a well-known indicator of stress in fish (Morgan and Iwama, 2011), was lower in the fish fed exclusively with *Azolla* and did not increase despite induced transport-related stress. Fish fed with 100% commercial pellets and those fed with 26% *Azolla* showed higher SSI. Significantly higher SSI in fish can be found in chronically stressed fish (Michel et al 2013; Xu et al 2018), but also during acute stress (Milla et al 2010). Altogether, our results suggest that giant gourami fed with high levels of *Azolla* may be less sensitive to stress than giant gourami fed on pellets. Previous studies conducted on the influence of plant feedstuffs on the fish diet highlighted that an increase in plant feedstuff inclusion is generally associated with either no effect or deterioration in welfare (e.g., Bonaldo et al 2016; Sadoul et al 2016; Knutsen et al 2019). Nevertheless, these studies mostly involved carnivorous fish species probably less adapted to plant feedstuffs. Interestingly, our findings corroborate traditional giant gourami farming practices, which limit the use of commercial pellets and tend to use more raw plants, including macrophytes, as feed before harvest and transport of giant gourami (FAO 2019). Nevertheless, the underlying biological mechanisms and the discrepancies in the responses observed among fish species remain unclear and require further investigation.

In conclusion, given that few raw fish feed materials are available locally in Indonesia, the ecological intensification of fish aquaculture using floating macrophytes should be considered for omnivorous fish such as the giant gourami. In this study, we demonstrated that fresh *Azolla* can partially replace commercial feed, although fish may require a longer time to reach the commercial size. In this perspective, the replacement rate of commercial feed should not exceed 30%. However, better results could be obtained following specific treatments of *Azolla* such as ensilage, fermentation or drying as shown in several studies (Cruz et al 2011; Fasakin et al. 2001; Hundare et al 2018). Furthermore, the positive impact of *Azolla* on non-specific immunology and resistance to transport stress and opportunistic disease may be promising and requires further investigation of *Azolla* as a potential herbal preventive treatment.

Declarations

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Compliance with Ethical Standards

All experiments involving fish complied with animal welfare regulations (Ethical Approval No. 181-2020 IPB) and were carried out under the Indonesian accreditation SNI 01–6485.2-2000.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Domenico Caruso, Angela Mariana Lusiastuti and Jacques Slembrouck contributed to the study's conception and design. Material preparation, data collection and data analysis were performed by all the authors. The first draft of the manuscript was written by Domenico Caruso and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

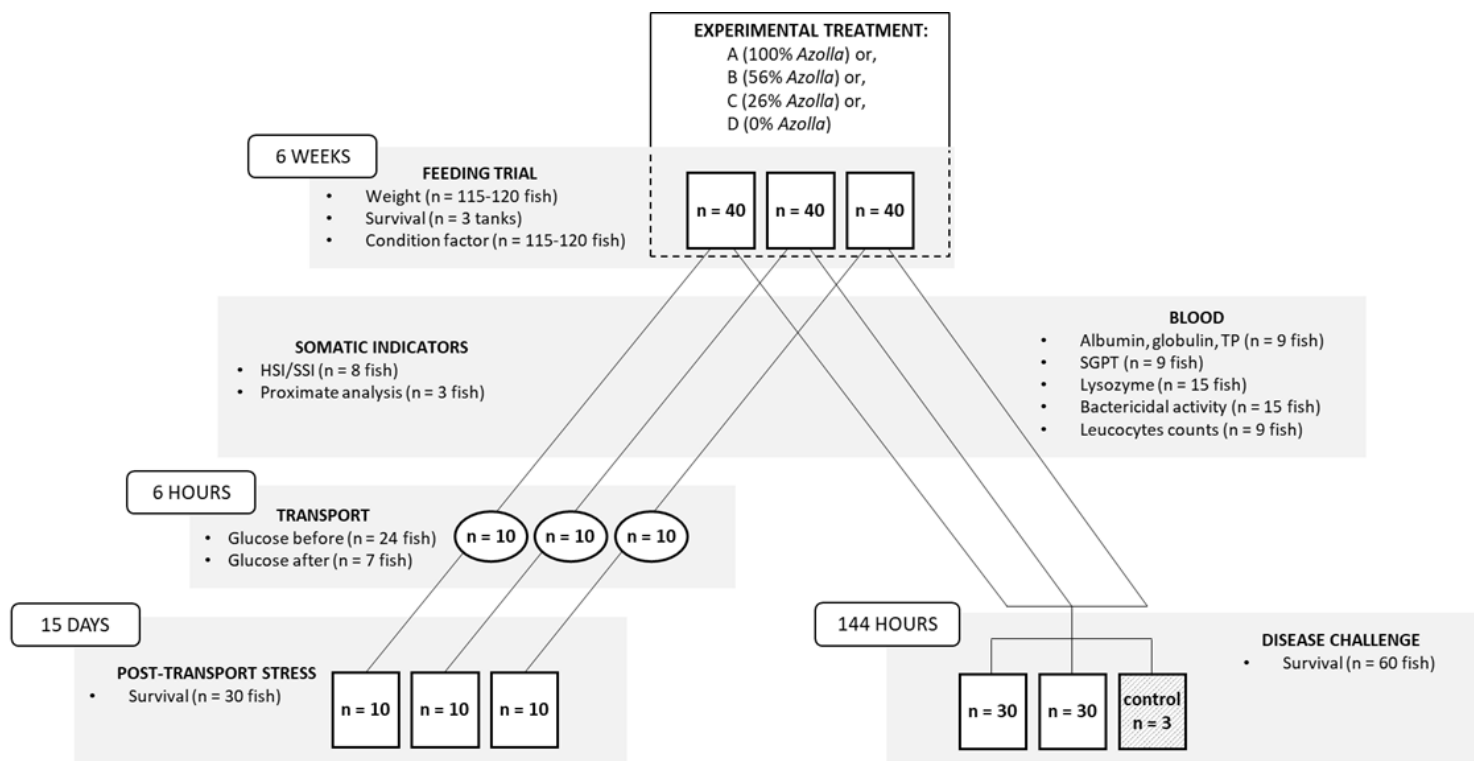


Figure 1

Schematic view of the experimental design. For clarity, the experiments performed are represented for only one feeding treatment, but the other treatments followed the same protocol. HSI/SSI: Hepatosomatic index/spleen somatic index, SGPT: Serum glutamic pyruvic transaminase, TP: Total proteins.

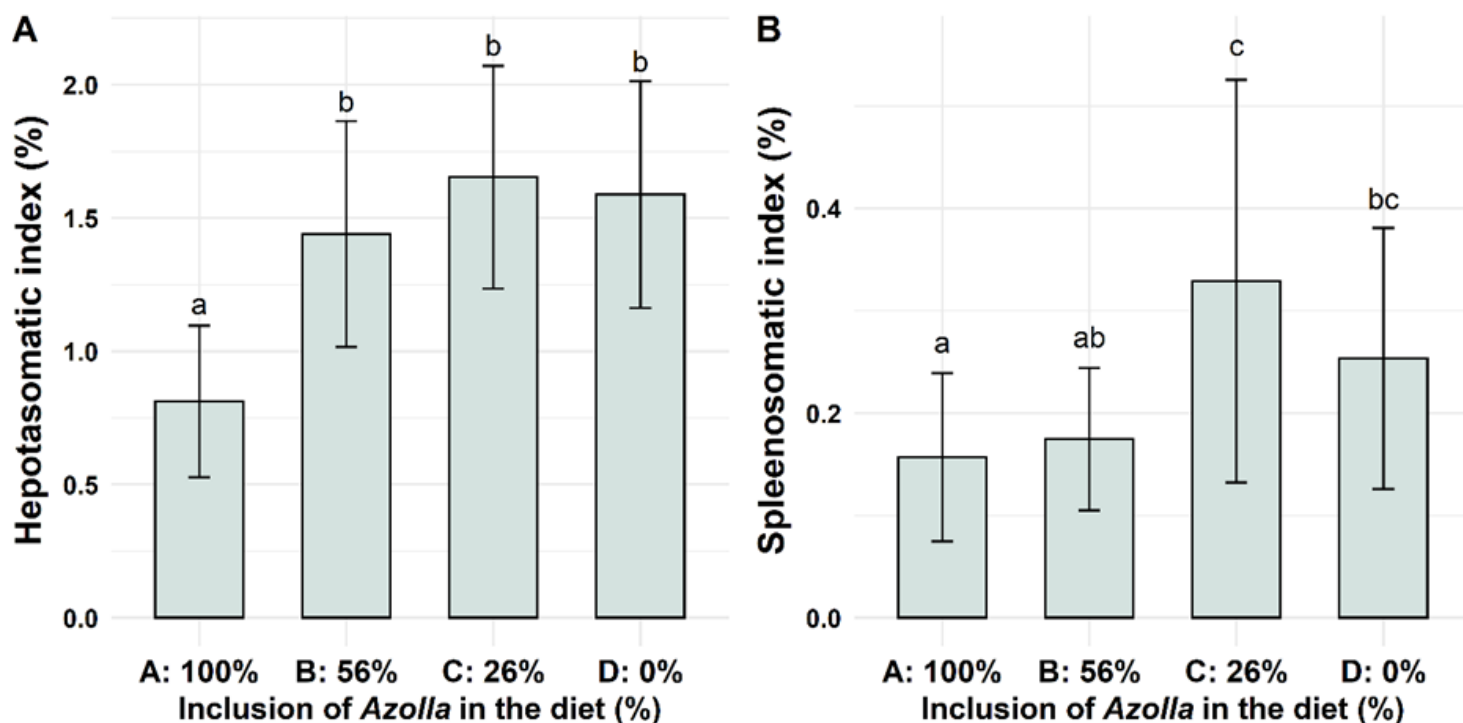


Figure 2

(A) Hepatosomatic index (%) and (B) spleen somatic index (%) in giant gourami fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively) at the end of the six-week experiment. Data are means $\pm$ SD (n = 24 per treatment). Different letters denote significant differences between the feeding regimes.

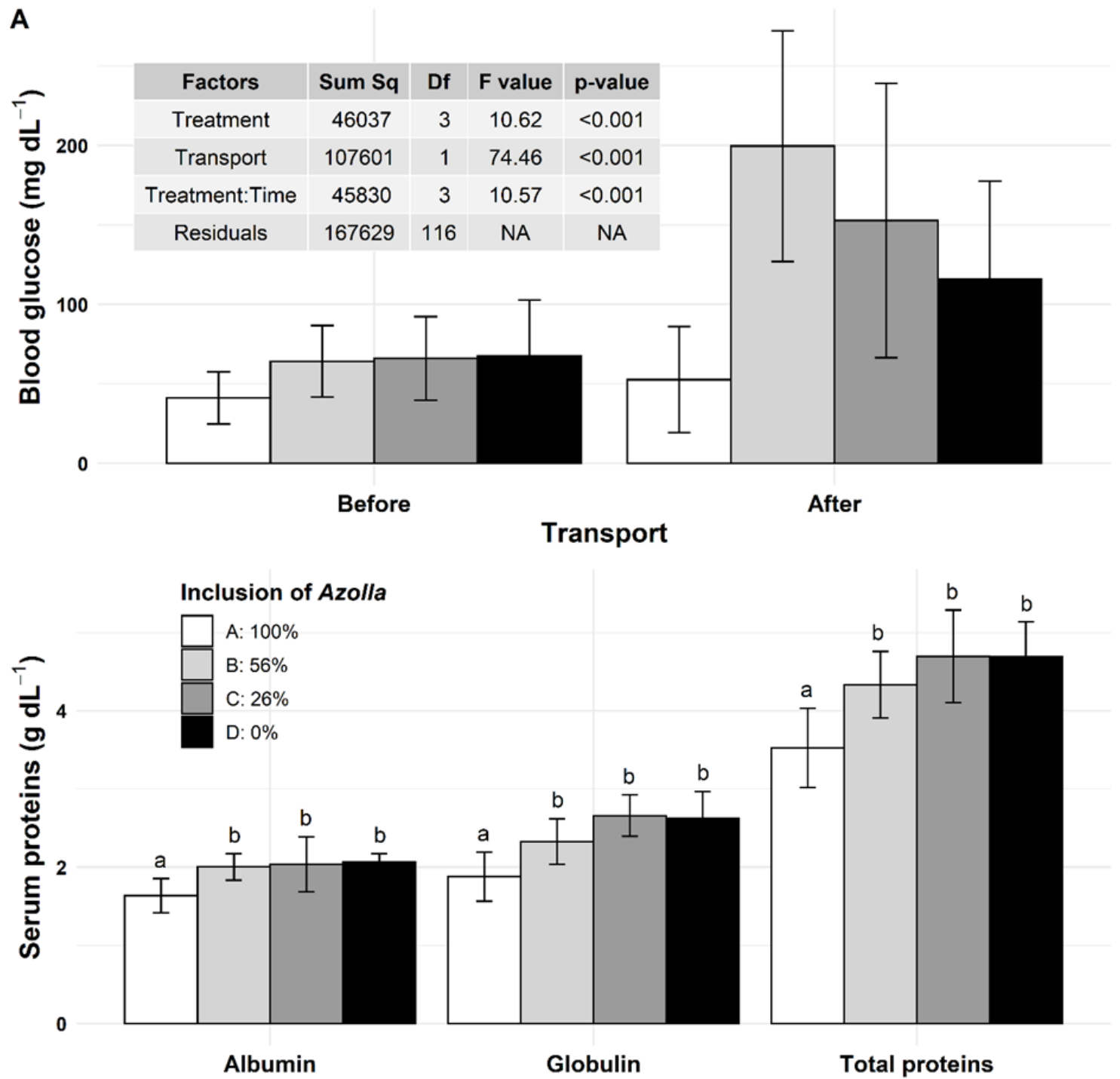


Figure 3

(A) Blood glucose concentration in giant gourami fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively) measured before (n = 24 per treatment) and after (n = 7 per treatment) the transport stress test. (B) Composition of serum proteins (total proteins, albumin, globulin) (n = 9 per treatment). Data are means $\pm$ SD. Different letters indicate significant differences between the experimental treatments.

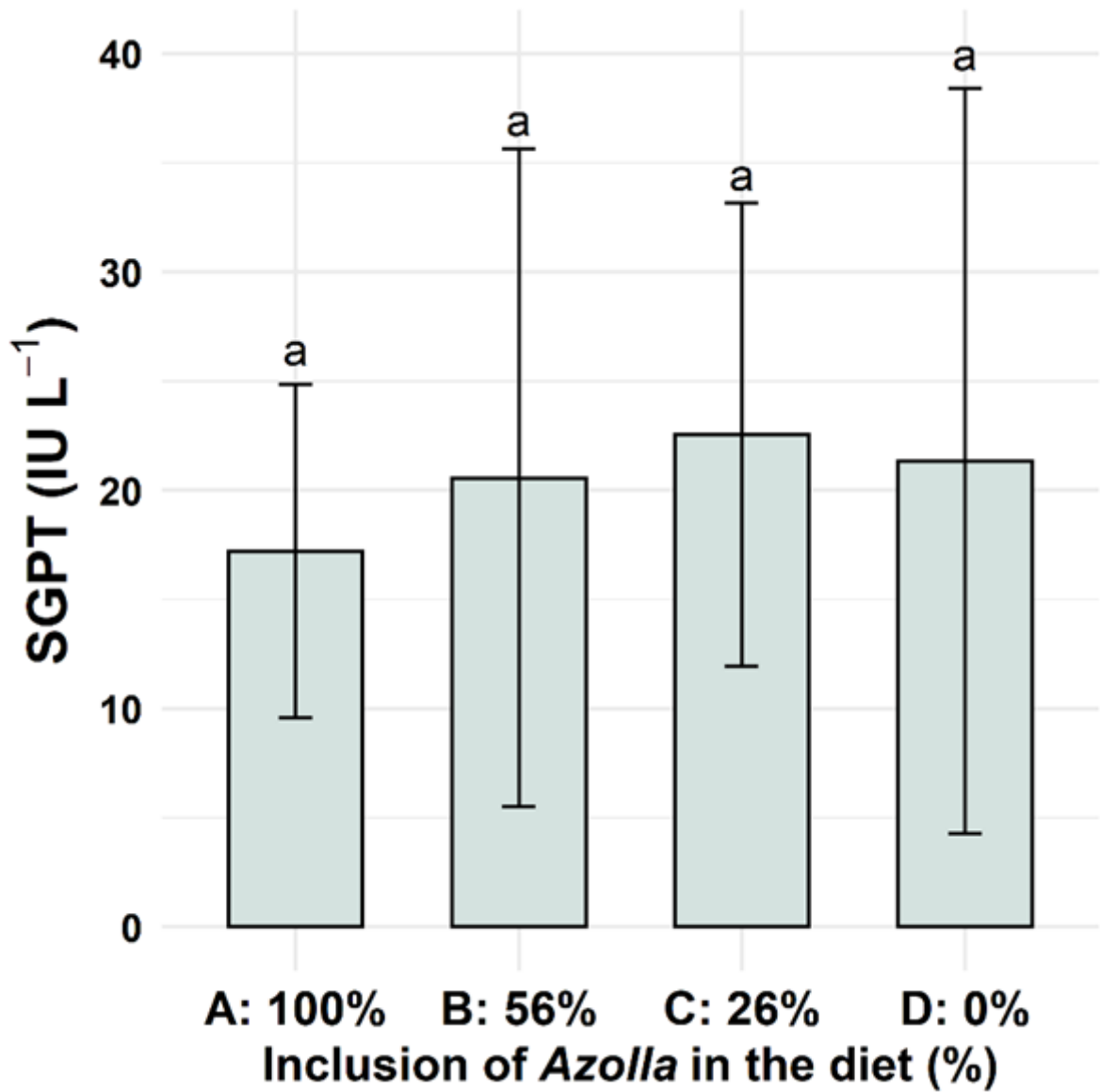


Figure 4

Serum glutamic pyruvic transaminase (IU L⁻¹) in giant gourami blood (n = 9 per treatment) fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively).

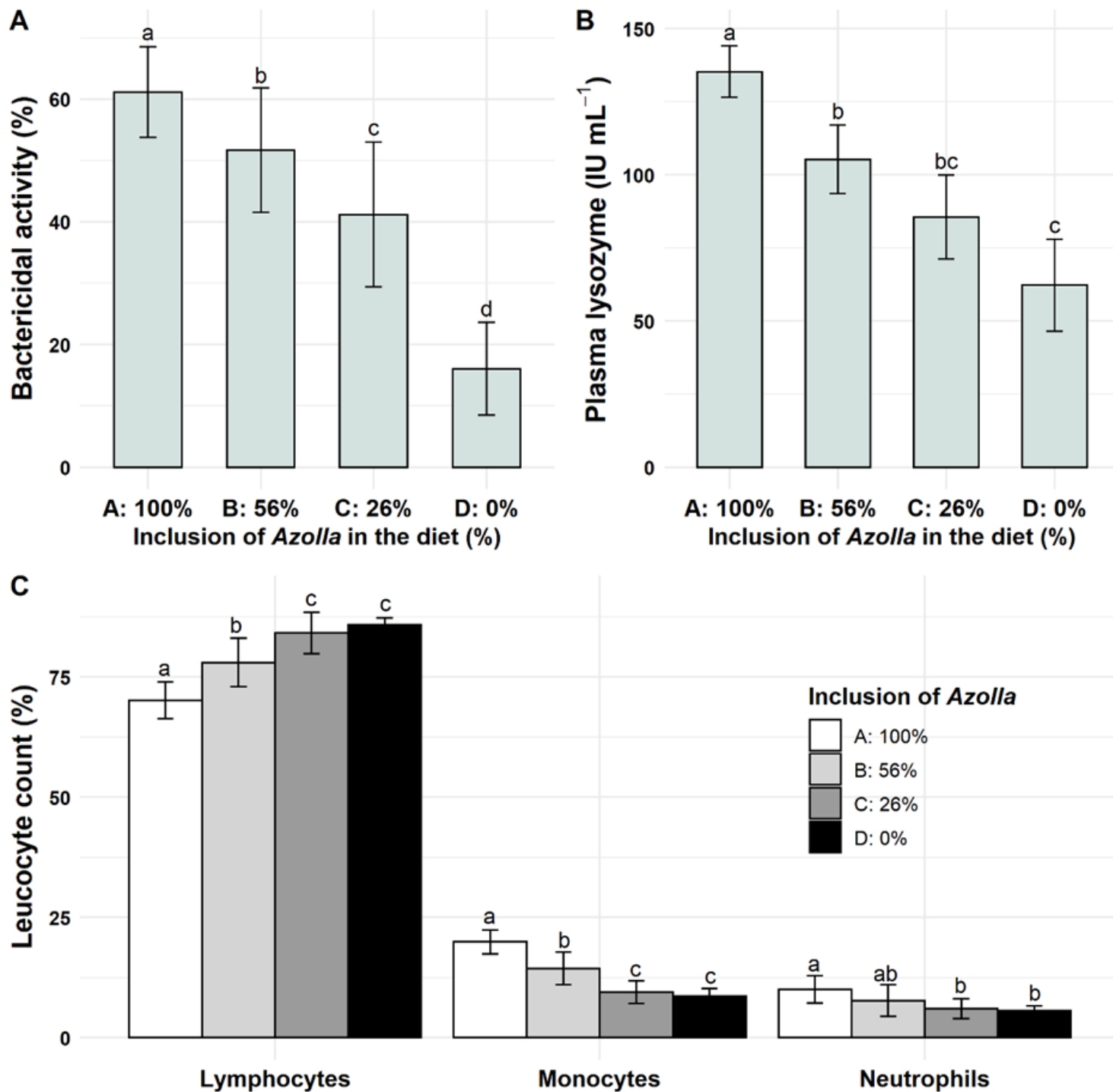


Figure 5

(A) Bactericidal activity in serum (expressed in % to blank control; n = 15 per treatment), (B) plasma lysozyme levels (IU mL⁻¹; n = 15 per treatment) and (C) leucocyte counts in giant gourami blood (%; n = 9 per treatment) fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively). Data are means $\pm$ SD. Different letters indicate significant differences between treatments.

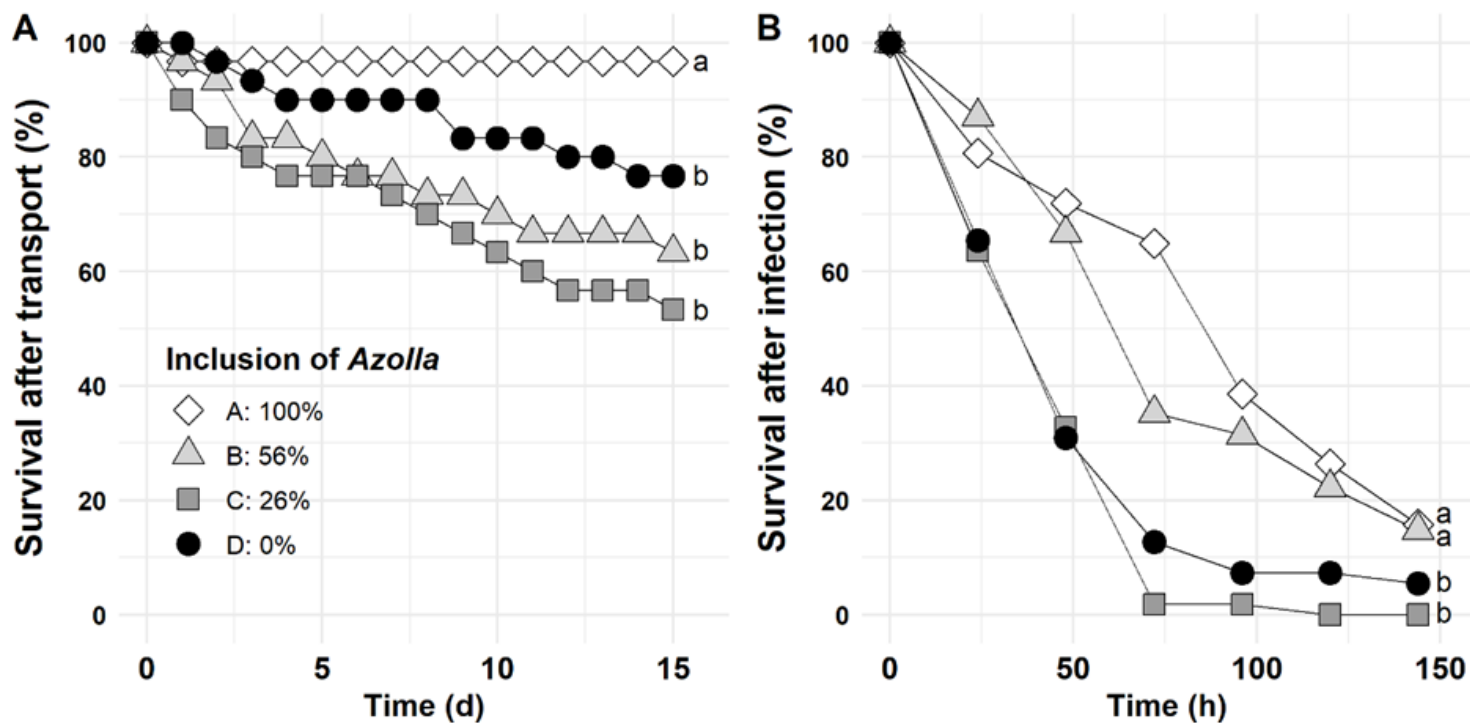


Figure 6

Kaplan-Meier survival analysis (%) of giant gourami fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively) (A) during the 15 days following the 6-hour transport stress test and (B) following 144 hours post-bacterial infection (*Aeromonas hydrophila*). Different letters indicate significant differences between treatments.