Aquat. Living Resour. 2023, 36, 9

https://doi.org/10.1051/alr/2023007

Aquatic Living Resources
Available online at:
www.alr-journal.org

RESEARCH ARTICLE OPEN ACCESS

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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Received 14 July 2022 / Accepted 17 March 2023

Handling Editor: Ryan B Carnegie

Abstract – Using *Azolla filiculoides*, a candidate macrophyte species for ecological intensification, in small-scale aquaculture requires the investigation of the potential effects of fresh plant material not only on fish growth but also on physiological status and responses to disease and stress. In this study, juveniles of giant gourami *Osphronemus goramy* reared into cages placed in an outdoor pond were fed for six weeks with different proportions of fresh *Azolla* in replacement of commercial pellets (A: 100%, B: 56%, C:26% and D: 0% of the feeding events). 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 from treatment A were significantly lower than in the other treatments. A decrease in lymphocytes was observed in treatments A and B ($p < 0.001$) and fish from these treatments had higher levels of monocytes ($p < 0.001$). Neutrophils were higher in treatment A only ($p = 0.012$). Plasma lysozyme levels and serum bactericidal activity increased with *Azolla* in the diet (both $p < 0.001$). Before transport stress, glycaemia was lower in fish from treatment A ($p < 0.001$) while after transport, glycaemia increased in all treatments excepted treatment A ($p < 0.001$) where survival was the highest after 15 days post transport. One week after infection the survival of fish was higher in fish from treatments A and B ($p < 0.001$). *Azolla* had positive effects on immunological indicators, and resistance to stress and disease but decreased growth. These findings suggest using *Azolla* at reasonable rate (i.e. <30% of the diet) to reduce pellets inputs while maintaining growth and providing other benefits to fish.

Keywords: *Azolla* / floating macrophytes / ecosystem resources / immunity / stress / *A. hydrophila*

1 Introduction

With a production of 4.3 million tons of farmed fish in 2020, Indonesia is the third-largest fish aquaculture producer country in the world (FAO, 2022). As in other Asian countries, farmed fish in Indonesia has become a cash crop, 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, 2020). Although the use of commercial feeds is the main driver of the development of aquaculture, this practice causes environmental damage (Aubin et al., 2019; Edwards, 2015). In addition, commercial feeds rely on terrestrial crops and wild fish, often consumed directly by humans and providing essential nutrition for low-income households. Their rising use in aquafeeds has the potential to increase price levels, worsen food insecurity among the most vulnerable populations and diminish the ability of aquaculture to add resilience to the global food system (Troell et al., 2014). 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).

In certain Asian countries, it is relatively widespread to use plants from ponds and the surrounding environment as fish feed. Among these plants, floating aquatic macrophytes may also help to improve aquaculture system productivity by

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producing fish feed as an alternative to commercial feeds (Fiogbé et al., 2004; Velásquez, 2016) while playing a role in the remediation of aquaculture effluents (Carlozzi and Padovani, 2016; Henry-Silva et al., 2006). Species such as *Azolla* sp. and duckweed *Lemna* sp have nutritional value for fish and can provide other beneficial ecological properties and ecosystem services making them good candidates 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 (Pouil et al., 2020; Wagner, 1997), *Azolla* has interesting nutritional properties, such as a high protein content (15–40% of its dry weight; Brouwer et al., 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 (Das et al., 2018; Datta, 2011; Gangadhar et al., 2015). 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 was higher in fish consuming 50 g *Azolla* kg^{-1} diet, declining with increased doses (Vasudhevan et al., 2013). Furthermore, *A. microphylla* extracts had 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; El-Sayed, 1992; Gangadhar et al., 2015) while Gangadhar et al. (2021) found that digestibility of protein, fat and nitrogen free extract (NFE) was reduced with increased dietary incorporation levels of *Azolla* in the diet of rohu *Labeo rohita*. 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. 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 values 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:

- 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.
- 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*).

We hypothesized that the use of *Azolla* as an alternative feed may provide health benefits to the fish while significantly reducing the dependence on commercial feeds.

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, 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 m^2 concrete pond until experiments started, approximately 9 months later. Ten-months old 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 m^2, 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 Tab. 1 for proximate composition). At the middle 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 while minimizing stressful handlings for the fish.

Water temperatures were monitored twice a day at 08:00 AM and 04:00 PM and varied between 25.1 and 26.9 °C. Water pH, dissolved oxygen, conductivity and turbidity were measured

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 ± SD.

Parameter	Commercial pellets (n = 3)	*Azolla* (n = 1)
Water content (%)	8.95 ± 0.21	89.69
Crude protein (%)	38.06 ± 0.74	28.28
Crude lipid (%)	4.67 ± 0.47	1.52
Ash (%)	8.78 ± 0.39	12.17
Crude fibre (%)	3.20 ± 0.19	10.84
[1]NFE (%)	45.30 ± 0.28	47.17
Gross energy (MJ kg^{-1})	19.23 ± 0.03	17.30

[1] NFE = Nitrogen-free extract represents carbohydrates.

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} O_2, 102–225 µS cm^{-1} conductivity and 20–44 nephelometric turbidity units (NTU). At the same time, water samples were collected and concentrations of ammonia (NH_3), nitrite (NO_2^-) and nitrate (NO_3^-) 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 NH_3 L^{-1}, 0.00–0.06 mg NO_2^- L^{-1} and 0.6–2.3 mg NO_3^- 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 × 5.5 × 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) as chicken manure has been evaluated as an efficient organic fertilizer for *Azolla* cultivation (Azab and Soror, 2020). One week later, *A. filiculoides* was seeded (400 g m^{-2}) and kept in the same conditions until the experiment began. The daily air temperatures ranged from 20 to 30 °C while water temperatures ranged from 25 to 27 °C. Light intensity reached peaks of >270 µmol 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% (Pouil et al., 2020; Slembrouck et al., 2018).

2.2 Experimental procedures

The course of the experiment as well as the different procedures performed are detailed in Figure 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. 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 $week^{-1}$. Feedings took place at the same time for all the treatments.

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 rinsed, carefully drained and weighed (± 0.1g) (Pouil et al., 2020). 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) (Tab. 1). At the end of the feeding trial, the fish remaining in each net cage were individually measured and counted to assess survival and growth.

Effects of *Azolla* in the diet of giant gourami on survival and growth were determined by calculating the following parameters for each experimental treatment.

Survival rates (SR), expressed as a percentage, were calculated by comparing the final number (N_f) with the initial number of fish (N_i): $SR\ (\%) = (N_f/N_i) \times 100$.

The specific growth in body weight (SGR, % d^{-1}) was calculated according to the following equation: $SGR = (\ln BW_f - \ln BW_i)/ED) \times 100$, where BW_i and BW_f are the initial and final body weights of fish (g) respectively, and ED is the duration of the experiment in days.

Fish biomass gain (BG, g) was calculated by weighing all fish from each net cage at the beginning and after a six-week trial. The feed conversion ratio (FCR) for commercial pellets was calculated using the following equation: $FCR = F/(BG\ g)$ where F is the total quantity of commercial pellets distributed during the whole rearing period.

The proximate composition of commercial feed, fish and *Azolla* were determined following AOAC methods (AOAC, 1999) (Tab. 2). 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 muscles 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 $GE = 5.7 \times g\ protein + 9.4 \times g\ crude\ lipid + 4.1 \times (g\ nitrogen\text{-}free\ extract\ (NFE) + g\ fibre)$ (Hall et al., 2013).

2.2.2 Transport stress test and disease challenge

A total of 30 fish from each of 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) to check glycaemia. The fish were released in net

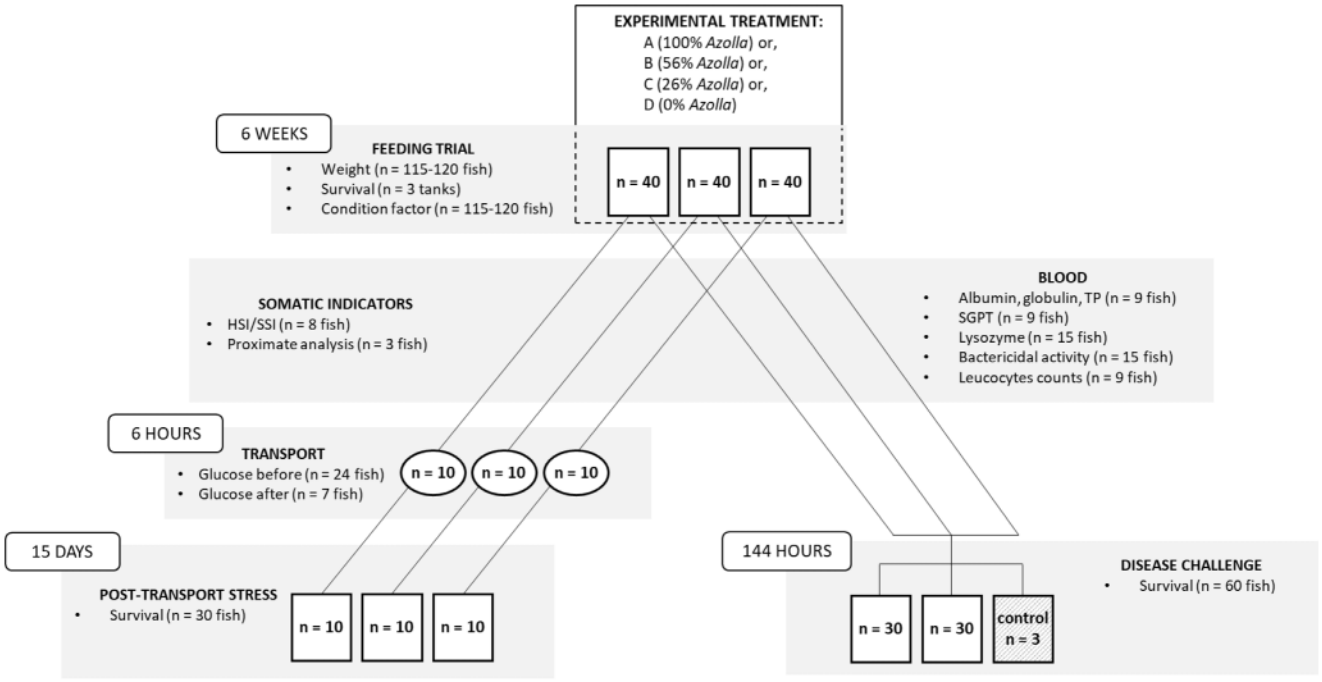

Fig. 1. Schematic view of the experimental design. For clarity, the experiments performed are represented for only one feeding treatment, but the other treatments followed the same protocol. HSI/SSI: Hepatosomatic index/spleen somatic index, SGPT: Serum glutamic pyruvic transaminase, TP: Total proteins.

Table 2. Experimental design for the different feeding treatments.

Treatments	Number of feedings		Ingested feeds (kg, wet weight[1])		Ingested proteins (g)	
	Commercial pellets	*Azolla*	Commercial pellets	*Azolla*	Commercial pellets	*Azolla*
A 100% *Azolla*	–	43	–	10.08 ± 0.40	–	294 ± 12
B 56% *Azolla*	19	24	1.40 ± 0.05	5.16 ± 0.45	486 ± 18	150 ± 13
C 26% *Azolla*	32	11	2.68 ± 0.13	1.94 ± 0.07	928 ± 46	57 ± 2
D (control. 0% *Azolla*	43	–	3.67 ± 0.04	–	1271 ± 12	–

[1] Estimated as described in Section 2.2.1.

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 (LD_{50}) 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 Figure 1.

2.3.1 Condition factor and somatic indicators

The condition factor (K_{Fulton}) was calculated according to the relationship K-BW (g)/TL (cm)$^3 \times 100$ 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.

Table 3. Growth and survival of giant gourami after 6 weeks of feeding with four levels of fresh *Azolla* in replacement of commercial pellets. Data are means ± SD per treatment. For each parameter, different letters indicate significant differences between treatments.

Parameters	A 100% *Azolla*	B 57% *Azolla*	C 26% *Azolla*	D 0% *Azolla*	*p*-values
Initial body weight (BW_i, *g*)	37.5 ± 7.0	36.2 ± 6.7	36.2 ± 5.5	36.8 ± 4.8	NS[1]
Final body weight (BW_f, *g*)	36.8 ± 6.8[a]	54.5 ± 9.6[b]	61.2 ± 10.3[c]	68.4 ± 10.0[d]	<0.001
Weight gain (g d^{-1})	–0.02 ± 0.01[a]	0.43 ± 0.04[b]	0.6 ± 0.05[c]	0.75 ± 0.06[d]	<0.001
Weight gain (%)	–1.8 ± 1.4[a]	50.3 ± 5.3[b]	69.1 ± 6.2[c]	86.3 ± 8.8[d]	<0.001
Biomass gain (g)	–77 ± 31.7[a]	638 ± 129.2[b]	958 ± 14.1[c]	1174 ± 97.5[d]	<0.001
SGR[2] (% d^{-1})	–0.04 ± 0.03[a]	0.97 ± 0.09[b]	1.25 ± 0.09[c]	1.48 ± 0.12[d]	<0.001
FCR[3]	–	2.3 ± 0.69	2.7 ± 0.12	3.1 ± 0;21	NS
Distributed pellets (g)	–	1433 ± 55[a]	2555 ± 128[b]	3663 ± 42[c]	<0.001
K_{Fulton}	1.46 ± 0.16[a]	1.56 ± 0.07[b]	1.64 ± 0.09[c]	1.69 ± 0.09[d]	< 0.001
Survival (%)	96.7 ± 2.9	95.8 ± 2.9	98.3 ± 2.9	96.7 ± 3.8	NS[1]

[1] NS = not significant.
[2] SGR = specific growth rate.
[3] FCR = feed conversion ratio calculated for commercial pellets.

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 µL of plasma from each sampled fish were added to 170 µL of an aqueous suspension of *Micrococcus lysodeikticus* (Sigma, 200 mg L^{-1} in 0.05 M NaH_2PO_4 at 25°C and 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 µL) 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 µL of the dilution was plated onto a nutrient agar plate and incubated at 32 °C for 24–36 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 Growth, feed efficiency and survival during feed trial

The replacement of commercial pellets with *Azolla* affected growth in giant gourami and decrease in growth was dose-related. At the end of the six-week experiment, the individual final body weight (BW_f) was significantly different ($p < 0.001$) in all the experimental treatments and the highest

Table 4. Proximate composition of the muscle 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. Results are expressed on a wet weight basis. Data are the means ± SD ($n = 3$) per treatment and different letters indicate significant differences between treatments.

Parameter	A 100% *Azolla*	B 56% *Azolla*	C 26% *Azolla*	D 0% *Azolla*
Water (%)	73.2 ± 0.45^{a}	70.7 ± 0.67^{a}	69.1 ± 1.72^{a}	68.4 ± 0.68^{a}
Crude protein (%)	18.0 ± 0.48^{a}	18.1 ± 0.65^{a}	18.9 ± 1.09^{a}	18.3 ± 0.39^{a}
Crude lipid (%)	2.5 ± 0.25^{a}	5.3 ± 0.36^{b}	5.3 ± 0.34^{b}	5.3 ± 0.53^{b}
Ash (%)	4.7 ± 0.22^{a}	4.2 ± 0.30^{a}	4.5 ± 0.48^{a}	4.4 ± 0.33^{a}
NFE[1] (%)	1.4 ± 0.06^{a}	1.5 ± 0.28^{a}	2.0 ± 0.61^{a}	3.3 ± 0.07^{b}

[1] NFE = Nitrogen-free extract,
[2] NS = non-significant.

BW_f was observed in treatment D without *Azolla* (Tab. 3). Other investigated indicators showed that *Azolla* affected growth performances and SGR and BG were negative in fish fed exclusively with fresh *Azolla* (Tab. 3). The mean FCR values calculated for fish fed with commercial pellets (treatments B, C and D) ranged from 2.3 to 3.1 and were not significantly different between the three groups ($p = 0.312$). Compared to the treatment D, the FCR of treatments B and C were reduced of 25% and 13%, respectively. 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 ± 0.16% in treatment A (100% *Azolla*) to 1.68 ± 0.09% in treatment D (control, 0% *Azolla*). The survival rates measured at the end of the six-week feeding trial were very high (96–98%) without any significant differences between treatments ($p = 0.281$).

3.2 Body composition and somatic indicators

The chemical composition of fish was slightly influenced by the inclusion of *Azolla* in the diet (Tab. 4). Significant changes in proximate composition in giant gourami muscle were observed for crude lipids and NFE. Crude lipids remained similar in fish from treatments B, C and D, but they were 2 times lower in fish fed exclusively with *Azolla* ($p < 0.05$). The opposite trend was observed for NFE, for which values were similar in the fish fed (at least partially) with *Azolla,* and values were more than 2 times higher in giant gourami fed exclusively with commercial pellets ($p < 0.05$). 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 ± 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 ± 0.08%, Fig. 2B).

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} ($p = 0.760$).

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 ± 7.6%, Fig. 4A). 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. 4B).

Leucocyte count (Fig. 4C) 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 ± 4% to 86 ± 1% of the leucocytes. Giant gourami from treatments A and B showed significantly higher ($p < 0.001$) levels of monocytes (17 ± 4% of the leucocytes) compared with the other treatments (9 ± 2 of the leucocytes). Neutrophils were significantly higher in fish from treatment A ($p = 0.012$) with a value of 10 ± 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. 5A). Fifteen days after the six-hour transport stress test, cumulative mortality differed significantly between the experimental treatments ($\chi^2 = 16.58$,

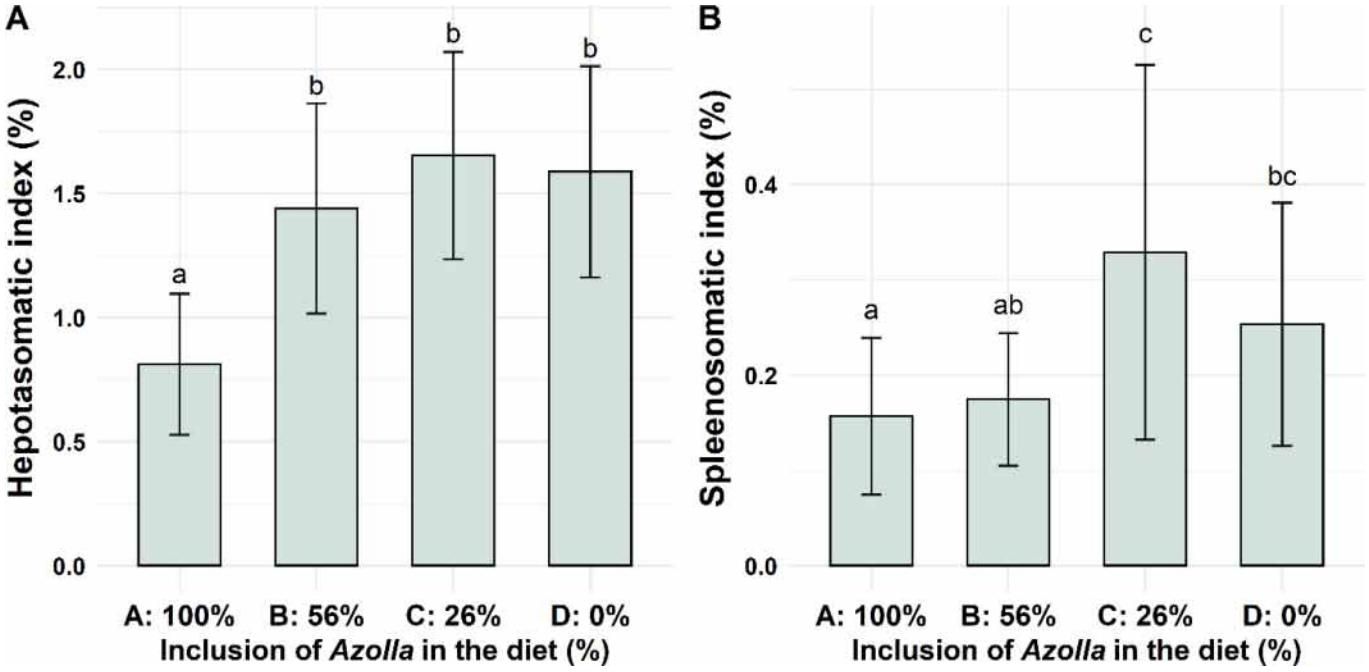

Fig. 2. (A) Hepatosomatic index (%. and (B) spleen somatic index (%. in giant gourami fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively. at the end of the six-week experiment. Data are means ± SD ($n = 24$) per treatment. Different letters denote significant differences between the feeding regimes.

$df = 3$, $p < 0.001$) 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. 5A).

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. 5B). 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. 4B). 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. 5B). 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 the one tested in the present study may be affordable alternatives for partial or total replacement of commercial feed of herbivorous or omnivorous fish species. 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

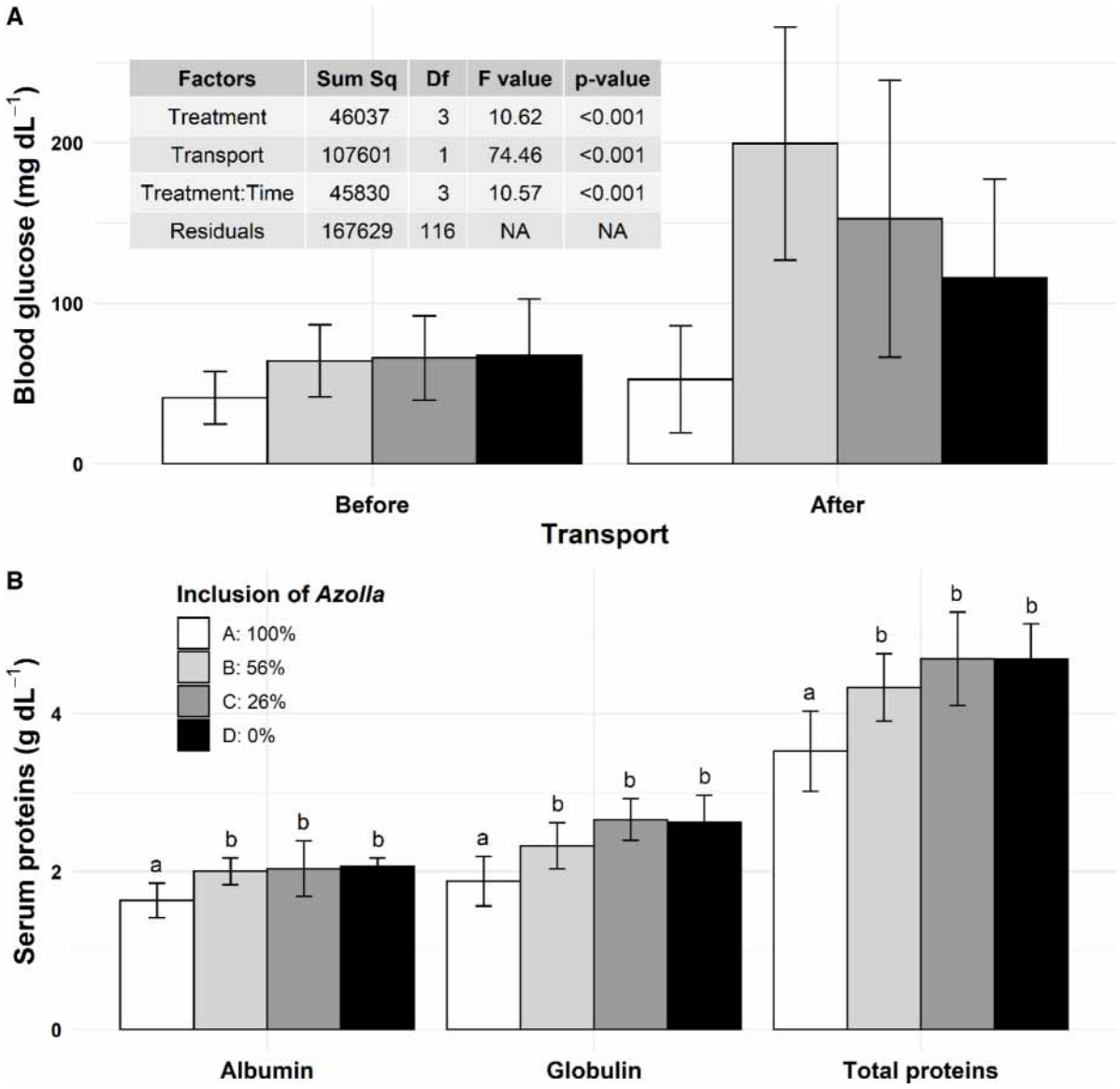

Fig. 3. (A) Blood glucose concentration in giant gourami fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively. measured before ($n = 24$) per treatment. and after ($n = 7$) per treatment. the transport stress test. (B) Composition of serum proteins (total proteins, albumin, globulin. ($n = 9$) per treatment. Data are means ± SD. Different letters indicate significant differences between the experimental treatments.

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 pinnata* supplementation can reduce oxidative stress in liver of tilapia (Attia et al., 2021).

The effects of *Azolla* on serum proteins in fish have been poorly investigated. Nevertheless, in the grass carp, there were 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*. In literature, there are contrasting observations on protein content in the body composition of tropical fish fed with fresh or dried *Azolla* (Datta, 2011, El-Sayed, 1992). Altogether, our findings on giant gourami fed exclusively with *Azolla* and to a lesser extent fed at high replacement rates were very close to those observed in fish

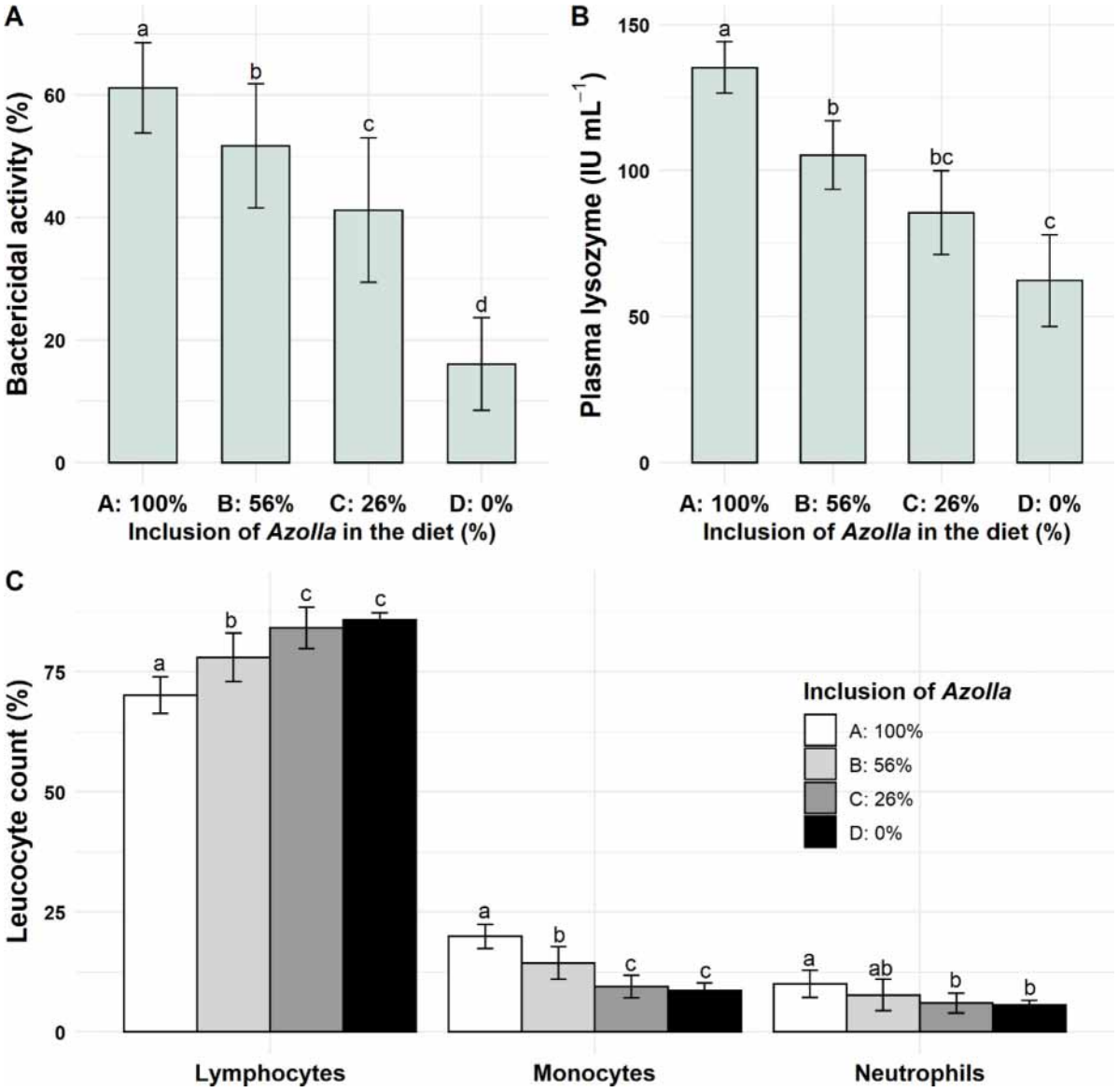

Fig. 4. (A) Bactericidal activity in serum (expressed in % to blank control; $n = 15$ per treatment., (B.) plasma lysozyme levels (IU mL^{-1}; $n = 15$ per treatment. and (C) leucocyte counts in giant gourami blood (%;$n = 9$ per treatment. fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively). Data are means ± SD. Different letters indicate significant differences between treatments.

during starvation. Indeed, in starved common carp, Varga et al. (2014) reported a reduction in HSI, condition factor and lipids in carcasses while, in grass carp, long-term food restriction reduced the accumulation of lipids, with down-regulation of lipogenesis genes and up-regulation of lipolysis genes (Gong et al., 2016).

Lysozymes play an important role in fish immune defences, inducing antibacterial activity in the presence of the complement system (Saurabh and Sahoo, 2008). 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).

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

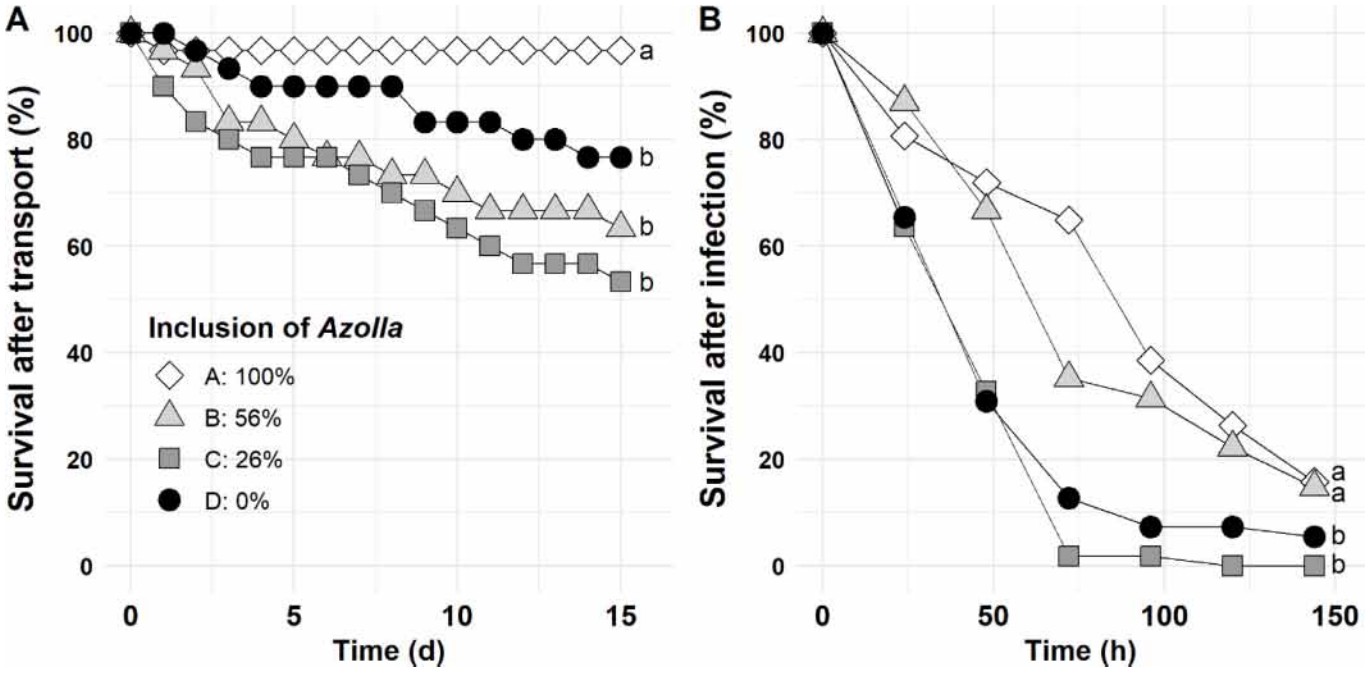

Fig. 5. Kaplan-Meier survival analysis (%. of giant gourami fed with four levels of fresh *Azolla* in replacement of commercial pellets (100%, 56%, 26% and 0% *Azolla* for treatments A, B, C and D, respectively. (A) during the 15 days following the 6-hour transport stress test and (B) following 144 hours post-bacterial infection *(Aeromonas hydrophila)* Different letters indicate significant differences between treatments.

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 (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 (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 rawer plants, including macrophytes, as feed before harvest and transport of giant gourami (FAO, 2019). However, several studies suggest that better results could be obtained following specific treatments of *Azolla* such as ensilage, fermentation or drying (Cruz et al., 2011; Fasakin et al., 2001; Hundare et al., 2018). Further investigations are required to fully understand the underlying biological mechanisms explaining the responses to *Azolla* diet and the discrepancies observed among fish species.

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%. 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.

Funding

This work was supported by the COFASP ERA-NET project “IMTA-EFFECT” funded by the European Union and the French National Research Agency.

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.

Acknowledgements. The authors acknowledge Imam Akhir Abdullah, a technician at the Cijeruk research station, and Ahmad Sihabuddin, Gusnia Sundari and Rully Apriani, technicians from IRD Indonesia for their technical assistance throughout the experiment. This is publication ISEM SUD.

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Cite this article as: Caruso D, Lusiastuti AM, Pouil S, Samsudin R, Arifin OZ, Slembrouck J. 2023. Can *Azolla filiculoides* be a complementary feed resource for ecological intensification in small-scale fish farming? Biological effects on giant gourami (*Osphronemus goramy*). *Aquat. Living Resour.* 36: 9