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Original Article

Prebiotic potential of *Azolla pinnata* (R.Br.) and dietary inclusion effect of pulverised azolla on the growth performance of milkfish fingerlings

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Abstract
The growth of the aquaculture sector leads to a demand for sustainable feed ingredients. Prebiotics are potential sustainable feed ingredients that can promote the improved performance of aquaculture species without the use of antimicrobials. This study investigated the potential of *Azolla pinnata* as a candidate prebiotic source. Prebiotic characteristics and dietary effect of commercial diet (CD) with varying pulverised azolla (PA) inclusion was evaluated. Results showed that PA (9.40% moisture) constitutes around 16.1% ash, 22.3% crude fibre, 20.9% crude protein, 1.7% crude fat and 29.6% nitrogen-free extract. Crude xylan and cellulose content of PA were 13.7% and 12.6% dry matter (DM) respectively. Growth of *Lactobacillus rhamnosus* in PA-supplemented MRS broth remarkably improved after 4 to 6 hours of incubation. Feeding trials results revealed that PA inclusion had no adverse effect on average survival of milkfish fingerlings, albeit significant improvement ($p < 0.01$) was noted in group fed with 2% PA-replaced CD in terms of the total weight gain (119.9%), specific growth rate (2.62% day^{-1}) and feed efficiency (79.9%). The present study demonstrated the prebiotic activity of PA, as well as its potential use as dietary feed component for improved growth and feed efficiency of cultured milkfish.

Keywords: *Azolla pinnata*; growth; milkfish; phytogenic; prebiotics; oligosaccharides

1 | INTRODUCTION

The global campaign against the use of antimicrobials as growth enhancer in food-animal production has necessitated the recent development of alternative strategies (Butaye *et al.* 2003). In this regard, applications of certain bioactive substances in the culture production of aquatic animals have been recently considered for their potential in enhancing the nutritive value of the aquafeeds (Glencross *et al.* 2019). Phytogenic feed additives, in particular, have been recently given attention for their functional roles in animal nutrition (Karásková *et al.* 2015; Caipang *et al.* 2019). Phytogenics are plant-based feed additives that are supplemented in optimum level, to specifically enhance the feed palatability and growth performance of farmed animals (Alemayehu *et al.* 2018). Indeed, research on alternative functional dietary components remains to be important approach towards sustainable aquaculture production (Labrador *et al.* 2016).

Many botanicals have been reported to confer beneficial health effect to cultured aquatic species, including improved intestinal physiology, enhanced resistance to stress and infectious diseases, immunomodulation, stimulation of appetite and selective growth stimulation of beneficial microorganisms and/or inhibition of the enteric pathogens (Reverter *et al.* 2017). Prebiotics are non-digestible dietary components and have been known to provide certain health benefits to the target host by encouraging the proliferation and activity of intestinal probiotics (Ringø *et al.* 2010). Prebiotics resist digestion by the hosts during transit through the GI tract, rather these are fermented by resident microbes, producing bioactive metabolites that lowers the gut pH which in turn, favours proliferation of probiotics such as bifidobacteria and lactobacilli (Vazquez-Olivo *et al.* 2018). Prebiotics may also act as immunostimulants, which improve the host's health via fermentative action by indigenous microflora (Ganguly *et al.* 2010; Song *et al.* 2014).

Azolla is a common macrophyte with a global distribution and grows in close association with the nitrogen-fixing blue-green algae, *Anabaena azollae* (Wagner 1997). This macrophyte contains significant amounts of crude protein and essential amino acids relative to most green forage crops and other aquatic plants (Panigrahi *et al.* 2014). Azolla is also rich in vitamins, minerals and growth-promoter intermediaries (Dhumal *et al.* 2009); hence its potential use as an alternative dietary supplement for cultured aquatic species (Shiomi and Kitoh 2000; Fiogbé *et al.* 2004; Gangadhar *et al.* 2015). Notwithstanding its attractive nutritional value and the relative ease to produce with minimal inputs (Brouwer *et al.* 2017; Mosha 2018), azolla remains to be underutilised in aquaculture production. In the Philippines, studies regarding the role of non-conventional plant materials as functional dietary feed components for improved aquaculture production of economically-important fishes are scarce. The present study was conducted to elucidate the potential of water fern (*Azolla pinnata* R.Br.) as candidate source of dietary prebiotics for cultured milkfish.

2 | METHODOLOGY

2.1 Azolla collection and identification

Fresh samples of *A. pinnata* growing in the earthen concrete fishponds at the Aquaculture Research Station, College of Fisheries – Laguna State Polytechnic University, were hand-collected, initially washed using tap water to remove the dirt and other unwanted debris, and finally rinsed with distilled water. Fresh azolla samples were taxonomically identified by the Plant Systematics Laboratory of the Institute of Biological Sciences, University of the Philippines Los Baños.

2.2 Analysis of hydrolysis products

2.2.1 Spontaneous fermentation

Sugar hydrolysis by means of spontaneous fermentation was carried out in 50-ml sterile Erlenmeyer flask using fresh azolla homogenate (FAH) as substrate. Exactly 1 g of FAH was fermented with or without the addition of 1% molasses (v/v) solution; another set-up containing 1% molasses only (i.e., without FAH) was also prepared and used as control. All fermentation set-ups were incubated at room temperature and intervallic monitoring of hydrolysis products was performed daily for 7 days (triplicate samples for each set-up were analysed per day). Detection of hydrolysis products was done by initially heating the hydrolysate at 80°C for 15 minutes to terminate hydrolysis. Unhydrolysed sugars in were precipitated by the addition of 3.0 ml of 95% (v/v) isopropanol, then subsequently removed via centrifugation at 7000 rpm for 10 minutes. The remaining clear supernatant was subsequently analysed for hydrolysis products.

2.2.2 Phenol-sulphuric acid assay

Quantitative analysis of simple sugars (i.e., xylose and glucose) was performed following DuBois *et al.* (1956), with some modifications. Calibration standards at concentrations of 50, 100, 150, 200, 250, 300 and 320 ppm were prepared for D-xylose and D-glucose as reference. To determine the relative concentration of xylose and glucose produced in the hydrolysate, 1 ml sample aliquot was taken and serially-diluted to 100-fold with sterile distilled water and exactly 2 ml of the diluted sample was transferred into a sterile screw-cap glass tube containing 1 ml of 5% phenol (C_6H_5OH) solution, to which 5 ml of concentrated sulphuric acid (H_2SO_4) was directly introduced as a rapid stream into the liquid. The acidified hydrolysate was stoppered, shaken vigorously for at least 5 minutes, then immediately heated in hot water bath for 15 minutes. The sample was soaked in iced water bath to allow for colour development. All samples were analysed in triplicate. The optical density (OD) at 475 nm for xylose and 485 nm for glucose (Rao and Pattabiraman 1989) was determined using a UV-Vis spectrophotometer (Series 1200, Cole-Parmer). The concentration (mg ml^{-1}) of xylose and glucose in the sample hydrolysate was approximated using the following equation where, C_{sugar} was the concentration of xylose / glucose; A_{nm} denotes the OD of the sample read; *df* for dilution factor used; and *b* and *s* represent the y-intercept and slope of the calibration curve respectively.

$$C_{sugar} = \frac{\left[\frac{(A_{nm}\text{-}b) \times df \times 1\ mg}{1000\ \mu g}\right]}{s}$$

2.2.3 Thin layer chromatography

The method modified from Kamble and Jadhav (2014) was adopted for the qualitative detection of hydrolysis products by thin layer chromatography. Briefly, sugar hydrolysates were spotted on TLC plates (silica gel, F 254, Merck, India) then the reaction was allowed to proceed within a chamber with n-butanol:ethanol:H_2O (5:3:2 v/v) as the solvent system. For subsequent detection of sugar oligomers, the TLC plates were initially immersed in 50% of H_2SO_4 solution in ethanol, then dried in hot-air oven at 150°C for at least 5 minutes. Xylose and glucose neotypes were identified by comparing the Rf values (i.e., distance travelled by the solute divided by the distance travelled by the solvent) of the samples with that of D-xylose and D-glucose as reference standards.

2.3 Nutritional component analysis

2.3.1 Preparation of pulverized azolla

Samples were air-dried in a well-ventilated room for at least three days, then oven-dried at 65°C for up to 48 hours or until the desired brittleness was achieved. Obtained DM was grounded using mortar and pestle, powdered using a kitchen blender equipped with sharp blades (NutriBullet®), then sifted through a conical nylon mesh screen with an opening size of ~180 µm, to obtain the pulverized azolla (PA). The PA samples were stored within sterile zip-lock plastic bags at room temperature.

2.3.2 Proximate analysis

Fresh and dried samples of azolla were subjected to proximate analysis i.e., moisture, ash, crude fibre, crude protein, crude fat and nitrogen-free extract (NFE), following standard methods (AOAC 2002).

2.3.3 Xylan and cellulose percent yield

Extraction of xylan was done according to the methods of Zilliox and Deheire (1998), Akpinar *et al.* (2007) and Dammström *et al.* (2009), with some modifications. Briefly, 10 g PA was initially treated with 200 ml of commercial bleach (pH adjusted to 5.0 using glacial acetic acid), then heated at 50°C for 1 hour to remove the lignins and other contaminants. The treated sample was filtered then the residue washed with distilled water to neutral pH. To extract xylan, the solid residue was soaked for at least 16 hours in 150 ml of 12% (v/v) sodium hydroxide (NaOH) solution, filtered, then ice-cold absolute ethanol was added to the filtrate at twice its volume. For complete precipitation, the sample was refrigerated at 4°C overnight. The precipitate was separated from the solution by filtration, then the residue was treated with 100 ml of 95% (v/v) ethanol, followed by washing twice with acetone. Crude xylan was obtained after the sample was oven-dried at 60°C until constant weight. For cellulose (ß-glucan) extraction, the procedure of Battegazzore *et al.* (2014) was adopted, with some modifications. Briefly, 10 g PA was initially treated with 150 ml of 3% H_2SO_4 solution, then the treated sample was heated at 90°C for about 2 hours (with intermittent stirring) to hydrolyse the hemicellulose, as well as remove the impurities (Krishnarao *et al.* 2001). The residue was separated via filtration, washed twice with distilled water, treated with 150 ml of 5% potassium hydroxide (KOH) solution, then heated at 90°C for 2 hours with intermittent stirring to extract silica. For removal of the amorphous cellulose and lignin, the residue was washed twice using distilled water, filtered, treated with 150 ml of 25% (v/v) sodium hypochlorite (NaOCl) solution (pH adjusted to 5.0 using glacial acetic acid), heated at 75°C for 4 hours (under constant stirring), then the residue was washed thrice with distilled water. Crude cellulose was obtained after the sample was oven-dried at 60°C until constant weight. Xylan/cellulose percent yield (%) was computed using the formula (Ratnadewi *et al.* 2016):

$$\text{Percent Yield (\%)} = \left(\frac{\text{DM}_{\text{xylan/cellulose (g)}}}{\text{DM}_{\text{pulverized azolla (g)}}} \right) \times 100$$

2.4 Prebiotic activity screening

Evaluation of prebiotic activity in vitro was done based on the procedure of Rubel *et al.* (2014), with few modifications. Briefly, 100 µL of overnight culture of *Lactobacillus rhamnosus* SRLPB77 was inoculated into 45 ml of MRS broth supplemented with PA. The *L. rhamnosus* SRLPB77 was isolated previously from cultured milkfish (*Chanos chanos*) and considered putative probiotic strain of lactic acid bacteria (LAB) by polyphasic identification. The MRS broth supplemented with glucose or inulin were also used as positive controls; an MRS broth without additional carbohydrate was used as negative control. Supplementation of basal MRS broth with additional carbohydrates was set at 1% (w/v) inclusion rate, which was recommended as the minimum amount that will elicit a growth-stimulating effect on probiotic bacteria (Li *et al.* 2008). All additives were sterilised via direct exposure to the UV light for at least 20 minutes, before these were added to the culture media. Cultures were incubated at 37°C for 12 hours and 1 ml sample aliquots were withdrawn from each culture at various times during incubation for measurements of pH and OD at 600 nm. All test assays were performed in triplicate. The prebiotic activity of PA and inulin (as reference prebiotic) was evaluated based on the relative growth ratio (RGR) of *L. rhamnosus*, which was calculated using the following equation where, ΔGR indicates the difference in the growth of the test probiotic (expressed in log CFU ml^{-1}) between time H (final) and time 0 (initial). A growth stimulating effect is substantiated when the average RGR was greater than unity, relative to glucose as the optimal carbon source.

$$\text{RGR} = \left(\frac{\Delta GR_{H\text{-}0} \text{ in prebiotic-enriched medium}}{\Delta GR_{H\text{-}0} \text{ in glucose-enriched medium}} \right)$$

2.5 Tank-scale feeding trial

Milkfish fingerlings were obtained from a local hatchery and these were allowed to acclimatise for 14 days in a concrete circular fish tank. Five dietary treatments were prepared using the commercial diet (CD) with varying PA inclusion levels: 100% CD (T1 – control); 1% PA-replaced CD (T2); 2% PA-replaced CD (T3); 1% PA-enriched CD (T4); and 2% PA-enriched CD (T5). A total of 225 fingerlings were distributed equally into 15 aerated 155L-capacity polyethylene tanks, following the completely randomised design (CRD). The fish were starved for 24 hours then the initial body weight (IBW) in each tank was determined before the experimental diets were applied. Feeding was done twice a day (09:00/16:00) for 4 weeks at 5% rate based on the IBW. Dissolved oxygen and temperature were measured daily using digital water quality sensors.

2.6 Data collection and analysis

At the end of the feeding trial, all fish from each tank were counted and bulk-weighed. The average survival rate (%), total weight gain (TWG), specific growth rate (SGR) and feed efficiency (FE) were calculated following standard equations (Sankian *et al.* 2017). One-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) for pairwise comparison were used to determine the statistical significance at 95% level. All statistical procedures were performed in IBM-SPSS 20 (SPSS Inc., Chicago, IL, USA).

3 | RESULTS

3.1 Sugar hydrolysis products

The production kinetics of xylose and glucose in fermented azolla hydrolysates are shown in Figure 1. The highest production of xylose and glucose were observed in set-ups enriched with molasses, peaking on the third (1.58 mg ml^{-1}) and second (5.18 mg ml^{-1}) day of fermentation respectively. Detectable amounts for both xylose (0.68 mg/ml) and glucose (2.13 mg ml^{-1}) were also noted in fermented azolla without molasses, albeit in lower amounts. Xylose was not detected in control set-up (molasses only) during the entire fermentation period; however, glucose in trace amounts (1.16 mg ml^{-1}) was detected.

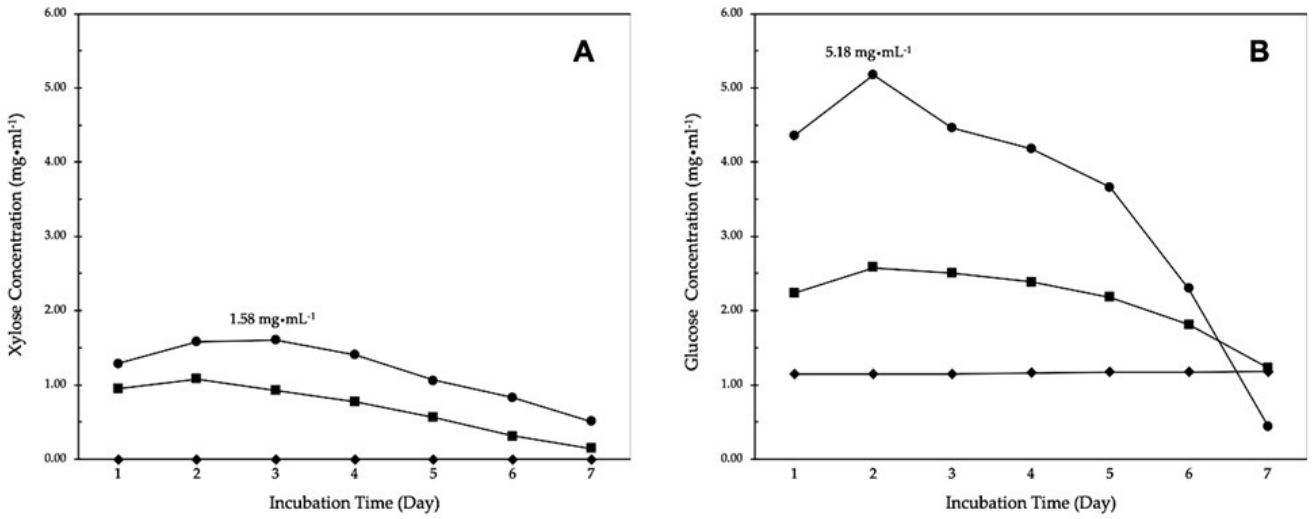

FIGURE 1 Production kinetics of (A) xylose and (B) glucose as hydrolysis products of fermented fresh azolla homogenate (FAH). Legend: [•] FAH with 1% molasses; [■] FAH only; [•] 1% molasses only.

The thin layer chromatograms shown in Figure 2 demonstrate the qualitative profiles of hydrolysis products, presumably from breakdown of the celluloses / hemicelluloses, as a result of endogenous cell-wall hydrolysis and / or spontaneous fermentation activity of indigenous microorganisms. The TLC spots shown on L1 and L2 denote the reference standards for D-xylose (Rf = 0.58) and D-glucose (Rf = 0.43) respectively. Apparent hydrolysis of xylan into xylooligosaccharides (XOS) and cellulose (ß-glucan) into ß-glucooligos- accharides (ß-GOS), in varying degree of polymerisation, possibly that of xylobiose, xylotriose, cellobiose, and cellotriose was confirmed in the present study. The TLC spots observed in set-ups containing fermented azolla (L4 and L5) indicate the presence of neotype xylose (Rf = 0.57) and glucose (Rf = 0.43), accompanied by two fractions of certain sugar oligomers, possibly that of disaccharides (Rf = 0.24) and trisaccharides (Rf = 0.07). Note that only three TLC spots were visible on L3 (control set-up containing 1% molasses only), apparently suggesting the absence of neotype xylose in the sample hydrolysates, which may be due to the negligible amount (or absence) of polymeric precursors such as xylan in molasses.

3.2 Nutrient composition

Results of the proximate analysis for fresh and dried sam-

ples of *A. pinnata* are given in Table 1. The average moisture in fresh azolla sample was estimated at 96.0%, which accounted for most of its total biomass; total ash (1.14%), crude fibre (0.71%), crude protein (0.86%), crude fat (0.12%), and NFE (1.17%) comprised the remaining 4% of *A. pinnata* wet biomass. Pulverized azolla (PA) had 9.4% moisture content, and the remaining DM was comprised of 16.1% total ash, 22.3% crude fibre, 20.9% crude protein, 1.70% crude fat and 29.6% NFE. The extraction yield (% DM) for crude xylan and cellulose (ß-glucan) in PA were approximately 13.7 ± 0.80% and 12.6 ± 0.60% respectively.

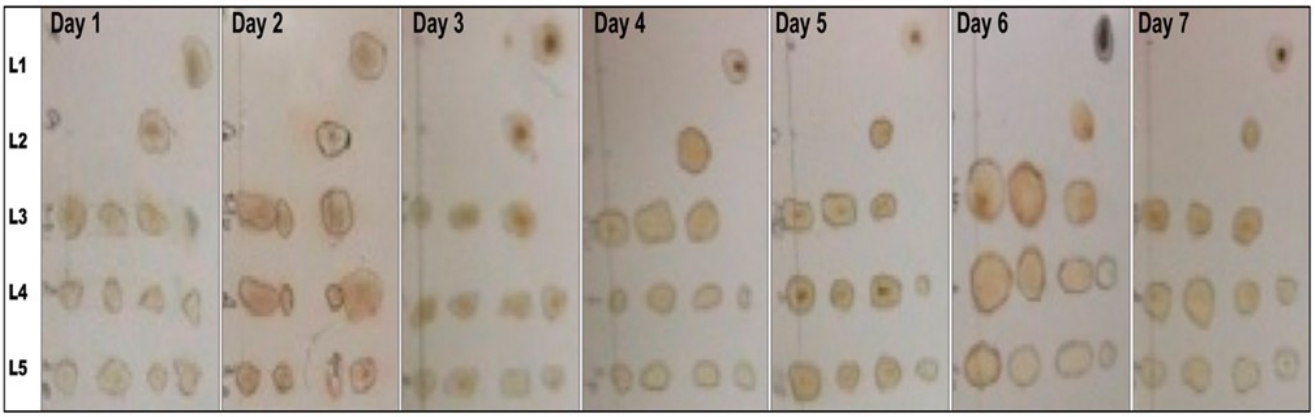

FIGURE 2 Thin layer chromatograms of hydrolysed sugars during spontaneous fermentation. L1, D-xylose; L2, D-glucose; L3, 1% molasses only; L4, fresh azolla homogenate (FAH) only; L5, FAH with 1% Molasses.

TABLE 1 Proximate nutritional components of *Azolla pinnata* in fresh (wet) and dried (pulverised) samples.

Component	Amount (%±SD)	
	Fresh sample	Dried sample
Moisture	96.0±0.06	9.40±0.32
Total ash	1.14±0.08	16.1±0.50
Crude fibre	0.71±0.05	20.9±0.84
Crude protein	0.86±0.08	22.3±0.78
Crude fat	0.12±0.03	1.70±0.04
Nitrogen-free extracts	1.17±0.07	29.6±0.82

3.3 In vitro prebiotic activity

The relative growth ratio (RGR) of *L. rhamnosus* within 12 hours incubation on MRS broth supplemented with PA or commercial inulin powder are shown in Figure 3. In general, growth of the test probiotic was lower on MRS broth containing PA, compared to the growth on inulin-enriched medium. It can be noted, however, that higher growth ratios of *L. rhamnosus* were observed within 4 (RGR = 4.21) to 6 (RGR = 1.07) hours of incubation, with respect to glucose as the optimal carbon source. Higher RGRs were also obtained for inulin (as reference prebiotic substrate) in comparison to glucose, during the 24-hour incubation period, particularly at 4 hours (RGR = 9.99) of incubation.

3.4 Performance of milkfish fingerlings

Results of the feeding trial are presented in Table 2. Survival rates were lower in groups on commercial diet containing PA; however, the mean values were not significantly different from control group ($p > 0.05$) fed with 100% commercial feeds. Meanwhile, fish given the commercial diet replaced with 2% (w/w) PA yielded significantly higher ($p < 0.01$) TWG (119.9 %), SGR (2.62 %/day), and FE (79.9 %), relative to other treatment groups. It can be noted that the group fed with 2% PA-enriched commercial diet consistently showed the lowest growth rates (TWG = 53.6%, SGR = 1.42% day^{-1}) and feed efficiency (35.8 %); albeit, the mean values did not differ significantly from control group ($p > 0.05$).

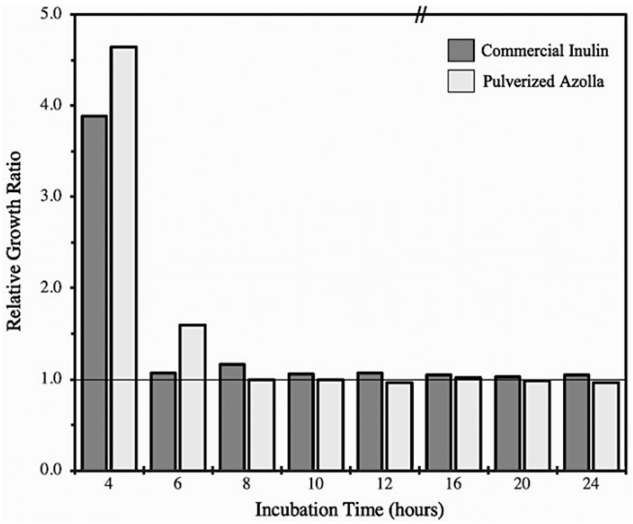

FIGURE 3 Relative growth ratio of *Lactobacillus rhamnosus* in MRS broth supplemented with PA and inulin powder at different incubation periods. The horizontal line represents the growth of *L. rhamnosus* on PA or inulin, equivalent to that attained with glucose.

TABLE 2 Performance of milkfish fingerlings fed with pulverized azolla (PA) in varying inclusion levels.

Treatment	Survival (%)	TWG[1]	SGR[2]	FE[3]
T1 – 100% commercial feed	82.2±10.2	77.1±13.0^{ab}	1.90±0.25^{ab}	51.4±8.62^{ab}
T2 – 1% PA-replacement	68.9±3.81	90.0±17.7^{bc}	2.13±0.30^{bc}	60.0±11.7^{bc}
T3 – 2% PA-replacement	73.3±6.65	119.9±20.1^{c}	2.62±0.29^{c}	79.9±13.4^{c}
T4 – 1% PA-enrichment	80.0±6.70	103.4±17.0^{bc}	2.36±0.28^{bc}	68.9±11.3^{bc}
T5 – 2% PA-enrichment	60.0±24.1	53.6±14.5^{a}	1.42±0.32^{a}	35.8±9.65^{a}

Mean values on the same column with different superscripts are significantly different at 95% confidence level.
[1] Total Weight Gain (%) = [(final body weight − initial body weight)/initial body weight] × 100
[2] Specific Growth Rate (% day^{-1}) = [(ln final body weight − ln initial body weight)/days] × 100
[3] Feed Efficiency (%) = (wet weight gain/feed intake) × 100

4 | DISCUSSION

This study demonstrated the potential use of *Azolla pinnata* as prebiotic source, having shown that azolla homogenate produced detectable amounts of xylose and glucose upon fermentation. Similarly, Miranda *et al.* (2016) reported that *A. pinnata* hydrolysate yielded 29.5 g L^{-1} of glucose in the presence of *Saccharomyces cerevisiae*. Acid hydrolysis of *Azolla* sp. also yielded 4 mg ml^{-1} of glucose (Hossain *et al.* 2010); likewise, *A. filiculoides* DM yielded about 0.15% and 0.70% (w/w) of xylose and glucose respectively, following hot water extraction (Brouwer *et al.* 2019). It can be presumed that the recovery of reducing sugars like glucose and xylose from azolla biomass can be attributed to the presence of polymeric sugar precursors like cellulose and hemicellulose which may accumulate up to 34 t dw ha^{-1} year^{-1}, under natural conditions (Miranda *et al.* 2016). Previous studies demonstrated the presence of hemicellulose / cellulose in azolla in varying concentrations. For *A. pinnata*, in particular, hemicellulose yield ranged between 12.8% and 19.6% the total DM; while cellulose ranged between 10.1% and 36.7% (Ghodake *et al.* 2011; Srinivas *et al.* 2012; Chatterjee *et al.* 2013; Miranda *et al.* 2016; Gupta *et al.* 2018). Therefore, content analysis of hemicellulose / cellulose appears to be an important step in the initial screening of lignocellulosic biomass as prebiotic sources. According to Yoo *et al.* (2012), the structural polysaccharides found in plant cell walls (PCW) are potential sources of novel prebiotic compounds. The polysaccharide constituents in PCWs are classified into cellulose, hemicellulose, and pectin (Vázquez *et al.* 2000; Scheller and Ulvskov 2010). The linear (1→4)-ß-D-glucan, cellulose, is considered to be the most abundant type of polysaccharides in plant fibres (Syntsya and Novak 2014); hemicelluloses comprise various types of polymeric sugars including xylan, xyloglucan, and (gluco) mannan (Yoo *et al.* 2012).

The present study elucidated the nutritional properties of *A. pinnata* in terms of proximate composition and xylan and ß-glucan content. For the proximate components, ash and crude protein in PA were comparable to other aquatic plants previously analysed (Boyd 1968); crude fibre (22.3%) was higher in PA, relative to the concentrations in leaf biomass of several aquatic plant species (Adelakun *et al.* 2016; Bahnasy *et al.* 2016; Akmal *et al.* 2014). The amount of crude fibre in *A. pinnata* varied as shown in previous studies, usually in lower concentration that ranged between 12.7% (Alalade and Iyayi 2006) and 14.7% (Cherryl *et al.* 2014). In this study, both xylan and cellulose content in PA were marginally higher, relative to the reported values of Miranda *et al.* (2016) at 10.1% for xylan as hemicellulose and 12.8% for cellulose. Gupta *et al.* (2018) also reported lower yield for xylan (11.1% as hemicellulose); but higher yield for cellulose (28.9%), compared to the values in this study. The observed difference in xylan and cellulose content can be attributed to several factors, including pretreatment and extraction method used and the variations with respect to the source locality, as well as ambient condition of the source environment (Nummi *et al.* 1985; Zhu *et al.* 2016). Xylan and cellulose content among different plant species also varies due to differences in the chemical compositions (Rabemanolontsoa and Saka 2013). For instance, xylan yield (% DM basis) was lower in duckweed (*Lemna minor)* at 3.5% (Zhao *et al.* 2012) and cassava peel at 4.83% (Ratnadewi *et al.* 2016). However, cellulose yield was remarkably higher (28.4%) in the water hyacinth (*Eichhornia crassipes*) as reported by Mishima *et al.* (2007) and duckweed at 20.3% (Ge *et al.* 2012), compared to the values in the present report. Recently, the study of Poeker *et al.* (2018) demonstrated the fermentation of ß-glucan and xylooligosaccharide (XOS) by indigenous microbiota in the host's gut. It was found that these carbohydrates were significantly associated with the presence of certain end metabolites, particularly short-chain fatty acids (SCFA). This underscores the potential of ß-glucan and XOS as prebiotic substrates for the selective stimulation of microbial gut fermenters with beneficial health effects (Carlson *et al.* 2017).

The prebiotic activity of PA was evaluated in vitro based on the growth of the test probiotic, *L. rhamnosus* SRLPB77. According to Rubel *et al.* (2014), the growth stimulating effect of prebiotic substrates can be ex-

pressed in terms of the relative growth ratio (RGR) of a probiotic, in comparison to glucose as optimal carbon source. In this study, the RGR of *L. rhamnosus* grown in MRS medium containing PA or commercial inulin was determined at different time intervals during the 24-hour incubation. Results showed that PA stimulated the growth of *L. rhamnosus* better than glucose within 4 to 6 hours of incubation; albeit, growth of the probiotic did not significantly improve thereafter. This result is similar to the findings of Lukova *et al.* (2018), in that, growth in vitro of *L. plantarum* strains in the culture medium containing 10% hydrolysates from dried leaves of *Plantago* spp. was significantly lower after 24 hours of incubation, as compared to the medium with glucose. The poor growth response of the studied lactobacilli was probably due to the presence of galactose and arabinose, which are difficult to metabolize (Lukova *et al.* 2018). In contrast, Chimtong *et al.* (2016) reported that oligosaccharides derived from hydrolysed spent tea leaves improved growth in vitro of *L. acidophilus* after 18 hours incubation, compared to glucose. According to Tarraran and Mazzoli (2018), lactic acid bacteria (LAB) including Lactobacillus strains cannot readily utilise lignocellulosic materials as carbon source; hence, efficient metabolism of oligosaccharides derived from partial hydrolysis of cellulose/hemicellulose is essential for optimal fermentation of these polysaccharides by LAB. On the other hand, the present study revealed that commercial inulin exerted improved stimulation on probiotic growth relative to both PA and glucose during the entire incubation period. This indicates the suitability of inulin as prebiotic substrate to improve the growth of *L. rhamnosus* SRLPB77 in vitro, highlighting the potential use of these biological materials in the development of functional dietary synbiotics. Indeed, the prebiotic activity of inulin towards several lactobacilli strains has been recognised in the recent study of Śliżewska and Chlebicz-Wójcik (2020), whereby, growth, metabolic profile and antagonistic activity towards pathogens of these probiotics were most favoured when 2% (w/w) of inulin was used.

Agricultural by-products have been recently tapped as potential sources of bioactive compounds that are supplemented in optimal amounts to the aquafeed (i.e., phytogenic feed additive), in order to improve health and growth performance of cultured aquatic species (Caipang *et al.* 2019). While the mechanism of action of phytogenics is not yet completely understood (Upadhaya and Kim 2017), these plant-based products have been implicated for various beneficial roles including among others, as antioxidant, antimicrobial / antiparasitic and anticarcinogenic agent, growth and / or appetite enhancer and stimulant of intestinal bile secretion and / or digestive enzyme activity (Asimi and Sahu 2013; Sutili *et al.* 2018). The present study examined the effect of PA as dietary component on performance of milkfish fingerlings. Results showed that partial replacement or enrichment of commercial diet with PA had no adverse effect on survival; however, significant improvements in terms of growth and feed efficiency were noted in fish fed with 2% (w/w) PA replacement. The potential use of dried azolla as alternative ingredient of aquafeed has been investigated in several aquaculture fishes (Mahraomai *et al.* 2018). Ebrahim *et al.* (2007) reported that dried *A. nilotica* at 31.8% dietary inclusion rate had no adverse effect on survival, growth, and feed efficiency of Nile tilapia (*Oreochromis niloticus*) fingerlings. A similar result was also reported by Mogouz *et al.* (2020), in that, *O. niloticus* fed a diet formulated to contain 10-20% of azolla meal had comparable growth rates, compared to the control. Also, survival and growth of *Labeo fimbriatus* given 40% sun-dried *A. pinnata* diet did not differ significantly from the fish on control diet (Gangadhar *et al.* 2015). The present findings demonstrated the possibility to re-constitute the basal diet of milkfish fingerlings to contain 2% (w/w) of PA, for improved growth and feed efficiency without negative effect on survival rate of the fish. The observed increase on the growth of the fingerlings can be due to the net effect on immune response and health, attributed to the optimal rate of PA inclusion in the diet. Yuan *et al.* (2008) showed that the common carp (*Cyprinus carpio*) fed with diets containing plant additives at 0.5 – 1% inclusion rates, improved macrophage phagocytic activity, respiratory burst and concentrations of total protein, albumin, globulin and nitric oxide synthetase activity in the serum. Polysaccharides from the root and stem of *A. membranaceus* were reported to halt reactive oxygen species (ROS) production, stimulate humoral and cellular immunity and thus possess immune-stimulating effects (Yuan *et al.* 2008). Moreover, use of pulverised *A. pinnata* as phytogenic dietary component in fish diet is also practical, as it contains essential amino acids, vitamins, growth promoter intermediaries, and minerals (Mahraomai *et al.* 2018).

5 | CONCLUSIONS

The present study demonstrated the potential of *A. pinnata* as source of prebiotic oligosaccharides. Analysis of fermented azolla hydrolysates revealed detectable concentrations of xylose and glucose, suggesting the presence of prebiotic sugar oligomers. The prebiotic activity of PA was also demonstrated by the improved growth in vitro of *L. rhamnosus* in PA-enriched medium within 4 to 6 hours of incubation. Results of the feeding trial also showed that milkfish fingerlings fed CD containing PA in varying levels had comparable survival rates, relative to the group on 100% CD; while the average TWG, SGR and FE were significantly improved in the group given CD replaced with 2% (w/w) PA, compared to the control group. In conclusion, dried *A. pinnata* biomass in pulverised form can be used as dietary feed component for improved production performance of cultured milkfish.

ETHICAL APPROVAL

All animal procedures performed in this study were reviewed and approved by the College Ad Hoc Animal Ethics Committee following the Guidelines for the Use of Fishes in Research (UFRC 2014).

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CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTION

CPdlC designed the research, analysed the data and prepared the manuscript. Technical assistance and expert advice were done by LPBA, EVM and RRR. PDHT conducted data analysis, technical assistance, proofreading, and manuscript preparation.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on a reasonable request from the corresponding author.

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