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RESEARCH PAPER

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Effectiveness of Galactooligosaccharide and Combination of Yeast + B-Glucan in Soybean Meal Diets on Innate Immune Response and Disease Resistance Against *Aeromonas hydrophila* in Striped Catfish (*Pangasianodon hypophthalmus*)

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Abstract

Feeding soybean meal (SBM) based diets affects fish gut immunity and causes significant tissue lesions that inhibit digestion and absorption. The addition of probiotic, prebiotics and their synbiotics used in SBM diets is assumed can mitigate the negative effect of SBM. This current study was carried out to investigate the effects of commercially available galactooligosaccharide (GOS) and combination of 1% yeast + 0.1% β-glucan (YβG) on haematological, immune response and disease resistance in striped catfish juvenile against *Aeromonas hydrophila*. Fish (average initial weight 16.45±0.07 g) were fed with four different diets up to 12 weeks containing control negative, control positive, 1 % GOS and combination of 1% yeast+0.1% β-glucan. After 12 weeks feeding trial, the fish were challenged intraperitoneally with 0.2×10^6 cfu/ ml of *A. hydrophila*. The haematological and immunological parameters were assayed both in pre-challenged and post-challenged groups. There was a significant improvement in red blood cell, haemoglobin concentration, white blood cell and granulocyte count in both supplemented groups over the control. Immunoglobulin level showed an increasing trend in pre-challenged fish but it was much higher in post-challenged fish. There was a significant increase in lysozyme activity and further significant increase in the levels of serum lysozyme occurred in fish injected with *A. hydrophila.* In conclusion, the supplementation of GOS and YβG could improve the health status of striped catfish based on the improvement of their haematological and immunological parameters as well as their ability to resist *A. hydrophila* infection.

Introduction

Aeromonas hydrophila is the causative agent of "Motile Aeromonad Septicaemia" (Newman, 1993) and considered as one of the important freshwater catfish pathogen mainly striped catfish, because of the severe mortalities caused by infection with this pathogen and the resulting economic impact among commercial aquaculture producers and conservation hatcheries (Crumlish et al., 2010). Control of bacterial disease caused by *Aeromonas* in aquaculture industry has been achieved by using some synthetic chemicals and antibiotics (Villamil et al., 2014). The use of those expensive chemotherapeutants for controlling diseases has been widely criticized for their negative impacts like accumulation of residues, development of drug resistance, immunosuppressants and reduced consumer preference for aqua products treated with antibiotics (Sahu et al., 2008).

Recently, use of probiotics and prebiotics is one of method that is gaining importance in controlling potential pathogens (Merrifield et al., 2010). There has

also been a growing interest to understand the effects of combined use of probiotic and prebiotic, known as synbiotic effect, in fish (Cerezuela et al., 2011). Several commercial probiotics and prebiotics products such as yeast, galactooligosacharides (GOS), and β-glucan have been showed positive effect on striped catfish performance (Sutriana et al., 2021). Yeasts have several attributes for consideration as good probiotic candidates such as not affected by anti-bacterial compounds, some strains have antagonistic activities against undesirable bacteria (Hatoum et al., 2012), can stimulate intestine maturation (Tovar et al., 2002), and modulate antioxidant enzyme in host fish (Tovar-Ramírez et al., 2010). Meanwhile, GOS and β-glucan are possessing dual functional properties that can activate the innate immune system not only by enhancing the growth of commensal microbiota but also by directly stimulating the immune system (Song et al., 2014).

Soybean meal (SBM) has been used as an alternative plant protein source in the fish farming industry because of its high protein content, comparatively well-balanced amino acid profile, relative ease of availability, and competitive price compared to fish meal (FM) (Biswas et al., 2007; Phumee, 2011). However, the use of high levels of SBM in the diet can cause a reduction in growth and feed utilization in rainbow trout (Oliva-Teles et al., 1994), Atlantic salmon (Refstie et al., 1998), and striped catfish (Phumee, 2011), as well as morphological and functional disruptions, such as enteritis, changes in absorptive cells, presence of inflammatory cells, shortening of the villi and microvilli in the intestines, and increased susceptibility to bacterial infection of Atlantic salmon (Van den Ingh et al., 1996; Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000), Rainbow trout (Rumsey et al., 1994) and common carp (Uran et al., 2008). The negative effects of SBM supplementation in fish feed are related to the antinutritional factors found in large amounts in soybean products such as soluble and insoluble fiber, phytic acid, trypsin inhibitors, hemagglutinins, lectins, saponins, phytoestrogens, phytosterols, oligosaccharides, β-conglycinin, glycinin, and isoflavone (Martins et al., 2017; Zhou et al., 2018).

Previous studies using prebiotic (GOS) and synbiotic yeast + β-glucan (YβG) in diet containing soybean meal, indicated that these supplementations can mitigate the negative effect of SBM and influenced growth performance, feed utilization, and intestinal microbiota of striped catfish (Sutriana et al., 2018). Based on the fact that supplementation of probiotics, prebiotics and their synbiotics used in SBM diet also improved immune response (Dhayanithi et al., 2015; Dou et al., 2023; Nakhei Rad et al., 2023) and survival rates of several fish species after challenged with various pathogens (Buentello et al., 2010; Sealey et al., 2007, 2015; Dou et al., 2023). In contrast to the progress made in other species, the effects of GOS and YβG in SBM diet on striped catfish have received little attention. Therefore, the present study was designed to investigate the influence of GOS and YβG in promoting health status of striped catfish (*P. hypophthalmus*) and its resistance to *A. hydrophila* infection.

Materials and Methods

Experimental Diets

Four diets were prepared to contain 30% protein and 12% lipid with a gross energy of 19 MJ/kg (Table 1). The control diet (FM) contained 100% protein from fish meal (FM) and the remaining 3 diets were formulated so that 45% of the protein was provided by SBM and 55% from FM (FM-SBM). Two of the latter diets were supplemented with 1% GOS (FM-SBM-GOS) and a combination of 1% yeast+0.1% β-glucan (FM-SBM-YβG), respectively. The selection of 45% protein replacement with SBM was based on the report by Phumee et al. (2011) who showed that inclusion of soybean meal protein beyond 30% compromised final weight, specific growth rate and protein efficiency ratio of juvenile striped catfish. GOS used in this study was Vivinal GOS (Friesland Foods), while β-glucan was from Biorigin (Macrogard) and *S. cerevisae* (Bakers yeast) from Sigma-Aldrich. All of the experimental diets were prepared and stored according to the method described by Sutiana et al. (2018).

Fish and Rearing Conditions

At the start of the experiment, a total of 360 fish (average initial weight 16.45±0.07 g) were randomly stocked into 12 circular fiberglass tanks (600 L). The respective experimental diets were fed to triplicate groups of fish (30 fish per tank) for 12 weeks. Continuous water was provided to each tank with a flow rate of 1.5 L min^{-1} was maintained throughout the experimental period. All groups were fed their respective diets twice daily at 3% body weight per day; this rate was adjusted biweekly according to fish body weight. During the experiment, temperature (27- 29°C), pH (6.2-6.5), and dissolved oxygen (5-6 mg L^{-1}) were monitored biweekly using a standard mercury thermometer, digital HI-98103 pH meter and digital DO-5509 meter, respectively.

Blood Collection and Analysis

Upon completion of feeding trial, 3 striped catfish per tank (9 fish per treatment) were sampled and anesthetized with Aquadine (Fish Stabilizer; International Fish S.O.S Association). Then, blood sample was immediately taken from the caudal vein using a 1 ml syringe with a 21-gauge needle for evaluation of haematological and immunological parameters.

The blood samples were divided in two parts, one part was transfer into a tube containing heparin (heparinized tube) as an anticoagulant, and this

heparinized blood was used to determine erythrocyte sedimentation rate (ESR), packed cell volume (PCV), haemoglobin (Hb), red blood cell (RBC), RBC indices, white blood cell (WBC) and differential leukocyte count as previously described by Al-Dohail et al. (2009) and Akter et al. (2019, 2023). The second part was transfer into tube without anticoagulant (a non-heparinized microtainer blood collection tube), allowed to clot at room temperature for 1 h and then kept at 4°C for 5 h. Serum was obtained after by centrifugation at 3000 xg for 15 minutes (Aly et al., 2008; Akter et al., 2019, 2023) and store at −20°C for further analysis.

Lysozyme Activity

Serum lysozyme activity was determined by the turbidity assay (Demers and Bayne, 1997 and Akter et al., 2019, 2023) based on the lysis of the lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma). The result was expressed in amounts of lysozyme (µg) per mL of sample calibrated to a standard curve.

Total Immunoglobulin

The total protein and immunoglobulin content in serum was determined according to the method described by Siwicki & Anderson (1993); Amar et al., (2000) and Akter et al., (2019, 2023). The total immunoglobulin value was expressed as (mg ml^{-1}), calculated according to the following formula:

Total Ig (mg ml^{-1})=Total protein in serum sample-Total protein treated with PEG

Challenge Test against *Aeromonas hydrophila*
Isolation, Identification and Pathogenicity Test of *A. hydrophila*

Aeromonas hydrophila used in this study collected form National Fish Health Research Center in Penang. After collection, the bacteria were injected into *P. hypophthalmus* maintained in the Fish Disease Laboratory at the Aquaculture Research Complex of Universiti Sains Malaysia. These bacteria were intraperitoneally injected into striped catfish to improved virulence. Typical clinical signs of *A. hydrophila* infection found in striped catfish was haemorrhage on the ventral surface of fish body and at the base of pelvic fins and abdominal distension.

The bacteria were then re-isolated from the infected kidney of *P. hypophthalmus* and grown on tryptic soy agar (TSA, Himedia, India) for 24 h at 30°C. Morphologically distinct and well isolated colonies were individually picked and transferred to new TSA plates by streaking until pure colonies were obtained. A Gram staining procedure was carried out to identify whether the bacterium is Gram positive or negative. Based on morphological and biochemical characteristics, a number of colonies representing all recovered aeromonads in this study were chosen for identification based on 16S rDNA analysis as described by Akter et al., (2018). The 16S rRNA gene sequences showed 100% similarity with *A. hydrophila* in the existing NCBI database (Accesion no KR067615.1).

Bacterial (*A. hydrophila*) cell free extract was then separated from the cells by centrifuging at 3000 xg for 10 minutes at 4°C. The cells were then washed two times

Table 1. Ingredients and proximate composition (g 100 g^{-1} dry matter) of the experimental diets

	Experimental Diets			
Ingredients	FM	FM-SBM	FM-SBM-GOS	FM-SBM-YβG
Fish meal[1]	38.25	21.10	21.10	21.10
Soybean meal	-	26.70	26.70	26.70
Corn starch	43.00	32.47	32.47	32.47
Fish oil	2.87	4.37	4.37	4.37
Soybean oil	5.88	5.36	5.36	5.36
Cellulose	2.00	2.00	2.00	2.00
GOS	-	-	1.00	-
Yeast	-	-	-	1.00
β-glucan	-	-	-	0.10
CMC[2]	3.00	3.00	3.00	3.00
Vitamin[3]	2.00	2.00	2.00	2.00
Mineral[4]	2.00	2.00	2.00	2.00
Cr_2O_3	1.00	1.00	1.00	1.00
Total	100	100	100	100
	Proximate composition (g 100 g^{-1}, dry matter matter basis)			
Moisture	8.03	8.24	7.96	8.69
Protein	30.62	30.96	31.30	30.58
Lipid	12.92	11.71	12.56	12.13
Ash	8.02	8.94	7.96	8.17
Fibre	2.41	4.22	4.96	4.90
NFE[5]	46.03	44.17	43.22	44.22
GE [6] (MJ/kg)	19.02	18.91	19.68	19.43

[1]Danish fish meal: crude protein, 72%; crude lipid, 5%

[2]CMC, carboxymethyl cellulose

[3]Vitamin Mix kg^{-1}(Rovitai Ltd 700/437 Chonburi Thailand**):** Vit.A 50 million i.u., Vit.D3 10 million i.u., Vit.E 130g, Vit.B1 10g, Vit.B2 25g, Vit.B6 16g, Vit.B12 100 mg, Biotin 500 mg, Pantothenic acid 56 g, Folic acid 8 g, Niacin 200 g, Anticake 20 g, Antioxidant 200 mg, Vit.K3 10 g and Vit.C 35g

[4] Mineral Mix kg^{-1}: Calcium phosphate(monobasic) 397.65g; Calcium lactate 327g; Ferrous sulphate 25g; Magnessium sulfate 137g; Potassium chloride 50g; Sodium chloride 60g; Potassium iodide150mg; Copper sulphate 780mg; Manganese oxide 800mg; Cobalt carbonate 100mg; Zinc oxide 1.5g and Sodium selenite 20mg

[5]NFE, Nitrogen free extract (100 – (protein+lipid+ash+fiber))

[6] GE, Gross energy (measured using bomb calorimeter, parr 6200 Bomb Calorimeter, USA)

with phosphate buffered saline (PBS, pH 7.4) and resuspended in the same buffer (Zheng et al., 2011). The absorbance at optical density of 600nm (OD600) was measured to obtain a value of 1, which corresponded to $1x10^8$ CFU ml^{-1} of bacterial suspension resulted from the plate counting. For plate counting, 1 ml of cultured bacterial cells was serially diluted to 10^{-7} in the same buffer. One hundred microliter of the sample was then spread onto the TSA plate in trilicate and incubated for 24h in an incubator at 30°C. The colony forming unit per milliliter of bacterial sample was counted manually.

To determine the optimum bacterial (*A. hydrophila*) cell concentration to be used in experimental challenge, groups of 210 fishes (50-55 g in average) were stocked in 21 aquaria (60cm x 30cm x 30cm) in disease lab and were fed with 30% protein commercial feed. The fishes were injected intraperitoneally with 0.2 ml of 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 CFU/ml of *A. hydrophilla* in phosphate buffered saline (PBS, pH 7.4). Control fish were injected with the same amount of sterile PBS. The mortality of fish was monitored regularly and continued for two weeks. The dose of $LD_{\wedge 50}$ was chosen for the experimental challenge.

Challenge Test

At the end of the feeding trial, a bacterial challenge test was conducted on each experimental group with *A. hydrophila* to evaluate the effects of GOS and YβG against bacterial infection. Total 45 fish from each treatment group were randomly selected (15 fish from each replicate tank) and distributed in glass tanks (60cm x 30cm x 30cm) in the closed recirculating system. Each group of fish was fed with the respective experimental diets that had been administered during the feeding trial. The fish were then challenged with *A. hydrophila* by intraperitoneal injection of 0.2 ml of 1×10^6 CFU ml^{-1} (result obtained from pathogenicity test) bacteria suspension per individual fish using a sterile syringe. The mortality was recorded daily for 3 weeks following injection. The blood was collected from three fish per replicate aquarium (nine fish per treatment) at the end of 1-, 2- and 3-week post infection. Haematological and immunological parameters during challenged test were analyzed following method described above.

Statistical Analysis

Normality and homogeneity of obtained data were tested employing Levene's and Shapiro-Wilks test in SPSS version 22. The results were analyzed statistically using one-way analysis of variance (ANOVA) and the mean differences among the four different treatments were tested with a significance level of $P<0.05$ using a Duncan's multiple range test (Duncan 1955). All statistical analyses were computed using SPSS software, version 22 for Windows. The data were presented as mean±SD.

Results

Haematological Parameters

The results of feeding different experimental diets on blood parameters after 12 weeks are presented in Table 2. ESR, PCV, RBC, Hb content, mean corpuscular volume (MCV), mean corpuscular haemoglobin content (MCH) and mean corpuscular haemoglobin concentration (MCHC) were not significantly ($P>0.05$) affected by dietary intake. However, WBC count was significantly higher ($P<0.05$) in the FM-SBM-YβG fed fish compared to the FM-SBM fed fish, but percentage of leucocytes types (lymphocyte, monocyte, granulocyte) for each treatment were unaffected.

Table 3 shows the haematological profile of striped catfish fed with experimental diets 1 week after infection with *A. hydrophilla*. Overall, fish fed FM-SBM diet exhibited higher ESR value, but lower PCV, RBC, Hb, WBC and granulocyte values than fish fed other diets. Supplementing the SBM diet with YBG and GOS improved the ESR, RBC, Hb, WBC and granulocyte values and was comparable to FM diet. While fish fed YBG diet showed significantly lower ESR and higher PCV, RBC, Hb, WBC and granulocyte values, only RBC, WBC and granulocyte were significantly increased in fish fed GOS diet compared to FM-SBM diet. Significant changes were not recorded in other blood parameters.

At 2-week post challenge, ESR value was significantly lower ($P<0.05$) in the FM-SBM-YβG fed fish compared with the FM-SBM group (Table 4). On the contrary, PCV value, RBC count, and Hb level was significantly increase in FM-SBM-YβG fish group than the FM-SBM group. Likewise, fish consumed diet supplemented with GOS also showed the higher RBC count and Hb level ($P<0.05$) compared to those fed FM-SBM diet. Fish fed FM diet had similar haematological parameters to the other diets except Hb value that significantly higher ($P<0.05$) than fish fed FM-SBM diet. The WBC values were found similar in all treatments.

Table 5 shows the haematological parameters of experimental fish on the 3rd week after being challenged with *A. hydrophila.* The results showed that the ESR and Hb values were similar among diets except fish fed FM-SBM-YβG diet which showed significantly lower ESR and higher Hb values compared with fish fed FM-SBM diet. Fish fed FM-SBM-GOS and FM-SBM-YβG diets also showed significantly lower percentage of granulocytes than FM-SBM diet. The other haematological parameters were not affected during this period.

Immunological Parameters

Lysozyme Activity

Lysozyme activity in fish before and after challenged with *A. hydrophila* is presented in Table 6. The results showed that the diet did not influence the

Table 2. Haematological parameters of striped catfish fed experimental diets for 12 weeks

	Diets			
Haematological parameters	FM	FM-SBM	FM-SBM-GOS	FM-SBM-YβG
ESR (mm h^{-1})	1.12±0.21	1.34±0.32	1.26±0.21	1.14±0.15
PCV (%)	38.45±4.33	37.03±2.89	38.53±2.48	39.71±1.89
RBC (x10^6 mm^{-3})	3.97±0.45	3.83±0.25	3.98±0.34	4.13±0.26
Hb (gdL^{-1})	14.19±0.35	13.96±1.34	14.48±0.67	14.59±0.85
MCHC (gdL^{-1})	37.27±3.69	37.70±2.52	37.64±1.47	36.72±1.85
MCH (pg $cell^{-1}$)	36.05±3.30	36.37±1.89	36.50±1.50	35.34±1.93
MCV (μm^3)	98.89±4.56	98.69±5.01	97.03±3.80	96.24±2.09
WBC (x10^4 mm^{-3})	4.26±0.53^{ab}	3.72±0.56^{a}	4.17±0.74^{ab}	4.60±0.46^{b}
Lymphocyte (%)	68.28±4.75	70.22±5.15	69.50±3.39	69.50±3.04
Monocyte (%)	5.11±1.24	5.78±2.33	5.56±1.40	5.61±1.39
Granulocyte (%)	26.61±4.63	24.00±3.61	24.94±3.64	24.89±3.10

Data presented as mean±SD, (n=9; 3 fish per replicate tank). Data with different superscripts in the same row indicate significant differences (P<0.05).

Table 3. Haematological parameters of striped catfish fed experimental diets after 1 week challenged with *A. hydrophila*

	Diets			
Haematological parameters	FM	FM -SBM	FM-SBM-GOS	FM-SBM-YβG
ESR (mm h^{-1})	2.18±0.59^{ab}	2.83±0.62^{b}	2.23±0.73^{ab}	2.01±0.80^{a}
PCV (%)	30.75±4.89^{ab}	30.32±4.89^{a}	33.42±5.68^{ab}	35.53±5.62^{b}
RBC (x10^6 mm^{-3})	3.32±0.53^{ab}	3.4±0.37^{a}	3.56±0.54^{b}	3.62±0.45^{b}
Hb (gdL^{-1})	11.43±2.14^{ab}	10.22±1.60^{a}	11.31±2.02^{ab}	12.48±2.21^{b}
MCHC (gdL^{-1})	37.11±2.89	34.12±3.37	34.11±5.06	35.20±3.29
MCH (pg $cell^{-1}$)	34.42±2.74	33.60±3.62	31.73±3.04	34.38±2.67
MCV (μm^3)	92.86±5.19	98.39±4.54	93.69±5.58	98.08±8.48
WBC (x10^4 mm^{-3})	5.38±0.59^{ab}	4.83±0.25^{a}	5.74±0.41^{bc}	5.92±0.66^{bc}
Lymphocyte (%)	66.33±3.25	67.44±3.52	65.39±2.42	64.61±1.64
Monocyte (%)	5.06±1.78	5.72±2.91	5.61±1.41	5.67±1.15
Granulocyte (%)	28.61±3.40^{ab}	26.83±2.68^{a}	29.90±2.60^{b}	29.42±1.44^{b}

Data presented as mean±SD, (n=9; 3 fish per replicate tank). Data with different superscripts in the same row indicate significant differences (P<0.05).

Table 4. Haematological parameters of striped catfish fed experimental diets after 2 weeks challenged with *A. hydrophila*

	Diets			
Haematological parameters	FM	FM -SBM	FM-SBM-GOS	FM-SBM-YβG
ESR (mm h^{-1})	1.67±0.38^{ab}	2.04±0.72^{b}	1.63±0.67^{ab}	1.41±0.38^{a}
PCV (%)	34.24±5.35^{ab}	31.54±4.97^{a}	35.34±5.39^{ab}	37.91±4.28^{b}
RBC (x10^6 mm^{-3})	3.65±0.52^{ab}	3.39±0.48^{a}	4.02±0.59^{b}	4.01±0.33^{b}
Hb (gdL^{-1})	12.16±1.43^{b}	10.39±1.34^{a}	11.94±0.99^{b}	13.50±2.38^{b}
MCHC (gdL^{-1})	35.75±2.47	33.08±1.64	34.35±3.33	35.42±3.13
MCH (pg $cell^{-1}$)	33.53±2.64	30.85±3.55	30.33±2.29	33.43±3.55
MCV (μm^3)	93.88±5.88	93.18±8.32	88.46±7.41	94.27±5.46
WBC (x10^4 mm^{-3})	5.06±0.71	4.69±0.64	5.23±0.63	5.10±0.53
Lymphocyte (%)	67.44±2.49	67.83±3.51	67.67±3.41	68.50±2.94
Monocyte (%)	5.17±1.50	5.94±2.35	4.78±1.72	4.39±0.96
Granulocyte (%)	27.39±3.11	26.22±3.38	27.56±3.30	27.11±2.67

Data presented as mean±SD, (n=9; 3 fish per replicate tank). Data with different superscripts in the same row indicate significant differences (P<0.05).

lysozyme activity in pre-challenged fish. The differences of lysozyme activity occurred after the challenge test which recorded an increase of lysozyme activity in fish fed FM-SBM-GOS and FM-SBM-YβG diets at first week post-challenge compared to FM and FM-SBM diets. After 2-weeks challenged, fish fed diet FM-SBM-YβG had significant higher lysozyme activity (P<0.05) compared to FM and FM-SBM diets, but similar value compared to fish fed FM-SBM-GOS diet. The lysozyme activity decreases at 3-week post-challenged with not significantly different observed in all dietary treatments.

Total Immunoglobulin

Analysis of total immunoglobulin showed that fish fed FM-SBM diet had significantly lower total immunoglobulin (P<0.05) in pre-challenged fish (Table 7) compared to fish fed others diet. Supplementation of YBG in the FM-SBM diet showed the higher total Ig content than others, except fish fed FM-SBM-GOS diet. Similar trend was also observed in post-challenged fish. Regardless post-challenge period (1, 2, 3 weeks post-challenge) the total immunoglobulin were

Table 5. Haematological parameters of striped catfish fed experimental diets after 3 weeks challenged with *A. hydrophila*

	Diets			
Haematological parameters	FM	FM -SBM	FM-SBM-GOS	FM-SBM-YβG
ESR (mm h^{-1})	1.41± 0.34^{ab}	1.79±0.67^{b}	1.53±0.40^{ab}	1.26±0.20^{a}
PCV (%)	36.95±5.31^{ab}	34.20±4.35^{a}	37.56±5.51^{ab}	39.53±4.44^{b}
RBC (x10^{6} mm^{-3})	3.89±0.47	3.68±0.54	3.98±0.43	4.06±0.47
Hb (gdL^{-1})	12.37±1.37^{ab}	11.16±1.66^{a}	12.41±2.10^{bc}	14.18±1.78^{c}
MCHC (gdL^{-1})	33.74±2.57	32.65±2.60	32.34±2.57	37.55±4.20
MCH (pg $cell^{-1}$)	32.19±1.58	32.65±2.60	34.30±4.06	35.70±3.74
MCV (μm^{3})	94.90±2.99	93.24±5.21	95.44±7.16	97.30±5.33
WBC (x10^{4} mm^{-3})	4.41±0.87	4.20±1.15	4.50±0.95	4.83±0.84
Lymphocyte (%)	68.22±4.06	67.33±5.40	71.33±3.76	71.56±4.79
Monocyte (%)	5.11±1.11	6.06±1.67	5.61±2.41	5.00±1.20
Granulocyte (%)	26.67±3.39^{b}	26.61±2.22^{b}	23.06±3.05^{a}	23.44±2.11^{a}

Data presented as mean±SD, (n=9; 3 fish per replicate tank). Data with different superscripts in the same row indicate significant differences (P<0.05).

Table 6. Lysozyme activity (µg ml^{-1}) of striped catfish fed experimental diets in pre- and post-challenged with *A. hydrophila*

	Diets			
Challenge Periods	FM	FM-SBM	FM-SBM-GOS	FM-SBM-YβG
Pre-challenged	5.26±2.75	5.17±1.86	6.13±2.81	7.96±3.72
1-week Post-challenged	16.30±1.63^{b}	13.82±1.99^{a}	18.42±1.86^{c}	21.03±2.38^{d}
2-week Post-challenged	13.76±1.07^{a}	13.04±1.63^{a}	14.43±1.64^{ab}	16.64±3.54^{b}
3-week Post-challenged	6.30±1.93	6.18±3.35	6.89±1.23	7.38±1.56

Data presented as mean±SD, (n=9; 3 fish per replicate tank). Data with different superscripts in the same row indicate significant differences (P<0.05).

significantly higher (P<0.05) in fish fed FM-SBM-YβG diet than other diets; except fish fed FM-SBM-GOS diet in post 2 weeks challenged, which were not different statistically.

Fish Resistance to *A. hydrophila*

Mortality of the challenged fish was observed on the first day after the challenge. Following a 21-day challenging trial, the lowest survival rate (62.22±3.85%) was noted in fish fed the FM-SBM diets and the highest survival rate (88.89±3.85%) was recorded in group fish fed FM-SBM-YβG diet. Dietary FM-SBM resulted in a significant decrease in the survival rate (P<0.05) while supplementation of GOS and YBG in the FM-SBM diet increase fish survival (Table 8).

Discussion

The limitation of soybean as a dominant protein source in fish feeds is well documented and in some fish species this disadvantage can be overcome by the inclusion of probiotics and prebiotics, for example, red drum (*Sciaenops ocellatus* Linnaeus) (Burr et al., 2008), Atlantic salmon (*Salmo salar*) (Grisdale-Helland et al., 2008), sharpsnout seabream (*Diplodus puntazzo*) (Picollo et al., 2013), and rainbow trout (Sealey et al., 2010). Those studies also indicate that the influence of these supplements depends on the species as well as probiotics and prebiotics used. In striped catfish, previous studies showed that feeding striped catfish with SBM based diets containing GOS and YβG respectively resulted in significantly better growth and feed utilization compared to the FM-SBM diet but were similar to the FM diet (Sutriana et al., 2018).

Haematological parameters are regarded as one of the most important indicators to assess fish health (Azarin et al., 2015; Ferguson et al., 2010; Akter et al., 2019, 2023) and can be influenced by factors such as species, stress, physiology, nutritional status and environmental conditions (Moradi et al., 2013). These parameters have been studied in many fish species to determine the normal range and any variation from these ranges are indicative of physiological and pathological abnormalities in fish (Ranzani-Paiva et al., 2000).

An assessment of fish haematology in the present study indicated a significant increase in WBC in fish fed FM-SBM-YβG diets for 12 weeks. The increases in WBC upon intake of YβG observed in this study are similar to previous work in Nile tilapia fed with *Saccharomyces cerevisiae* as a whole yeast cell (probiotic), its extract (mannan-oligosaccharide - Prebiotic) and Pre-Probiotic mixture (Synbiotic) (Abu-Elala et al., 2013; Munni et al., 2023). The increased of WBC level following prebiotics and probiotics feeding have also been reported in snakehead fed 1 % *Lactobacillus acidophillus*, 1 % yeast, and 0.1 % β-glucan (Talpur et al., 2014) and carp fed yeast glucan (Selvaraj et al., 2005). The WBC count in response to dietary YβG could be attributed to the presence of β-glucan which was available in both its pure form (β-glucan) and as a component of yeast cell wall as well as the presence of specific receptors for β-glucan on phagocytic cells such as heterophiles and monocytes (Sang and Fotedar, 2010). β-glucan binds to receptor molecules on the surface of circulating and tissue phagocytes and such binding will increase the phagocytic activities in engulfing, killing and digesting bacteria. Concurrently, upon binding, cytokines will be secreted and this in turn stimulates the formation of

Table 7. Immunoglobulin content (mg ml^{-1}) of striped catfish fed experimental diets in pre- and post-challenged with *A. hydrophila*

	Diets			
Challenge Periods	FM	FM-SBM	FM-SBM-GOS	FM-SBM-YβG
Pre-challenged	18.09±1.72^{b}	16.33±1.86^{a}	18.23±2.00^{bc}	19.83±1.05^{c}
1-week Post-challenged	16.37±1.01^{b}	15.25±1.34^{a}	16.59±1.01^{b}	18.95±0.96^{c}
2-week Post-challenged	16.53±0.69^{b}	14.92±1.06^{a}	17.48±0.44^{c}	18.08±0.63^{c}
3-week Post-challenged	14.74±0.79^{b}	13.04±1.30^{a}	15.43±0.64^{b}	16.68±1.30^{c}

Data presented as mean±SD, (n=9; 3 fish per replicate tank). Data with different superscripts in the same row indicate significant differences (P<0.05).

Table 8. Survival rate of striped catfish after challenged with *A. hydrophila*

	Diets			
Parameters	FM	FM-SBM	FM-SBM-GOS	FM-SBM-YβG
No of injected fish	45	45	45	45
Dosage of bacteria injected	0.2 ml of 10^{6}CFU/ml	0.2 ml of 10^{6}CFU/ml	0.2 ml of 10^{6}CFU/ml	0.2 ml of 10^{6}CFU/ml
Survival rate (%)	73.33±13.34^{ab}	62.22±3.85^{a}	80.00±6.67^{b}	88.89±3.85^{b}

Data presented as mean±SD, (n=3; total fish live per 3 replicate tank). Data with different superscripts in the same row indicate significant differences (P<0.05).

new white blood cells and contributes to the remarkable increase in white blood cells in the absence of any infection (Raa, 2015).

Supplementation of GOS and YβG in SBM diet also affected the haematological parameters after the challenge test in which notable changes occur in ESR, PCV, and Hb. Regardless of the post-challenge period, ESR values were significantly higher in fish fed FM-SBM diet compared to fish fed FM-SBM-YβG diet. This observation could be due to the reduced capacity to resist infection in fish fed the FM-SBM diet. Higher ESR values noted in this study could be related to damage of red blood cell due to bacterial infection as a result of RBC swelling during infection (Haney et al., 1992; Harikhrisnan et al., 2003). The decrease in ESR values when feeding the fish with GOS and YβG supplemented diets is an indication of reducing the risk of infection or inflammation associated with the fish. Similarly, Talpur et al., (2014) observed a decrease in ESR in fish after feeding of 1% *lactobacillus acidophillus*, 1% yeast, and 0.1% β-glucan in the diet of snakehead and challenged with *A. hydrophilla*.

Aeromonas hydrophila produces endo- and exotoxins which cause lysis of red blood cells (Chopra et al., 2000), hence could explain the decline in PCV, Hb and RBC levels observed in this study. In the study by Harikrishanan et al. (2010), red blood cells significantly decreased after the challenge test with *A. hydrophila* and the simultaneous decrease in PCV, Hb and RBC in infected-fish indicated that red blood cells was destroyed. Campo et al. (2008) stated that haemoglobin plays a role in fish resistance because it works to bind oxygen in the blood thus increasing its oxygenated blood supply and the haemoglobin level is related to RBC level in the blood.

White blood cell (WBC) is one of the non-specific immune systems in the cell which is produced in high numbers when infection occurs in the body and it is related to the immune system working against infection (Uribe et al., 2011). It has been reported that the level of WBC is related to the presence of pathogen and general health status of fish (Harikhrisnan et al., 2011) and the increasing number of WBC in infected fish may serve as a protective barrier against pathogenic infection (Talpur and Ikhwanuddin, 2013). In this study, WBC count in fish fed FM-SBM-GOS and FM-SBM-YβG diets was significantly higher up to 1 weeks after infection compared to the fish fed FM-SBM diet. In agreement with our results, a similar increase of WBC was also observed when rainbow trout infected with *A. hydrophila* following treatment with immunogen (Yar-Ahmadi et al., 2016). On the contrary, significantly lower levels of leukocytes count were observed in the case of Nile tilapia challenged with either *A. hydrophila* and *P. fluorescens* (Sirbu et al., 2022).

Phagocytosis is the first defense of the cellular responses, which is carried out by monocytes (macrophages) and granulocytes (neutrophils) (Rodriguez-Estrada et al., 2013), and these cells capable of killing a variety of pathogens including bacteria (Wijendra and Pathiratne, 2007). In comparison of post-challenge periods, supplementation of YβG in this study showed the highest granulocyte count compared to FM-SBM after 1 week infection with *A. hydrophila* and the value decreased after 3-week challenged whereas granulocyte level in fish fed FM-SBM remained higher. This observation indicated that the immune system was successful in controlling the bacterial infection in YβG treatments, returning the percentage of granulocyte back to normal condition after 3 weeks infection.

Lysozyme level or activity is used as an important indicator of innate immune response in fish (Tort et al., 2003, Abreu et al., 2009) which acts against invasive microorganisms through lytic activity against bacteria (Ellis, 2001). Lysozyme splits the β 1, 4, glycosidic linkages between N-acetylmuramic acid and N-acetylglucosamine in the bacterial cell walls, causing cell lysis, thus preventing them from invading (Alexander

and Ingram, 1992; Paulsen et al., 2003; Akter et al., 2019, 2023). The present study indicated no significant influence in lysozyme activity when the fish were not infected with *A. hydrophila,* however, fish infected with *A. hydrophila* showed the decreased level of lysozyme activity but the supplementation of GOS and YβG in SBM diet resulted in increasing of lysozyme activity up to 2 weeks infection. It has been reported that immunostimulant can enhance or restore immune responses to normal levels after bacterial, viral and parasite infection (Jagruthi et al., 2014; Dhayanithi et al., 2015). In line with our finding, lysozyme activity increased significantly when rainbow trout fed with 2 g/kg immunogen supplemented diet after challenged with *A. hydrophila* (Yar-Ahmadi et al., 2016) and in genetic improvement of firmed tilapia when fed with 0.4-0.8% β-glucan supplemented diets (Dou et al., 2023). A significantly higher lysozyme activity was also reported in the serum of snakehead fingerlings fed with either probiotic or prebiotic diets after 2 weeks infected with *A. hydrophila* (Talpur et al., 2014). Moreover, the feeding of probiotic and synbiotic diets also increased the serum lysozyme activities of rainbow trout (Newaj-Fyzul et al., 2007; Nakhei Rad et al., 2023), orange-spotted groupers (Chiu et al., 2010), and olive flounder (Harikrishnan et al., 2010) that were induced with different pathogens. On the contrary, significantly higher level of lysozyme activity was reported when zebra fish fed with prebiotic (mushroom), probiotic (*S. cerevisiae*) and synbiotic (mushroom+*S. cerevisiae*) diets (Hosseini et al., 2024).

Immunoglobulin content in the blood serum is a major humoral component of the immune system (Giri et al., 2012) and plays a significant role in recognizing and counteracting foreign organism including bacteria and viruses (Cerezuela et al., 2012; Akter et al., 2019, 2023). The increased immunoglobulin activity in post-challenged fish indicates that a defense capability of fish against induced pathogen was activated (Talpur et al., 2012). Several studies reported that feeding the fish with prebiotic and synbiotic supplemented diet had a positive effect on the production of Ig content in pre- and post-challenged fish (Giri et al., 2012; Akrami et al., 2012; Talpur et al., 2014; Dou et al., 2023; Nakhei Rad et al., 2023), which is also proven in this study.

The enhancement of some haematological and immunological parameters in fish fed the FM-SBM-GOS and FM-SBM-YβG diets contributed to the least percentage of mortality following *A. hydrophila* infection observed in fish fed the corresponding diet in the present study. In line with this study, the supplementation of prebiotic and probiotics in SBM diet improved survival rates of several fish species after challenged with various pathogens (Buentello et al., 2010; Sealey et al., 2007, 2015; Dou et al., 2023). The higher mortality observed in fish fed FM-SBM diet indicated that fish fed this diet more susceptible to *A. hydrophila,* which was associated with the immune capacity suppression in soybean meal-fed fish (Akter et al., 2015; Sealey et al, 2015). Decreasing of immune capacity in SBM fed fish were also observed in Atlantic salmon (Bakke-McKellep et al., 2000), rainbow trout (Burrells et al., 1999), and Westslope cutthroat trout (Sealey et al., 2015).

The observed improvement of haematological and immunological parameters as well as the lower mortality resulting from the pathogenic *A. hydrophila* infection appears to be signs of enhanced health status of the SBM-YβG fed fish. Yeast, *Saccharomyces cerevisae* contains various immunostimulating compounds such as β-glucan, nucleic acids as well as mannanoligosaccharides which have the capability to enhance immune responses (Ortuno et al., 2002; Abdel-Tawwab et al., 2006). β-glucan either in pure form or as yeast cell wall component can modulate the activity of phagocytes and other components of the innate immune system and has shown the ability to stimulate defense mechanisms in vivo and in vitro (Nayar et al., 1998). In addition, β-glucan can also directly stimulate both specific and non-specific immunity (Vetvicka et al., 2013) and resulted in resistance against certain pathogens (Ellis, 2001). Therefore, the combined uses of yeast and β-glucan possess the better effect and resulted in better haematological and immunological response as well as resistance against *A. hydrophila* infection.

Although it is known that prebiotic oligosacharide has immunomodulatory actions in freshwater fish (Staykov et al., 2005; Petterson et al., 2010; Soleimani et al., 2012), only few studies demonstrate the effect of GOS on fish immune system. Several studies demonstrate the effect of GOS on the immune system originated from human and animal models. This effect could either be direct in the form of interactions with immune, mucosal or epithelial cells and/ or indirect through the species or strain selective modulation of the microbiota and their metabolic products (Anthony et al., 2006; Vulevic et al., 2008). Previous studies evaluated the efficacy of Bimuno, a mixture of galactoligosaccharide, against *Salmonella typhimurium* and indicated that Bimuno suppressed the ability of *S. typhimurium* to colonize and cause disease in mice (Searle et al., 2009). This mechanism of protection is might be by acting as a receptor on the host epithelial cell surface that can be recognized by the intestinal pathogens (Shoaf et al., 2006), thus blocking the adherence and subsequent invasion of *S. typhimurium* (Searle et al., 2009). However, whether this kind of protection also establish in striped catfish against *A. hydrophilla*, require further studies to test this assumption.

Several commercial probiotics and/or prebiotics have been suggested to be an alternative to vaccines and chemotherapeutics in fish disease control by stimulating the immune response and enhancing the health status of fish, which were also observed in striped catfish fed GOS and YβG in this study. Although there are added costs associated with using these products,

improvement of reducing disease incidence as evident in this study, may balance such costs. In this regard, Yousefian and Amiri (2009) state that when fish resistance to the disease increase and more fish survive until they reach marketable size, then the subsequent cost of medication and overall production costs would be remarkably reduced (Yousefian and Amiri, 2009).

Conclusion

In conclusion, supplementation of prebiotic GOS and symbiotic YβG in SBM based diets have improved the health status of striped catfish based on the improvement of their haematological and immunological parameters as well as their ability to resist *A. hydrophila* infection.

Ethical Statement

The handling, maintenance and killing of animals procedures applied in this study complied with the guidelines of the Animal Ethics Committee of Universiti Sains Malaysia.

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Author Contribution

Amalia Sutriana: Conduct the experiment, data collection and analysis.

Mst. Nahid Akter: Helped to conduct the experiment, data collection, analysis, manuscript writing.

Roshada Hashim: Oversight and leadership responsibility for the research activity, planning and execution, including mentorship, and manuscript editing.

Siti Azizah Mohd Nor: PhD co-supervisor, helped in experimental design, data analysis and manuscript editing.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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