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Safmannan-Supplemented Diet Ameliorated Serum Lysozyme Activity and Intestinal Bacterial Colonization and African Catfish Fingerlings

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Abstract: A 45-day feeding trial was conducted to study the effects of Safmannan as feed additive for African catfish (*Clarias gariepinus,* Burchell) fingerlings. Total of 270 catfish fingerlings were randomly distributed into nine experimental aquaria. Three complete diets were used in this study: Diet 1, feed from company A (control); Diet 2, feed from company B; Diet 3, feed from company B supplemented with Safmannan. Each of the diets was fed to triplicate groups of fish. The results showed that fish growth was not significantly ($p>0.05$) affected by diets, even with the supplementation of Safmannan. The feed conversion ratio of catfish fed with Diet 1 was significantly ($p<0.05$) lower than those fed with Diet 2 but not significantly different from catfish fed with Diet 3, reflecting the competitiveness of Safmannan-supplemented feed to the control feed. Fillet of catfish fed with Diet 2 had significantly higher crude protein content than those fed other two diets while significantly lower crude lipid content was resulted in the fillet of catfish fed with Diet 2 and Diet 3. Interestingly, Safmannan supplementation significantly lowered the colony forming unit count and increased serum lysozyme activity. In conclusion, Safmannan could be used as an immunostimulant for catfish farming.

INTRODUCTION

Fish is an essential protein source for mankind. It offers about 16% of the animal protein throughout human consumption[1]. People's demand on fish as foodstuff opens up the development of aquaculture, including the farming of other aquatic organisms. Aquaculture is presently contributing half of the global fish supply and projected to grow sustainably in meeting the increasing human demand[2]. Particularly in Malaysia, the annual amount of fish consumed per capita was recorded as the second highest among Asian countries[3].

Among the food fish species in Malaysia, the farming of African catfish, *Clarias gariepinus,* has drawn great attention with total production of 28,464.07 tonnes recorded in the year of 2019. The reasons for this fish species to be in such high demand are mainly due to their capability of accepting artificial feeds, ability in

producing good quality of flesh, tolerance to wide range of environmental conditions and resistance to disease[4]. In fact, African catfish is claimed to be one of the most vital tropical catfish species from the aquaculture context[5]. In addition, to the desired characteristics of African catfish that contribute to its high demand, African catfish also possess conveniences in high stocking densities, relative rapid growth rate, high fecundity and palatability[6]. However, matter that give rise to farmers' agony is that, African catfish fry are often plagued by high mortality, particularly during the first fourteen days after hatching, possibly due to their relatively higher susceptibility towards disease.

Along with the development of aquaculture industry, administration of antibiotics had effectively revolutionised aquatic health by curing infections. Nevertheless, owing to the constantly evolving natural system, pathogenic organisms commence defense through resistance[7]. People tend to cope with stronger antibiotics which eventually been claimed as reckless efforts. Therefore, farmers and growers that tend to rely on antibiotics to deal with the losses eventually causes negative impact not only on African catfish, but also on consumer health arising from the drug residue as well as the contaminated environment. One critical issue is the development of antibiotic resistance from bacteria as a result of excessive application[8]. Due to the restriction on antibiotic administration, interest on immunostimulant as an alternative to antibiotic is on the rise. Immunostimulant is described as a substance, either chemical or biological, that improve the innate immune response, via. specific interaction with cells within the system[9]. Some examples of immunostimulant that have been used in aquaculture industry are muramyl dipeptide, chitin, chitosan, letinan, oligosaccharide and yeast derivatives[10].

The Safmannan-enriched product appears to be a possible alternative to supply as feed additive to play a part as immunostimulant for improvement of catfish survival. Safmannan is characterised by its layer of mannoproteins which is the major source of Mannan-Oligosaccharides (MOS) that remarkably highlighted with the pathogen-binding capacity of it. MOS plays the role as effective ligand, dedicating competitive binding site for Gram-negative bacteria like *Salmonella* and *Escherichia coli*[11]. Therefore, pathogenic microorganisms with mannose-specific Type-1 fimbriae will eventually bind to MOS. This action beneficially preventing attachment of pathogens to intestinal epithelial cells, thereby impeding intestinal bacteria colonization which then favours catfish with the raise of natural self-defence in term of immune response and improvement in intestinal health with better capacity of nutrients absorption.

In view of the limited information available on the supplementation of Safmannan as an immunostimulant in the catfish farming, Safmannan-supplemented feed was examined in this study for its potential in enhancing the survival of catfish fingerlings before reaching the juvenile stage, meanwhile acting in a more conductive manner to human health and thereby maximizing the production yield, profitability as well as sustainability of catfish culture industry. Thus, this present study was aimed to evaluate the beneficial effects of Safmannan supplemented feed to the growth performance of catfish fingerlings; to determine the effect of Safmannan as immunostimulant to enhance the survival of catfish fingerlings and to study the feed performance of current market formulation and supplementation of Safmannan.

MATERIALS AND METHODS

Experimental diets: Three complete diets were used in this study. Diet 1, the premium commercial catfish feed in the local market (company A) was purchased from a fish feed retailer and acted as the control feed; Diet 2 and 3, containing no added Safmannan or 800 ppm of Safmannan were produced and provided by Cargill Feed Sdn Bhd (company B). Each of the diets was fed randomly to assigned triplicate groups of African catfish. The details of the feeds are shown in Table 1.

Experimental fish and management: Catfish fingerlings were purchased from a local supplier and kept in the stocking tank upon arrival at Aquaculture Facilities, Universiti Tunku Abdul Rahman. The catfish fingerlings were conditioned for 1 week by feeding them with control diet. A total of 270 healthy and similar size of catfish fingerlings (initial weight of 2.63±0.01 g) were selected and randomly distributed into a series of 9 experimental aquaria that applied with 3-in-1 filters to provide aeration and water circulation. The catfish fingerlings were then hand-fed with assigned diet twice daily, until apparent satiation, for 45 days. The feed consumption was recorded daily, where water parameters including Dissolved Oxygen (DO), pH and temperature of the experimental water were measured on weekly basis to maintain constant water quality (pH range of 6 to 7; temperature range of 26-30°C) for all aquaria. Weekly sampling was carried out by batch-weighing all catfish from respective aquarium to monitor the fish growth performance.

Sample collection: After 45 days of feeding trial, all experimental fish were anaesthetized with tricane methane sulphonate (MS222) and the body weight and total length of all catfish were measured and recorded individually. For each replicate, blood sample was collected from 4 sampled catfish into heparinized tubes and micro-centrifuged at 4000×g for 5 min. The percent haematocrit value was determined by measuring relative volume of the packed red blood cells volume. Another set

Table 1: Information of three complete diets used in the feeding trial

Dietary treatment (Diets)	Price (per 20 kg)	Source (Company)	Notes
1	RM 69.95	A	Deemed as best feed in the market, used as control diet
2	RM 68.00	B	Without Safmannan supplementation
3	RM 68.35	B	With Safmannan supplementation at 800 pp

of blood sample of catfish was collected and transferred into 1.5 mL Eppendorf tubes contained no anticoagulant for lysozyme assay. To harvest the serum, blood samples were allowed to clot at 4°C for 10 h and then centrifuged at 3000×g for 15 min. The collected serum will be stored at -80°C for further analyses. The collected serum was stored at -80°C for immunological analysis. Six fish were dissected and filleted, the fillet was pooled, wrapped and stored at -20°C for chemical analysis. Tissue samples including liver, viscera, gonads and intraperitoneal fat were excised and weighed for determination of Hepatosomatic Index (HSI), Viscerosomatic Index (VSI), Gonadosomatic Index (GSI) and Intraperitoneal Fat index (IPF). The remaining fish were used for subsequent microbiological analysis. Fish growth performance were determined in regard to weight gain and Specific Growth Rate (SGR). HSI, VSI, IPF, GSI and condition factor (K) were applied to determine the nutritional status of the fish. These body indices were calculated as a percentage of organ or tissue to the whole-body weight of individual fish. Feed utilization efficiency was determined by Feed Conversion Ratio (FCR).

Microbiological analysis: From the remaining fish, 4 fish per replicate were sterilized with 70% ethanol to avoid cross contamination. After dissection, approximately 0.3 g of empty gut was collected from 4 sampled catfish and homogenised in 2.7 mL of sterile distilled water. Serial dilution was done by diluting homogenates to 10^{-4} in 2.7 mL of sterile distilled water and 0.1 mL of the final dilution was spread onto nutrients agar plates. After incubation period of 48 hours, agar plates with 30-300 Colony Forming Units (CFU) were enumerated. The final result was expressed as Log_{10} CFU mL^{-1}.

Immunological analysis: The immunological analysis was conducted using a Lysozyme Detection kit (Sigma-Aldrich). For preparation of solutions needed, 800 µL of *Micrococcus lysodeikticus* cell suspension was pipetted into one cuvette for blank, one for a control and one for each serum sample. All the cuvettes were equilibrated to 25°C. A 30 µL of reaction buffer was added to blank cuvette; 30 µL of lysozyme to control cuvette; and 30 µL of serum sample to remaining cuvettes. The cuvettes were covered with parafilm, inversed and placed in the spectrophotometer for determination of absorbance where the decrease in absorbance at 450 nm for five minutes was recorded. One unit produces a A_{450} of 0.001 per minute at pH 6.24 at 25°C, using a suspension of *M. lysodeikticus* as substrate, in a 2.6 mL reaction mixture (1 cm pathlength).

Chemical analysis: The proximate compositions of experimental feeds and fillets of catfish were assayed by undergoing proximate analysis using standard AOAC methods[12]. Determination of crude protein, crude lipid, crude fibre, ash and moisture were conducted for experimental feeds whereas determination of crude protein, crude lipid and moisture were performed for fillets.

Statistical analysis: All data were presented as mean ± standard error. All collected data were subjected to Analysis of Variance (ANOVA) using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) to determine if significant difference occurred among dietary treatments. The means were compared using Duncan's multiple range test and treatment effects were considered at the $p<0.05$.

RESULTS

Proximate composition of the three diets were determined and shown in Table 2. Crude protein ranging from 39.99-42.51%; crude lipid ranging from 5.85-8.01%; dry matter ranging from 91.93-93.29%; the ash content ranging from 6.71- 8.33% and the crude fibre ranging from 2.97-5.36%.

All experimental catfish gained weight by over 600% after 45 days of feeding trial (Table 3). No significant differences ($p>0.05$) were recorded for total length, final weight and SGR but significant differences ($p<0.05$) were noted in IPF and GSI where Diet 1 resulted in highest value while Diet 2 resulted in lowest value for both IPF and GSI. The condition factor of fish ranged from 0.64-0.67 where haematocrit ranged from 19.50-24.67%. Survival rate of catfish is ranging from 96.67- 98.89%. In regard to FCR, Diet 1 achieved the lowest value of 1.00 which significantly lower than that of Diet 2 (1.06) but not significantly different from Diet 3 with value of 1.04.

In regard to total viable bacterial count, significant difference was noted among the treatments (Table 4) where fish fed with Diet 3 had significantly lowest bacterial count (5.834 CFU mL^{-1}) in fish gut, followed by Diet 2 and the highest value was recorded in fish gut of those fed with Diet 1. Fish fed diet 3 recorded the highest serum lysozyme activity with the value of 366.67 units mL^{-1} which significantly higher than that of other two diets (Table 5). For the proximate composition of catfish fillet, significant differences were noted for crude protein where fish fed with Diet 2 contained the highest crude protein; meanwhile fish fed with Diet 2 and 3 showed significantly lower crude lipid as compared to those fed with Diet 1 (Table 6).

Table 2: Proximate composition of the three diets[1]

	Dietary treatment[2] (Diets)		
Parameters	1	2	3
Dry matter (%)	91.93±0.02	91.16±0.04	93.29±0.02
Ash (%)	7.42±0.41	8.33±0.20	6.71±0.53
Crude protein (%)	39.99±0.40	42.51±0.17	41.69±0.27
Crude lipid (%)	8.07±0.73	7.39±0.09	5.85±0.43
Crude fibre (%)	2.79±0.07	5.31±0.26	5.36±0.03

[1]Values are mean±SE of triplicate of diets; [2] See Table 1

Table 3: Growth performance, biological indices and FCR of catfish fed with different dietary treatments[1]

	Dietary treatment[2] (Diets)		
Parameters	1	2	3
Final total length (cm)	14.71±0.07	14.53±0.06	14.65±0.29
Final weight (g)	19.65±0.56	20.20±0.18	20.36±0.64
Weight gain (%)	634.78±9.28	640.63±9.66	651.72±23.33
SGR[3] (%/day)	4.46±0.05	4.52±0.03	4.56±0.10
HSI[4]	1.84±0.18	1.38±0.03	1.57±0.28
VSI[5]	1.66±0.27	1.37 ± 0.12	1.48±0.08
IPF[6]	0.89±0.08[b]	0.50±0.06[a]	0.52±0.09[a]
GSI[7]	0.39±0.07[b]	0.18±0.05[a]	0.20±0.03[ab]
Condition factor[8]	0.66±0.01	0.67±0.02	0.65±0.01
Haematocrit[9] (%)	19.50±2.32	24.67±2.16	24.08±0.36
Survival rate[10] (%)	98.89±1.11	96.67±0.00	96.67±1.93
FCR[11]	1.00±0.01 [a]	1.06±0.01[b]	1.04±0.01[ab]

[1]Values are mean±SE of triplicate groups of fish. Different superscripts in the same row indicate significant difference at p<0.05; [2] See Table 1; [3] SGR = Specific Growth Rate = [(ln final mean weight-ln initial mean weight)/ days of feeding trial]×100; [4] HSI = Hepatosomatic Index = [liver weight (g)/ body weight (g)]×100; [5] VSI = Viserosomatic Index = [visceral weight (g)/body weight (g)]×100; [6]IPF = Intraperitoneal fat index = [intraperitoneal fat weight (g)/body weight (g)]×100; [7]GSI = Gonadosomatic index = [gonad weight (g)/body weight (g)]×100; [8]Condition factor = [final body weight (g)/(total length (cm))^3]×100; [9] Haematocrit = [height of packed red cells (mm)/height of packed red cells and plasma (mm)]×100; [10]Survival rate = (final fish number/ initial fish number)×100; [11] FCR, feed conversion ratio = total dry feed fed (g)/wet weight gain (g)

Table 4: Total viable bacterial count (Log_{10} values) of catfish empty gut[1]

Dietary treatment[2] (Diets)	Log CFU/mL
1	6.229±0.03[c]
2	5.959±0.01[b]
3	5.834±0.02[a]

[1]Values are mean±SE of triplicate groups of fish. Different superscripts in the same column indicate significant difference at p<0.05; [2] See Table 1 footnote

Table 5: Serum lysozyme activity of catfish fed with different dietary treatments[1]

Dietary treatment[2] (Diets)	Lysozyme contained in serum (units/mL)[3]
1	233.33±19.25[a]
2	200.00±19.25[a]
3	366.67±19.25[b]

[1]Values are mean±SE of triplicate groups of fish. Different superscripts in the same column indicate significant difference at p<0.05; [2]See Table 1 footnote; [3]One unit produces a A_{450} of 0.001 per minute at pH 6.24 at 25°C, using a suspension of *Micrococcus lysodeikticus* as substrate, in a 2.6 mL reaction mixture (1 cm pathlength)

Table 6: Fillet proximate composition of catfish fed with different dietary treatments[1]

Dietary treatment[2] (Diets)	Dry matter (%)	Crude protein (%)	Crude lipid (%)
1	21.32±0.35	16.24±0.39[a]	3.85±0.21[b]
2	21.00±0.53	18.81±0.09[c]	2.20±0.18[a]
3	21.43±0.17	17.48±0.03[b]	2.38±0.09[a]

[1]Values are mean±SE of triplicate groups of fish. Different superscripts in the same column indicate significant difference at p<0.05; [2] See Table 1 footnote

DISCUSSION

Throughout this present study, the results showed that catfish fed with Safmannan-supplemented feed had numerically highest total weight gain and SGR. This is in agreement with previous studies on broilers[8, 13, 14], juvenile white leg shrimp[15] and *Nile tilapia*[16]. This could be explained by that dietary Safmannan can promote function of intestine by improving nutrients uptake of intestine[17]. Moreover, a study on broilers suggested that oligosaccharides contained in Safmannan can help to gain appetite and lead to higher feed consumption[14]. Meanwhile, significant differences were detected on the IPF and GSI but not on HSI and VSI, this showed that the dietary composition, to certain extent, influenced fish biological indices. Particularly, the HSI and VSI results are in agreement with a study stating broiler whereby Safmannan did not influence HSI and VSI of broilers[13]. Nevertheless, catfish fed with Diet 2 and 3 had significantly lower IPF, this is probably due to the lower crude lipid content of the respective diet. Generally, lower reading of IPF expresses a good sign for consumer as the fish is less susceptible to lipid oxidation that gives rise to spoilage. Therefore, denotation regarding feed from company B able to yield catfish that possess lower percent IPF can be made. Furthermore, GSI recorded for catfish fed with Diet 1 was significantly higher than those fed with Diet 2, but not significantly different from Diet 3. Despite the survival rate of experimental catfish fed with respective three diets was not significantly different from each other but catfish fed with Diet 1 possessed numerically highest survival rate. In contrary, previous studies on common carp[17], *Nile tilapia* [16] and white leg shrimp[18] showed that dietary inclusion of Safmannan can enhance survival rate of the aquatic organisms. Indeed, cannibalism is the sole factor contributing to the mortality in this study. Catfish fed with Diet 2 and 3 were observed to perform more cannibalistically than those fed with Diet 1. The possible reason that led to high cannibalistic behavior could be the size heterogeneity of cultured catfish in this present study. Therefore, proper management on fish size variation can enhance survival rate of catfish[19].

Catfish fed with Diet 3 showed a comparable FCR as those fed with Diet 1 in this present study. This is in the agreement with previous studies on broilers[8, 13, 14] and

layers[20]. Moreover, positive effect of Safmannan on the FCR of common carp has also been proven[21]. These studies suggested that dietary inclusion of Safmannan can reduce FCR significantly. Besides, feeds from company B with higher protein content yielded catfish with higher protein content in fillet. Moreover, the crude lipid contained in fish fillet was found to have direct connection with the dietary crude lipid. This is in line with previous studies that proximate composition of fish fillet highly reflects the dietary composition[5].

Results from microbiological analysis indicated that supplementation of Safmannan significantly reduced the bacteria count within the fish gut. This is in the agreement with study from on zebrafish[22] and common carp[21] which observed that MOS effectively plays a role in regulating the intestinal microorganisms. In addition, it has been reported that MOS successfully reduced the intestinal colonization of pathogenic microbes[23]. Where similar conclusion was made by another study on the reduction of *Salmonella* spp.[19] when they included MOS in the diet of broilers. In fact, Safmannan-supplemented feed plays two vital roles in regulating the intestinal microbes' population. Besides reducing the pathogens present within the gastrointestinal tract with its pathogen-binding capacity, Safmannan, or more specifically refer to MOS on the other hand, offering a suitable condition for the establishment of beneficial bacteria, thereby arousing the event of competitive elimination[24]. Therefore, based on the results collected in this present study, Safmannan supplementation exert its influence on microbe populations and further microbiological works can be done to determine the composition and nature of the bacteria and to evaluate if Safmannan can reduce the pathogenic bacteria or forge ahead the beneficial bacteria.

In the present study, supplementation of Safmannan significantly increased the serum lysozyme activity of catfish. This is in the agreement with a previous study in which dietary MOS resulted in higher amount of serum lysozyme activity in broilers[17] suggesting that stimulation of phagocytes occurs when serum activity increases. Lysozyme provides protection to catfish by breaking the pathogen's peptidoglycan wall and thus preventing the occurrence of infections[25].

CONCLUSION

This present study suggested that supplementation of Safmannan significantly reduced the intestinal bacterial colonization and improved the serum lysozyme activity of catfish. Besides, Safmannan-supplemented feed was found to outperform the control feed with the comparable FCR, lower IPF, higher protein content in fillet as well as lower cost price. In light of the above, Safmannan as a feed additive retains its potential to serve as an immunostimulant for catfish culture. Therefore, the optimal inclusion of Safmannan in feed for enhancing the growth performance and immune system of African catfish is an important mean to maximize the production yield of catfish farming industry.

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