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Inclusion of cricket (*Gryllus bimaculatus*) meal in African catfish (*Clarias gariepinus*) feed influences disease resistance

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Abstract

In our present study, we observed the effect of cricket meal (CM) on disease resistance of African catfish. Fish were fed diets containing 350 g kg^{-1} and 400 g kg^{-1} of CM and 350 g kg^{-1} of fishmeal as control. The fish were divided into triplicates treatments of ten fish per replicate, weighed 22.5±0.6 g and fed with experimental diets for 40 days before being challenged against *Aeromonas hydrophila*. Relative percentage of survival (RPS) was recorded over 12 days post-challenge. White blood cell count, total protein, globulin and lysozyme showed significantly increasing levels in those fed with cricket meal diet compared to the control group. Mortalities at 12-day post-challenge significantly decreased to 30% (RPS: 66.7%) and 27% (RPS: 70%) for 35% and 40% CM respectively compared to 90% mortality in control group. Intestines and liver tissues of infected fish were dissected for pathogenic confirmation. The intestines of control diet showed the highest bacterial load (58.2×107 cfu g^{-1}) compared to CM diets. The current study indicates that dietary CM could enhance the innate immune system and disease resistance of African catfish.

Keywords: Immunostimulant; bacteria challenge; fishmeal; fish feed; cricket meal; *Aeromonas hydrophilla*

1 | INTRODUCTION

Interest in African catfish, *Clarias gariepinus* has markedly increased over recent years in many regions especially in Southeast Asia (Skelton 2001). Due to its high fecundity and palatability as well as tolerance to a wide range of environmental conditions, this species has become one of the most popular fishes in markets for human consumption in Malaysia (DOF 2014). Although this species is cultured commercially in Malaysia, the feed given is still limited to fishmeal based diets. Due to insufficient supply, increasing price and high demand for fishmeal (Mohsin *et al.* 2012a, 2012b; Galib *et al.* 2013), many studies have been focusing on searching for a suitable substitute for fishmeal as a protein source for animal feed. Growing interest towards finding fishmeal replacement has led to many studies in insect meal for fish and terrestrial animal feed (Jabir *et al.* 2012; Van Huis *et al.* 2013; Barroso *et al.* 2014; Gasco *et al.* 2016; Taufek *et al.* 2016a). However, studies related to crickets as a protein source for fish is

scarce but latest study by Taufek *et al.* (2016b) has demonstrated that cricket meal could replace up to 100% of fishmeal without adverse effect on growth and oxidative stress, besides showing high nutrient digestibility in African catfish (Taufek *et al.* 2016a).

The disease outbreak is commonly recognized as potential restriction in aquaculture industry. The most frequent encountered pathogenic bacteria is motile *Aeromonas* species (MAS) or better known as the aerobic bacteria organism namely *Aeromonas hydrophila*, *A. caviae* and *A. sobria*, which are associated with most hazardous infection disease in African catfish (Janda and Abbott 2010). Studies have proven that fish infected with MAS led to skin lesion and haemorrhage septicaemia (Law 2001; Ahamad *et al.* 2013; Anyanwu *et al.* 2015) and resulted in massive mortalities as well as financial loss to fish farmers worldwide. Numerous reports on the MAS outbreak on African catfish have been reported in different countries of the world (e.g. in Indonesia, Kusdarwati *et al.* 2017; Bangladesh, Rahman and Chowdhury 1999; Malaysia, Laith and Najiah 2013).

In order to treat the bacterial disease, the use of antibiotics is the common approach. However, antibiotics resistance may encourage development of pathogens, resulting in a negative impact on the fish due to the accumulation of antibiotic residue (McPhearson *et al.* 1991; Depaola *et al.* 1995; Schmidt *et al.* 2000). Besides, the expanding use of antibiotics in fish and consequently the environment has led to the imposition of strict regulation to limit the use of antibiotics (Alderman and Hastings 1998). One of the promising alternatives as preventive measures for fish disease management is immunostimulant. The production of feed as immunostimulant for preventing bacteria disease has been in demand as it offers alternative to antibiotics. Immunostimulant could improve immune status of fish by enhancing both specific and non-specific defence mechanism of fish and other animals (Siwicki *et al.* 1994).

Chitin, which is a natural polymer found in crustacean shells has been widely studied in aquaculture as an immunostimulant to protect salmonid, white shrimp and carp against bacterial disease (Siwicki *et al.* 1994; Wang and Chen 2005; Mari *et al.* 2014; Henry *et al.* 2015). Crickets have been known to contain a significant amount of chitin and are proven to give positive response in growth for poultry (Wang *et al.* 2005). Therefore, it is possible that crickets could be a potential protein source in animal feed and may be potential as a protection against bacterial diseases. Hence, the current study attempts to investigate the efficiency of dietary cricket meal on non-specific immune response and disease resistance against *A. hydrophila* in African catfish.

2 | METHODOLOGY

2.1 | Experimental diet

Adult live field cricket used in the formulated diet were purchased from a local field cricket farm. The cricket were fed on crumble chicken feed throughout their life cycle. They were then transported to the laboratory and refrigerated at –20°C before being dried in an oven at 60°C. The dried cricket were then grounded with dry feed grinder and kept in a cold room (4°C) prior to proximate analysis. All the raw materials for the ingredients including fishmeal, corn meal, rice bran, soybean, vitamins, mineral and di calcium phosphate (DCP) were purchased from a local livestock feed centre.

Formulation and chemical composition of all the experimental diets and feed ingredients were noted (Table 1) while the compositions of fishmeal and cricket meal were also recorded (Table 2). Three formulated diets were used in the feeding trial with different crude protein (CP) levels, which involved 350 g kg^{-1} cricket meal (35% CM), 400 g kg^{-1} CM (40% CM) and 350 g kg^{-1} fishmeal (35% FM) diet as control. These diets consisted of CP level of 35% for dietary 35% CM and control while dietary 40% CM contains 40% CP level.

The Winfeed 2.8 version software was used to establish the formulated feed. All dry ingredients were grounded in a hammer mill (Disk Mill, FFC 454). Vitamins, minerals and DCP were mixed thoroughly with the dry ingredients and water was added to the mixture prior to being pelleted into sizes of 1 mm diameter using a mini pelleting machine (KCM, Y132M-4). The wet pellets were then dried in an oven at 70°C for 24 h and later stored in a cold room (4°C) until used for feeding.

2.2 | Experimental fish and set-up

African catfish were acquired from local farmers on the 22nd of December 2014 and transported to the Freshwater Aquarium Laboratory located in the Institute of Biological Sciences, Faculty of Science, University of Malaya. The feeding trial was conducted in triplicates with 10 individuals (mean [±SE] weight of 22.5±0.6 g). All the fish individuals were acclimatized to natural environment condition for two weeks prior to the feeding trials and fed with commercial diet twice a day during the acclimatization and experimental periods. Water quality was monitored regularly and mortality was recorded. Nine plastic tanks (3×2×1 inches) with a capacity of 100 L of water with closed recirculation system were used in these feeding trials. The tanks were equipped with top filter pump at a flow rate of 20 L min^{-1} and aeration diffuser was provided in each tank for circulation of dissolved oxygen (DO). Approximately 20–30% of dechlorinated water was replaced in every other day.

TABLE 1 Formulation and composition of the experimental diets

Ingredients (g kg^{-1})	*Control*	*35% CM*	*40% CM*
Fishmeal	350	0	0
Cricket meal	0	350	400
Soybean meal	184.7	195.1	287.5
Corn starch	114.3	234.6	254.7
Rice bran	336	205.3	42.8
Vitamin premix[a]	2	2	2
Mineral premix[b]	3	3	3
DCP	10	10.0	10
Total	*1000*	*1000*	*1000*
Nutrient level determined by as is basis (% dry matter)			
Dry matter	94.28	94.42	94.06
Crude protein	36.55	34.7	40.2
Crude fat	10.03	11.82	11.76
Crude ash	9.00	9.70	8.18
Crude fiber	3.50	4.90	5.77
Gross Energy (kJ g^{-1})[c]	18.93	18.86	19.24
NFE	35.2	33.3	28.15

[a]*Vitamin premix 100 g^{-1} diet: vitamin A, 500 IU; vitamin D3, 100 IU; vitamin E, 0.75 mg; vitamin K, 0.02 mg; vitamin B1, 1 mg; vitamin B2, 0.5 mg; vitamin B3, 0.3 mg; vitamin B6, 0.2 mg; vitamin B12, 0.001 mg; vitamin C, 0.1 mg; niacin, 0.2 mg; folic acid, 0.1 mg; biotin, 0.235 mg; pantothenic acid, 1.0 mg; and inositol, 2.5 mg.*

[b]*Mineral premix kg^{-1} diet: selenium, 0.2 mg; iron, 8 mg; manganese 1 mg; zinc, 8 mg; copper, 0.15 mg; potassium chloride, 0.4 mg; magnesium oxide, 0.6 mg; sodium bicarbonate, 1.5 mg; iodine, 1 mg; and cobalt, 0.25 mg.*

[c]*as per Schulz et al. (2005)*

The feed were given at 3% of the body weight (BW; Wilson and Moreau 1996) at 0900 and 1600 h throughout the experiment in two equally sized portions for 40 days. Uneaten feed was collected by using small-meshed fish net 30 minutes after feeding and weighed to determine the total amount of feed consumed. The water quality parameters for all tanks were measured (APHA 1992). Water temperature and pH varied from 28–29°C and 6–6.8 respectively. Whereas, DO level was >4.5 mg L^{-1}. Ammonia and nitrate were determined weekly and the levels varied from 0.8 mg ml^{-1} and 1.9 mg ml^{-1} respectively.

2.3 | Proximate and chemical analysis

The experimental diets and ingredients were analysed for proximate composition according to Association of Official Analytical Chemist method (AOAC 2003). Kjedahl method was used to analyse CP after acid digestion (FOSS Tecator Digestor Auto). Moisture and dry matter were measured by drying in an oven at 105°C to constant weight. Meanwhile, ash was determined by combustion in a muffle furnace (Naberthem) at 600°C. Soxhlet method with petroleum ether extraction (FOSS Soxtec 2055) was used to measure crude lipid content. Chitin was estimated by using the acid detergent fibre (ADF) and protein residue of ADF according to Marono *et al.* (2015).

Nitrogen free extract (NFE) by using the following equation

NFE = % dry matter - (% crude protein + % crude fat + % crude ash +% crude fibre)

Whereas gross energy for every diet was calculated by using the following factors: CP (23.9 kJ g^{-1}), crude lipids (39.8 kJ g^{-1}) and NFE (17.6 kJ g^{-1}) (Schulz *et al.* 2005).

The experimental trial was conducted for 40 days from 5 January – 13 February 2015.

TABLE 2 Chemical composition of the fishmeal and cricket meal used in the experimental diets (% dry matter basis)

Components	*Fishmeal (%)*	*Cricket meal (%)*
Dry matter	85.59	95.18
Crude protein	53.61	57.02
Crude lipid	2.96	10.90
Crude ash	19.30	4.83
Chitin	-	7.15

2.4 | Blood sampling

After 40 days of feeding trial, blood samples were randomly collected from five fish specimens from each tank. The individuals were anesthetized with clove oil before blood collection. The blood was drawn from caudal vein by using 1.0 ml syringe and 22G×1½ inch gauge needle and transferred into tubes coated with thin layer of EDTA as anticoagulant. Plasma samples were separated by centrifugation at 3000 rpm for 15 min and plasma from the same replicate were pooled before being stored at –80°C for biochemical and immunological assays.

2.5 | Haematology and biochemical analysis

Prior to centrifugation, 100 µl of the whole blood were separated and stored at 4°C for white blood cell (WBC) count. The WBC (10^3 mm^{-3}) were counted manually using haemocytometer. The Turk's solution (3% acetic acid, 1% gelatin violet and a drop of methylene blue) were mixed for WBC diluting fluid. The blood samples were diluted at 1:20 with triplicate counts for each sample and counted by using Neubaeur haemocytometer. Biochemical parameters of plasma total protein were analysed following the Bradford assay (Bradford 1976) whereas albumin content was determined after Doumas *et al.* (1971) and globulin was determined by substracting albumin from the total protein value.

2.6 | Lysozyme assay

Lysozyme activity was assayed spectrophotometrically (Shugar 1952) with slight modification by using 0.01%

(w/v) lyophilized *Micrococcus lysodekticus* (A_{450}) as the substrate in phosphate buffer (66 mM, pH 6.24). A 100 µl of plasma sample was added to 2.5 ml substrate suspension. The absorbance was measured at 450 nm at room temperature for 10 min and the initial and final absorbance were recorded. A lysozyme activity unit was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min^{-1}.

2.7 | Bacterial pathogen and experimental challenge

Aeromonas hydrophila used in this study was obtained from the National Fish Health Research Center (NaFish), located in Batu Maung, Penang, Malaysia. The bacteria were cultured in Tryptone Soy Broth (TSB) overnight at 30°C. Subsequently, the bacterial suspension was washed with phosphate buffer saline (PBS) and diluted to get the desired concentration for the bacteria challenge.

Before performing the bacteria challenge to the fish under study, the LD50 dose of the bacteria was established by challenging another group of fish to the unrelated but same bacteria species according to the method of Oliveira *et al.* (2011). Ninety individuals were divided into nine tanks (=10 in each tank). The nine treatments composed of 1.6×10^4 to 10^{12} colony forming unit per ml. Mortalities were observed in within 96 h and water variables were monitored during the experiment. The LD50 calculated from this experiment was 1.6×107 cfu ml^{-1}. This concentration was used for the subsequent challenge.

After 40 days of feeding trial, all 10 individuals per tank were challenge by intra-muscularly injected 0.1 ml of *A. hydrophila* suspension. The fish continued to receive their respective feed while morphology disabilities as well as mortalities were monitored for over 12 days. The relative percentage of survival (RPS) of the fish was calculated according to following formula (Amend and McDowell 1983).

$$RPS = 1 - \left(\frac{\%\ mortality\ in\ the\ cricket\ meal}{\%\ mortality\ in\ the\ control\ group} \times 100 \right)$$

2.8 | Confirmation of pathogenicity

Mortalities were inspected twice a day and three dead fish from each replicate were collected and immediately dissected to isolate *A. hydrophila* in the liver and intestine. The fish were dissected aseptically whereby the samples were homogenized in 1:9 (w/v) of physiological saline. The homogenized solution was diluted serially and 100 µl from each serial dilution were spread onto selective media, which contained Mueller-Hinton agar supplemented with 10% defibrinated sheep blood and 30 µg ml^{-1} ampicillin (Misra *et al.* 1989). These were incubated at 30°C for 12 h and the colonies formed were calculated according to Rashid *et al.* (1994).

$$Bacterial\ cfu\ g^{-1}\ of\ fish\ organ = No.\ of\ colonies\ counted\ in\ the\ plate \times 10^{n} \times 100$$

Where *n* is the dilution factor.

2.9 | Statistical analysis

All data were subjected to homogeneity of variance and normality tests before employing one-way analysis of variance (ANOVA) using SPSS version 21.0 (SPSS Inc., Chicago IL, USA). The differences between means were compared by Duncan's post hoc test considering an α level of significance of 0.05.

3 | RESULTS

In the present study, the WBC count of fish fed control diet was significantly lower than CM fed fish (Table 3). However, the values did not differ among fish fed with CM diets. A significant reduction ($P < 0.05$) in total protein was observed in control group while there were no significant differences ($P > 0.05$) among the CM groups. On the other hand, the level of albumin did not show any significant difference among all the diets (all $P > 0.05$). The globulin concentration significantly decreased in control group although no significant difference was observed in both CM fed fish. Similarly, lysozyme activity of those fed the control diet reduced significantly ($P < 0.05$) when compared to CM diet.

TABLE 3 Biochemical parameters, WBC and lysozyme activity of African catfish fed with experimental diets (mean ± SEM; *n* = 3)

Parameters	*Control*	*35% CM*	*40% CM*
WBC (10^3 mm^3)	13.6±0.86[a]	14.5±0.75[a]	19.2±0.13[b]
Total protein (mg dL^{-1})	53.4±5.59[a]	59.7±7.81[b]	61.1±6.15[b]
Albumin (mg dL^{-1})	1.9±0.12[a]	2±0.16[a]	2.2±0.22[a]
Globulin (mg dL^{-1})	51.5±5.49[a]	57.7±7.76[b]	59±6.08[b]
Lysozyme (U ml^{-1})	8.4±1.16[a]	19.6±2.71[b]	22.2±2.54[b]

Mean values in the same row with different superscript letters are significantly different (P < 0.05)

The intramuscular injection resulted in 90% mortalities in fish fed with FM diet within 8 days on post inoculation whereas the fish fed with 35% CM and 40% CM caused 30% (after 5 days) and 27% (after 8 days) mortalities respectively. The relative percentage of survival (Figure 1) suggested that fish fed with diet containing CM showed no significant difference ($P > 0.05$) and they were more tolerant to the bacteria challenge since higher survival were recorded in both diets than control.

To confirm the pathogenicity of *A. hydrophila*, the bacteria were isolated from liver and intestines of experimentally infected fish. The highest bacterial load was found to

be 58.2×10^7 cfu g^{-1} in the intestine of the control group while the lowest was 0.13×10^7 cfu g^{-1} in the liver of fish fed with 35% CM diet which did not differ significantly from fish fed 40% CM (Table 4).

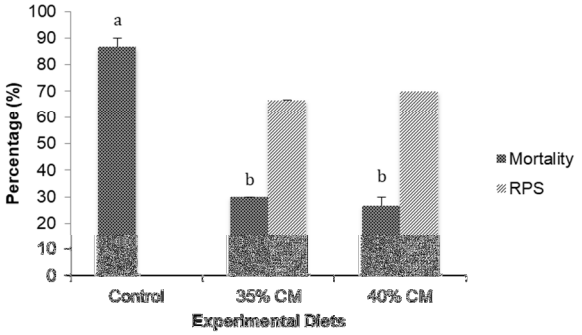

FIGURE 1 Mortality and relative percentage of survival (RPS) of fish in 12 days post-challenged with *Aeromonas hydrophila*. Values are the mean (± SEM) and means with different letters are significantly different ($P < 0.05$) from each other

TABLE 4 Bacterial load (×10^7) in liver and intestine of fish challenged with *Aeromonas hydrophila* (mean ± SEM; $n = 3$). Liver and intestine were excised from dead and infected fish

Diets	Control	35% CM	40%CM
Liver (cfu g^{-1})	0.625±0.03^b	0.134±0.01^a	0.151±0.01^a
Intestine (cfu g^{-1})	58.2±2.11^b	3.2±0.22^a	4.1±0.35^a

Mean values in the same row with different superscript letters are significantly different (P < 0.05)

4 | DISCUSSION

The use of immunostimulant, which contains bioactive compounds could improve health, increase innate immune response and prevent as well as treat various diseases (Gannam and Schrock 1999; Jadhav *et al.* 2006; Barman *et al.* 2013). Hence, the present study investigated the effect of CM diet as an immunostimulant in African catfish. The experimental diets consisted of different levels of CM as well as crude protein levels (35% CM with 35% CP and 40% CM with 40% CP) to establish their effect on immune response and disease resistance against *A. hydrophila*.

White blood cells play an important role in non-specific immunity and indicator of fish health status. This study demonstrated that WBC count showed a slightly increased level in the fish fed with 40% CM. Hence, the increases in WBC's count in 40% CM diet support the fact that CM contains anti-infection properties for African catfish. This finding is consistent with other works that found an increase in WBC count in *Labeo rohita* juveniles when fed with levamisole and ascorbic acid (Choudhury *et al.* 2005) and also garlic peel in African catfish diet (Thanikachalam *et al.* 2010).

The plasma or serum protein particularly albumin and globulin play a major role in maintaining immune response of fish. In the present study, increase plasma protein and globulin of fish fed with CM suggested a stronger immune response of fish fed with this diet. Previous research by Siwicki (1990) has reported an elevated level of serum total protein when include β-glucan (0.2%) and chitosan (0.5%) in common carp diet. Besides, the dietary inclusion of chitosan in olive flounder has been shown to improve the total protein, albumin and globulin (Dautremepuits *et al.* 2004).

Lysozyme activity has been primarily used as defense mechanism of nonspecific humoral immunity that could disrupt the cell wall of harmful pathogens invaders particularly parasites, bacteria and virus (Ellis 1999). It has been reported to have anti-bacterial activity, which could cause lysis and stimulate the phagocytosis in bacteria (Ellis 1999). The increased of lysozyme activity in fish fed with cricket meal suggested that the presence of bioactive substances including chitin could enhance the lysozyme activity in fish therefore can be considered to be a natural protective mechanism in fish.

A number of studies indicate that chitin and chitosan supplementation could elevate lysozyme activity in fish. Diet enriched with 1% chitin has been reported to increases lysozyme activity in *Cirrhina mirgal*a (Mari *et al.* 2014) while Esteban *et al.* (2001) reported no significant differences between 100mg kg^{-1} chitin and control diet fed to gilthead seabream. Based on previous studies in *Cyprinus carpio*, dietary inclusion of chitosan induced significantly higher lysozyme activity in chitosan fed fish followed by levamisole and chitin (Gopalakannan and Venkatesan 2006). On the other hand, African catfish fed supplemented indigenous plant such as neem (*Azadirachta indica*) has also been reported to increase lysozyme activity (Thomas *et al.* 2013).

Disease resistance was measured by discovering the survival of animal after being challenged with certain pathogen (Palti *et al.* 1999). Fish are most susceptible to bacteria and virus due to direct contact with the environment. The mucus and skin or scales are natural barriers to foreign substance and act as non-specific or innate defence mechanism thus suppressing the colonization of fish pathogen. These mechanisms prevent the attachment, invasion or multiplication of the invaders on or in the tissues. In the current study, RPS of fish fed with cricket meal was higher (66.7–70%) up to 12 days post-injection in comparison to fishmeal diet. Relative percentage survival values exceeding 50% indicates positive effect of the immunostimulant (Amend and McDowell 1983).

From the observation after the challenge, all fish developed clinical sign such as loss of balance, spreading of greyish – white lesion on the surface of the body up to caudal fin and the fin bases become reddish in colour. The wound on their body were assessed by evaluated the depth of the wound which is the distance from visible surface to the deepest area. These clinical symptoms were observed in all fish. However, the group fed with CM showed wound recovery after six days post-challenge with no loss in appetite and hence consumed their respective feed up until 12 days after the challenge. The mortality rate reduced significantly compared to control group and it reached plateau after 8 and 5 days post-infection for 40% CM and 35% CM respectively. This situation might be due to enhancement of the non-specific immune system of the fish by bioactive compound in CM such as chitin. On the other hand, the control group exhibited highly stressed condition due to the infection and consequently most of them died after 12 days post-challenge.

Wang *et al.* (2005) have reported that field cricket (*Gryllus testaceus*) contains 8.7% chitin while the result of this study indicates that CM (*G. bimaculatus*) composition consists of 7.15% chitin (Table 2). Several studies have revealed the potency of crustacean chitin as immunostimulant that could enhance immune response, disease resistance and survival of fish and shellfish (Sakai *et al.* 1992; Kawakami *et al.* 1998; Esteban *et al.* 2001). Mari *et al.* (2014) have observed approximately 70% reduction of mortalities of *C. mrigala* against *Aphanomyces invadans* bacteria when fed 10% chitin supplemented diet. Diet containing 0.75% chitin shows a significantly higher RPS (63.16%) in *Macrobrachium rosenbergii* compared to chitin-free diet when challenged against white tail disease viruses (Kumar *et al.* 2015). Besides, chitin also have the ability to improve growth performance of snow trout and golden mahseer (Mohan *et al.* 2009).

Although CM contains significant amount of chitin, it may have consisted unknown bioactive substance that might have immunostimulant activity on the fish. One of the compounds is dipterose, a water-soluble polysaccharide, which have been identified in melon fly, *Bactrocera cucurbitae*, and found to activate innate immune response in mouse macrophage (Ohta *et al.* 2014). On the other hand, gut microorganisms of the insect have been shown to stimulate the modulation of immune response as well as protection from parasites and pathogen which might contribute to the enhance of disease resistance in fish fed with cricket meal (Engel and Moran 2013).

Studies by using insect meal to evaluate the effects on immunostimulant are very limited. At present, housefly, *Musca domestica* have been found to provide an increased protection against *Edwardsiella trada* and significantly enhance peritoneal phagocytic activity in red sea bream (Ido *et al.* 2015). According to Ji *et al.* (2015), 50% replacement of silkworm pupae meal did not affect growth performance as well as health status of *C. carpio*. In addition, Yixiang *et al.* (2013) summarized that diet supplemented with 25 g kg^{-1} maggot meal and 150 mg kg^{-1} L-carnitine not only promote growth but also improved non-specific immunity and enhanced fish resistance against *A. hydrophila* in black carp.

The intestine and liver tissues of African catfish infected with *A. hydrophila* were cultured to confirm death as a result of *A. hydrophila*. The pathogenicity confirmation test was also conducted to determine the bacterial loads isolated in both livers and intestines of fish fed the experimental diets. Based on the bacterial isolation from the fish after the challenge, the intestine showed higher bacteria load than liver. This was also observed in the climbing perch with 9.4×10^{8} cfu g^{-1} and 2.9×10^{6} cfu g^{-1} in intestine and liver respectively (Hossain *et al.* 2013). In addition, Asian stinging catfish, *Heteropneustes fossilis* also exhibited higher levels of bacteria accumulation in intestine (1.8×10^{9} cfu g^{-1}) than liver (6.46×10^{8} cfu g^{-1}) (Mostafa *et al.* 2008). However, a finding by Sarkar and Rashid (2012) indicated that walking catfish, *Clarias batrachus* shows a higher bacterial load in the liver (6.5×10^{8} cfu g^{-1}) compared to intestine (5.6×10^{7} cfu g^{-1}). Thus, it was proven that *A. hydrophila* was pathogenic to African catfish, which caused 90% mortalities if the fish were only fed with fishmeal without any immunostimulant.

These findings suggested that in general, supplementation of 35 g kg^{-1} CM with 35% CP level was able to reduce disease resistance against pathogenic *A. hydrophila* as it enhances the non-specific immunity of African catfish. It also indicates that different CP level may not affect the immune response as well as their efficiency in protection against bacteria disease in African catfish. The bioactive substance present in the CM plays a role in conferring significant protection against *A. hydrophila* infection on catfish. Thus, CM can act as an immunostimulant in African catfish diet. Further studies need to be carried out to isolate and characterize the active compounds in CM including chitin that was responsible for antibacterial activity of the fish against *A. hydrophila*.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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