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Frass from Black Soldier Fly Larvae, *Hermetia illucens*, as a Possible Functional Dietary Ingredient in Channel Catfish Feed

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Abstract: Frass–the by-product of the larva meal industry–was recently shown to be a sustainable ingredient for use in diets of several fish species. Additionally, because of its possible immunomodulatory activity and anti-microbial properties, frass may have great potential as an organic method of pathogen control in aquaculture. Five diets containing 0, 5, 10, 20 and 30% frass from black soldier fly larvae, *Hermetia illucens*, were fed to channel catfish (5.24 ± 0.04 g) in quadruplicate aquaria to apparent satiation twice daily. At the end of the 10-week feeding trial, blood samples were collected from all groups to measure hematological and immune parameters, and to determine the effects of dietary frass on resistance to *Flavobacterium covae* infection. Hematological parameters (red blood cell count, hemoglobin, and hematocrit)—but not white blood cell count—were improved with the inclusion of frass. Serum glucose levels were significantly lower in fish on the diet with frass than fish on the diet without frass. Fish fed the highest dietary levels of frass (30%) had a significantly higher serum cholesterol level than fish on the control diet. Serum complement activity was significantly higher in fish on diets containing frass at levels of 10% and 20%. No significant differences were observed in other measure serum components including albumin, alkaline phosphatase, alanine aminotransferase, amylase, calcium, phosphorus, potassium, total protein, globulin, thyroxine and lysozyme activity. Even though overall mortality was low (0–17%), fish on the diets containing frass at levels 20% or more showed significantly higher survival than that of control fish or fish on lower levels of dietary frass. The use of frass in the catfish diet may prove beneficial by improving hematological parameters, and select serum immune effectors, and the overall resistance of juvenile channel catfish against *F. covae* infection.

Keywords: insect larvae frass; alternative feeds ingredient; catfish; immune responses; disease resistance

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1. Introduction

The channel catfish, *Ictalurus punctatus*, industry is a well-established aquaculture industry in the United States [1]. Economically, the channel catfish industry alone contributed about $447 million USD in sales in 2022 [2]. Catfish are relatively hardy and highly adaptable to a wide range of environmental conditions and culture systems. However, increasing the production of channel catfish and other farmed aquatic animals for human consumption leads to intensification and the subsequent magnification of fish stressors, which can heighten the risk of disease. Disease-related mortalities are considered a major threat to aquaculture production and account for tremendous annual economic losses [3,4]. The interplay between nutrition and the immune system is well recognized. Therefore, improved feeding regimes, together with the continuous enhancement of fish immunity, will have a great impact on fish growth and profitability throughout the production cycle [5].

In recent years, insects have received wide attention as a potential source of protein both for humans and livestock. Insect-based protein meals can be used as a more sustainable alternative to conventional protein (fish or plant protein meals) used in aquaculture [6–9]. The most promising, well-studied candidates for industrial feed production are black soldier fly larvae, yellow mealworms, silkworms, grasshoppers, and termites [10,11]. The

production of insect-based feed requires less space and natural resources than traditional farm animals, while also valorizing organic wastes, which reduces landfill usage and greenhouse gas emissions [12]. Their successful use as a partial replacement for fishmeal has been studied in several fish species [13–17]. Following the harvest of the larvae, considerable amounts of the larval by-product, "frass", is left behind. We recently demonstrated that larval frass from the by-product of black soldier fly larvae (BSFL) has a growth-promoting effect on channel catfish through increasing palatability and feed intake [18]. Besides its promising value as a feed ingredient for several fish species, such as channel catfish (*Ictalurus punctatus*) [18,19], hybrid tilapia (*Oreochromis niloticus* × *O. mozambique*) [20], and Florida Pompano (*Trachinotus carolinus* L.) [21], frass contains abundant nutrients, chitin—a naturally occurring biopolymer from invertebrate shells—and beneficial microbes [22,23]. In fact, the use of frass in tilapia diets have shown to be beneficial by improving innate immune components and subsequently, the resistance of hybrid tilapia against bacterial infection [20] as well as increasing the serum antibacterial effect of Pacific white shrimp, *Litopenaeus vannamei*, against *Vibrio parahemolyticus* [24].

Due to concerns regarding antibiotic resistance, costs, and the residue accumulation of antibiotics, the past decade has seen a search for alternatives to antibiotics. Numerous strategies have been evaluated to limit antibiotic usage, some of which may serve as functional dietary supplements in commercial fish feeds. Many substances from a variety of sources (bacterial components, chemical agents, animal, plant extracts, etc.) have been studied as prospective immunostimulants for fish [25,26]. Recent advances in immune-nutrition studies have revealed that some nutrients are linked to the immune status of fish [27]. Non-specific defense mechanisms are rapidly activated by immuno-stimulants and can be rapidly readied to protect fish against pathogens [28]. Thus, one of the most promising methods for controlling diseases in a sustainable aquaculture industry involves strengthening the defense mechanisms of fish through the prophylactic administration of immunostimulants.

The current manuscript is an extension of a growth trial of dietary larval frass that was previously reported [18]. Five diets containing 0, 50, 100, 200 and 300 g frass per kg diet as partial replacements of a combination of soybean meal, wheat short and corn meal on an equal protein basis were fed to channel catfish (5.24 ± 0.04 g) in quadruplicate aquaria twice daily to apparent satiation. Besides its promising value as a feed ingredient for several fish species, including catfish, frass could serve as potential immunomodulatory functional feed ingredient due to the presence of bioactive components and beneficial microbes. This article expands on that trial to test the effect of the dietary inclusion of BSFL frass on the hematology, blood chemistry, immune system and susceptibility of channel catfish to columnaris disease.

2. Materials and Methods

2.1. Experimental Fish

Marion strain channel catfish, *Ictalurus punctatus*, fingerlings were spawned and maintained at the USDA-ARS, Aquatic Animal Health Research Laboratory (Auburn, AL, USA) on commercial fry and fingerling diets and were acclimated to the experimental basal diet for 2 weeks before stocking. At the end of the acclimation period, fish (average weight of 5.24 ± 0.04 g) were randomly stocked into 20, 110 L aquaria at a density of 50 fish per aquarium. The aquaria were supplied with flow-through dechlorinated, heated (28 °C) city water with a flow rate of about 0.7 L/min. Water was continuously aerated using air stones. Water temperature and dissolved oxygen in three randomly chosen aquaria were measured once every other day in the morning, using a YSI model Pro DO meter (Yellow Spring Instrument, Yellow Spring, OH, USA). During the trial, the water temperature averaged 26.8 ± 1.12 °C, and the dissolved oxygen averaged 6.35 ± 0.53 mg/L. The photoperiod was maintained at a 12:12 h light/dark schedule.

2.2. Experimental Diets, Feeding and Sampling

A nutritionally complete, practical basal diet was formulated to contain approximately 31.5% crude protein and 6.2% lipid based on feedstuff values reported in NRC [29] (Table 1). Five diets containing frass (0, 5, 10, 20 and 30%) as partial replacements of a combination of soybean meal, wheat short and corn meal on an equal protein basis were prepared. Frass from black soldier fly, *Hermetia* illucensas, fed dried distiller's grain with solubles (DDGS), was donated from EnviroFlight LLC, Yellow Springs, OH, USA. Carboxymethyl cellulose (CMC) was added to all diets as a binding agent. Dry ingredients were thoroughly mixed for 10 min in a Hobart mixer before the oil was added. After the oil was diffused, approximately 300 mL of deionized water per kg of the diet was added. The moist mixture was extruded through a 3 mm diameter die in a Hobart meat grinder. The resulting moist pellets were air-dried at room temperature to a moisture content of about 10%. Pellets were ground into small pieces, sieved to obtain approximate sizes and stored frozen in plastic bags at −20 °C until fed. Nutritive value and fatty acid profile of black soldier fly larval frass used in the experiment is shown in Table 2. Fish in four randomly assigned aquaria were fed one of the five experimental diets twice daily (between 07:30 and 08:30 h, and 15:00 and 16:00 h) to apparent satiation for 10 weeks.

Table 1. Percentage composition of experimental diets.

	Experimental Diets (%) [1]				
	1	**2**	**3**	**4**	**5**
Menhaden fish meal	8	8	8	8	8
Soybean meal	45	44	43	41	39
Frass	--	5	10	20	30
Wheat short	24	20.4	16.9	9.8	2.5
Corn meal	14	13.8	13.5	13.0	12.8
Corn oil	4	3.8	3.6	3.2	2.8
Dicalcium phosphate	1	1	1	1	1
CMC	3	3	3	3	3
Vitamin premix [2]	0.5	0.5	0.5	0.5	0.5
Mineral premix [3]	0.5	0.5	0.5	0.5	0.5

CMC = carboxymethyl cellulose. Frass is a by-product of the black soldier fly (*Hermetia illucens*) larva meal industry. [1] Diets 1, 2, 3, 4 and 5 contained 0, 5, 10, 20 and 30% frass, respectively. [2] Vitamin premix, diluted in cellulose, provided by following vitamins (mg/kg diet): vitamin A (520,400 IU/g), 7.7; vitamin D3 (108,300 IU/g), 18.5; vitamin E (250 IU/g), 200; vitamin K, 10; thiamin, 10; riboflavin, 12; pyridoxine, 10; calcium pantothenate, 32; nicotinic acid, 80; folic acid, 2; vitamin B12, 0.01; biotin, 0.2; choline chloride, 400; and L-ascorbyl-2-polyphosphate (35% vitamin C activity), 172. [3] Trace mineral premix provided by following minerals (mg/kg diet): zinc (as $ZnSO_4\_7H_2O$), 150; iron (as $FeSO_4\_7H_2O$), 40; manganese (as $MnSO_4$_7H2O), 25; copper (as $CuCl_2$), 3; iodine (as Kl), 5; cobalt (as $CoCl_2\_6H_2O$), 0.05; and selenium (as Na_2SeO_3), 0.09.

Table 2. Nutritive value and fatty acid profile of experimental *Hermetia illucens* larval frass.

	Nutritive Value (%)
Moisture	7.2
Protein	21.6
Fat	6.3
Ash	9.3
Fiber	7.0
Starch	35.0
	Fatty acid profile (% of crude fat)
C12:0 Lauric acid	0.2
C14:0 Myristic acid	0.1
C16:1 Palmitic acid	13.7
C17:0 Margaric acid	0.1
C18:0 Stearic acid	2.5
C18:1 Oleic acid	28.3

Table 2. *Cont.*

	Nutritive Value (%)
C18:2 Linoleic acid	50.5
C18:3 Alpha Linoleic acid	1.3
C20:0 Arachidic acid	0.5
C20:1 Eicosenoic acid	0.7
C20:2 Eicosnoic acid	0.3
C20:3 Eurcostrienoic acid	0.2
C20:5 Eicosapentaenoic acid	0.2
C24:0 Lignoceric acid	0.3
C22:6 Docosahexaenoic acid	0.2

2.3. Hematological Assay

At the end of the feeding period, four fish were randomly chosen from each tank and netted into a sedating dose of 150 mg/L tricaine methanesulfate (MS-222) for 1–5 min. Blood samples were collected from the caudal vasculature with dried heparinized (100 IU) tuberculin syringes (22–26 gauge, 3/8″ needle) for hematological assays. Red and white blood cell counts were performed in duplicate for each sample by diluting whole blood (1:10,000) and enumerating in a Spencer Bright Line hemacytometer. The hematocrit of each fish was determined using the microhematocrit method [30]. Hemoglobin was determined using the cyanomethemoglobin method (Point Scientific, St. Louis, MO, USA). Hemoglobin values were adjusted using the cyanomethemoglobin correction factor for channel catfish described by Larsen [31]. After the blood sampling, fish were killed in 300 mg/L MS-222.

2.4. Serum Biochemical Parameters

An additional four fish per tank were bled using non-heparinized tuberculin syringes and allowed to clot at 4 °C overnight. Serum samples were collected following centrifugation and stored at −80 °C until used for the determination of serum biochemical parameters (albumin, alkaline phosphatase, alanine aminotransferase, amylase, calcium, phosphorus, glucose, potassium, total protein, globulin, cholesterol and thyroxine) and nonspecific immune parameters (lysozyme assay and complement activity assay). Serum chemistry was performed using an Abaxis, VetScan VS2 analyzer (Zoetis, Parsippany, NJ, USA). T4/cholesterol profile rotors were used to assay the serum cholesterol and thyroxine levels. For the rest of the plasma chemistry profiles, comprehensive diagnostic reagent rotors were used.

2.5. Nonspecific Immune Responses

Lysozyme activity was performed as described in Yildirim-Aksoy et al. [32]. Serum from each of the four fish per tank was assayed in duplicate for lysozyme activity. Serum lysozyme activity was determined by the method of Litwack [33], as modified by Sankaran and Gurnani [34], by measuring the lytic activity of the catfish serum against bacterium *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO, USA).

Serum natural hemolytic (alternative pathway) complement activity was adapted from Sunyer and Tort [35] and modified for use in microtiter plates as described in Yildirim-Aksoy et al. [32], except 0.85% phosphate-buffered saline (PBS) containing $MgCl_2$, $CaCl_2$ and gelatin (PBS^{3+}) was used instead of GVB^{2+} as the assay solution. This assay is based on the hemolysis of sheep erythrocyte (Remel Inc., Lenexa, KS, USA) by the complement present in fish serum. Briefly, sheep erythrocytes were washed and standardized to 5×10^7 cells/mL in PBS^{3+} before use. The sheep erythrocyte cell suspension was added to the serially diluted (1:2) serum in PBS^{3+} in a round-bottom 96-well microtiter plate. Positive (100% lysis) and negative controls (spontaneous lysis) were also processed in each plate by replacing serum with distilled water and buffers, respectively. Samples were incubated at room temperature (22 °C) for 1 h with regular shaking. The plates were centrifuged at 800× *g* for 10 min at 4 °C to avoid unlysed cells. Supernatants were transferred to a

flat-bottom microtiter plate, and the absorbance measured at 415 nm using an enzyme-linked immunosorbent assay plate reader. Complement hemolytic activity is expressed as an ACH_{50} value (Alternative Complement Hemolysis), which represents the volume of serum necessary to produce lysis of 50% of the target cells under standard conditions, and the results are presented in units/mL.

2.6. Bacterial Challenge

A standard frozen glycerol stock of ALG-00-530 strain of *Flavobacterium covae* (formerly known as *F. columnare*), a genomovar II isolate, originally isolated from a diseased channel catfish in Alabama, was used. *F. covae* the primary causative agent of columnaris disease in catfish. The frozen material was inoculated into 25 mL modified Shieh broth and grown at 28 °C for 24 h with shaking at 135 rpm. Following 24 h culture, 200 µL of the culture was inoculated into 200 mL of broth and cultured at 28 °C (135 rpm) until optical density reached 0.3 at 540 nm. The challenge doses were determined by diluting and plating the culture, enumerating colonies, and calculating the CFU/mL, following standard practices.

To determine the optimum bacterial cell concentration to be use in the experimental challenge, groups of 15 fish that were held in separate aquaria and fed the control diet for 8 weeks, were challenged with 0; 2.5×10^8; 5×10^8; 1×10^9; 2×10^9; and 4×10^9 *F. covae* cells/mL. For bacterial exposure, fish were held in 15 L of aerated water containing the challenge inoculum for 30 min before water flow was restored at 0.5 L/min. Fish were monitored twice per day, at which time moribund and dead fish were removed. The LD_{50} (50% lethal dose) was calculated to be 1×10^9 cells/mL and utilized for challenging. At the end of the 10-week feeding period, 15 fish from each aquarium with 4 replicate tanks per diet were randomly selected and exposed to 1×10^9 cells/mL of *F. covae* by immersion, as described above. Each group of fish continued to receive their respective diets. Mortality was recorded twice daily for 7 days.

2.7. Statistical Analysis

Data were analyzed by one-way ANOVA using the general linear model. If there was a significant F-test, subsequent comparisons of treatment means were determined using the Dunnett's multiple range test. Differences were considered significant at the 0.05 probability level. All statistics were performed using Graphpad Prism 9.0 (San Jose, CA, USA).

Over-time survival data after bacterial challenge and the comparison of two group survival curves (control vs. each level of frass) were analyzed using Graphpad Prism by Kaplan–Meier Log Rank Survival Analysis.

3. Results

The inclusion of frass resulted in the improvement of hematological (red blood cell (RBC) count, hemoglobin, and hematocrit) parameters (Table 3). The RBC count of fish fed with 10% or higher frass diet was significantly ($p \leq 0.01$) higher than that of the control fish. Fish offered diets containing the highest level of frass had the significantly ($p \leq 0.001$) highest RBC counts. White blood cell (WBC; $\times 10^5/\mu L$) count was unaffected by dietary frass, irrespective of the inclusion level. Hemoglobin concentration and hematocrit increased at each incremental level of dietary frass, but the values were significantly ($p \leq 0.05$) higher only at the highest dietary frass level. Values for mean corpuscular volume (MCV; $p \leq 0.05$), which define the size of the red blood cell and mean corpuscular hemoglobin (MCH; $p \leq 0.01$) and quantifies the amount of hemoglobin per red blood cell, were significantly lower in fish fed dietary frass at levels from 10 to 30% than those of the fish fed the control diet. The mean corpuscular hemoglobin concentration (MCHC) was similar across all the treatments.

Table 3. Hematological values of channel catfish fed diets containing various levels of frass for 10 weeks [1].

Dietary Levels of Frass (%)	RBC $\times 10^6/\mu L$	WBC $\times 10^5/\mu L$	Hb (g/dL)	Ht (%)	MCV [2] (fl)	MCH [2] (pg)	MCHC [2] (%)
0	2.28	1.81	7.22	30.50	138.50	32.70	23.80
5	2.75	2.56	7.34	32.00	117.17	26.96	23.10
10	3.10 **	2.63	7.70	32.75	111.32 *	25.35 **	23.58
20	3.10 **	2.38	7.91	34.67	113.28 *	25.75 *	22.85
30	3.28 ***	2.73	7.83 *	36.17 *	111.23 *	24.03 **	21.66
Pooled SEM	0.399	0.363	0.369	2.229	11.277	3.191	0.798

[1] RBC = red blood cell count; WBC = white blood cell count; Hb = hemoglobin; Ht = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; and MCHC = mean corpuscular hemoglobin concentration. Values are means of one determination per fish, four fish per tank and four tanks per treatment. Asterisks indicate significant difference between the control and frass-fed groups. Number of asterisks represent degree of statistically significant difference from control: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. [2] MCV = Ht (%)/RBC ($\times 10^6$) $\times$ 10; MCH = Hb (g)/RBC ($\times 10^6$) $\times$ 10; MCHC = Hb (g)/Ht (%) $\times$ 100.

Serum levels of albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMY), calcium (CA), phosphorus (PHOS), potassium (K), and thyroxine (T4) were not significantly ($p \geq 0.05$) different from dietary frass levels (Table 4). However, serum glucose (GL) levels were significantly ($p \leq 0.01$) lower in fish fed a diet with frass than that of fish fed a diet without frass. Fish fed the highest dietary levels of frass (30%) had significantly ($p \leq 0.05$) higher serum cholesterol levels than fish fed a diet without frass (control diet) (Table 4). Both serum total protein (TP) and globulin (GLOB) levels were highest in fish fed highest the 30% dietary frass treatment; however, these differences were not significant (Figure 1).

Table 4. Serum chemistry values of channel catfish fed diets containing different levels of frass for 10 weeks [1].

Dietary Levels of Frass (%)	ALB g/dL	ALP u/L	ALT u/L	AMY u/L	CA mg/dL	PHOS mg/dL	GL mg/dL	K mm/L	Cholesterol mg/dL	T4 ug/dL
0	2.27	56.83	7.42	18.83	13.14	11.33	98.45	4.93	185.50	0.30
5	2.29	51.42	6.25	18.83	12.96	10.97	81.91 **	5.03	213.38	0.23
10	2.27	52.83	6.42	18.33	13.02	10.40	79.67 **	5.13	213.13	0.28
20	2.26	54.08	5.92	21.42	13.13	10.72	82.91 *	5.32	201.25	0.20
30	2.26	49.33	7.33	20.00	13.24	11.09	78.42 **	5.18	218.25 *	0.38
Pooled SEM	0.014	2.934	0.672	1.240	0.112	0.348	7.851	0.143	10.782	0.050

[1] ALB = albumin; ALP = alkaline phosphatase; ALT = alanine aminotransferase; AMY = amylase; CA = calcium; PHOS = phosphorus; GL = glucose; K = potassium; T4 = thyroxine. Values are means of one determination per fish, four fish per tank and four tanks per treatment. Asterisks indicate significant difference between the control and frass-fed groups. Number of asterisks represent degree of statistically significant difference from control: *, $p \leq 0.05$; **, $p \leq 0.01$.

There was no significant difference among the lysozyme activity (mg/mL) of fish in different treatments (Figure 2). Serum complement activity (unit/mL) was significantly higher in fish fed diets containing frass at levels of 10% ($p \leq 0.05$) and 20% ($p \leq 0.01$). All fish fed the diets containing 20% and 30% frass showed significantly better survival than those fed a diet with a lower dietary level of frass and control diet. Cumulative mortality *F. covae* post-challenge was not significantly ($p \leq 0.05$) different between the control diet and 10% or lower levels of dietary frass (Figure 3). However, the overall mortality was low, with mortality ranging from 0 to 20%.

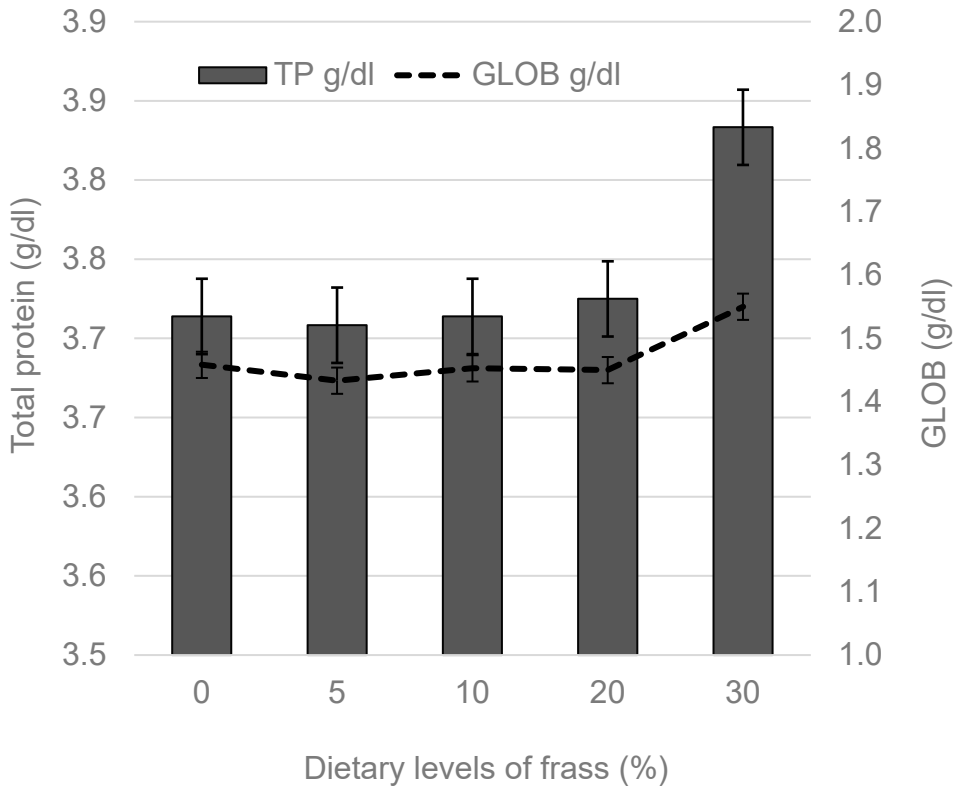

Figure 1. Serum total protein (TP) and globulin levels (GLOB) of channel catfish fed diets supplemented with different levels of frass for 10 weeks. Data are presented as mean ± standard error of means (SEM) from four replicates.

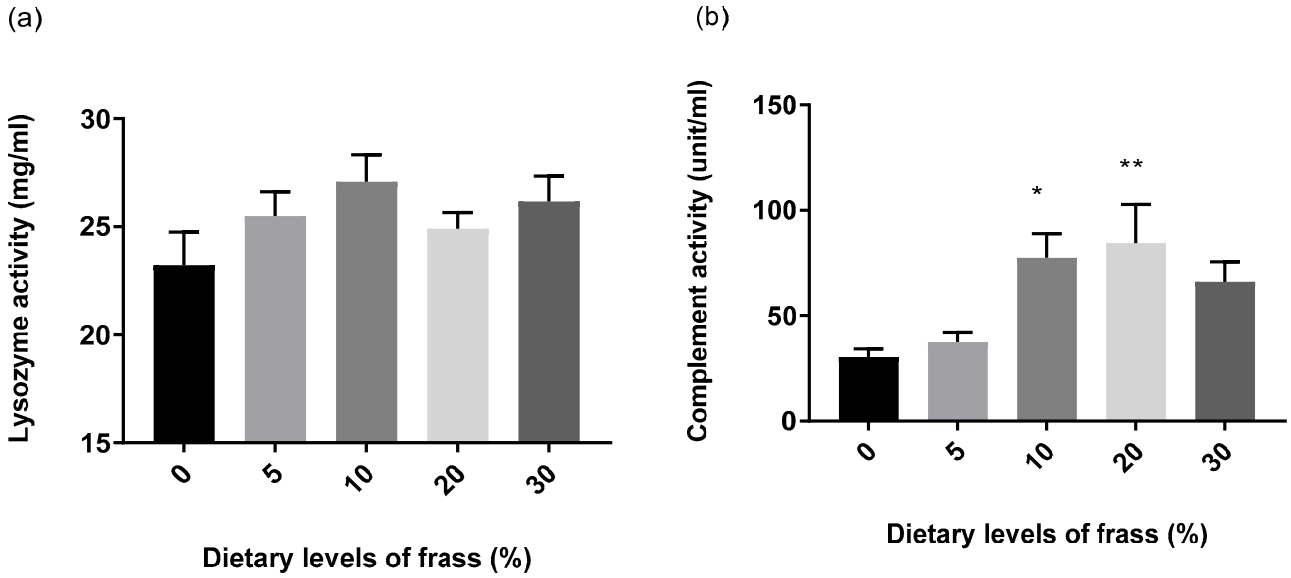

Figure 2. Lysozyme activity (**a**) and natural hemolytic complement activity (**b**) of channel catfish fed diets supplemented with different levels of frass for 10 weeks. Asterisks indicate significant difference between the control and frass-fed groups. Number of asterisks represent degree of statistically significant difference from control: *, $p \leq 0.05$; **, $p \leq 0.01$. Data are presented as mean ± standard error of means (SEM) from four replicates.

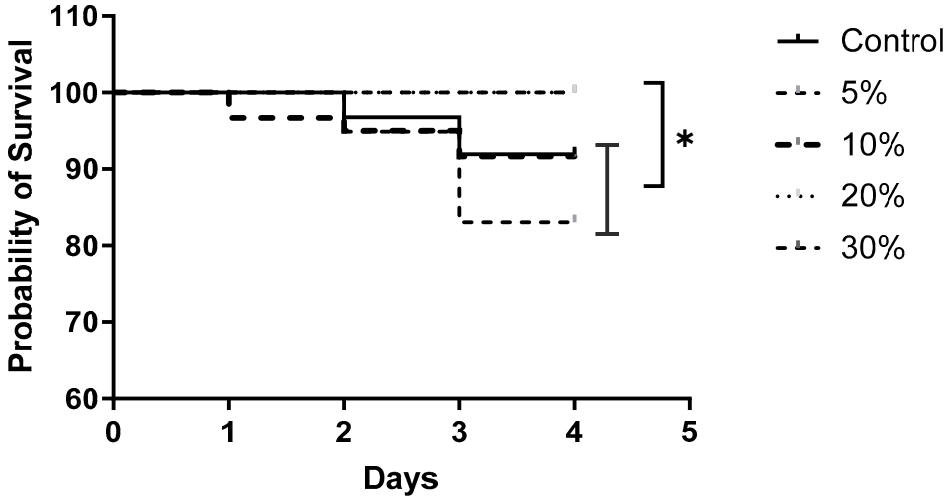

Figure 3. Percent survival of channel catfish challenged with *Flavobacterium covae* fed different levels of frass for 10 weeks. Values are means of four replicates per treatment. Asterisks indicate significant difference between the control and frass-fed groups at $p < 0.05$.

4. Discussion

Hematological, biochemical and immunological indexes could be utilized to recognize probable nutritional effects on the general health status and physiological stress responses of aquatic animals. The effects of insect meal inclusion in the fish diet on hematological and immunological parameters have been studied in several fish species. For example, Taufek et al. [36] reported significantly higher hemoglobin and hematocrit in African catfish, *Clarias gariepinus*, fed cricket meal than those of fish fed fish meal. However, there is no study that has examined the effects of feeding frass on the hematological parameters of catfish. Our results showed a clear and significant increase in hematological parameters (RBC count, hematocrit and hemoglobin). Mean corpuscular volume (MCV), which is the average size of the erythrocyte, and mean corpuscular hemoglobin (MCH), which is the quantity of hemoglobin present in a single red blood cell, values were lower in fish fed dietary frass. However, this difference was no longer significant when normalized to the hemoglobin content per unit volume of red blood cells (mean corpuscular hemoglobin concentration (MCHC)). Improved hematological values of fish fed dietary frass may be related to the increased iron level in the liver of catfish, as shown in our previous study [18]. These improved hematological values may allow blood to absorb more oxygen during periods of reduced oxygen to maintain optimum health. Similarly, the inclusion of mealworm frass to replace 25% commercial concentrate feed improved hematological parameters of sheep [37]. Studies with hybrid tilapia [20] and Nile tilapia [38] fed dietary frass of up to 30% and insect meal, however, showed no influence on hematological values.

Blood parameters are important for detecting the physiological stress response (due to factors such as temperature, photoperiod, density, salinity or nutrition) as well as the general health of the fish. Elevated levels of ALP and ALT in the blood are most commonly related to liver damage [39–42]. The present study showed that serum biochemical indices, except for glucose and cholesterol, were not affected by dietary frass treatments. The results of no influence on serum ALT and ALP activities by dietary frass suggested that frass might not cause negative effects to liver health. ALP and ALT are enzymes found in the liver that help break down proteins and convert proteins into energy for the liver cells, respectively [43]. Similarly, no negative effects to the liver of hybrid tilapia fed with up to 30% dietary frass was detected [20]. The glucose level of the treated groups was significantly lower than that of the control group. The level of carbohydrates and cholesterol in serum may be attributed to the level of glucose and cholesterol in diets, respectively. In general, foods that cause blood glucose levels to rise the most are those that are high in carbohydrates. The dietary influence in plasma biochemicals was also

reported between cultured seabass fed an artificial feed and wild sea bass fed on natural foods [44]. We reduced the vegetable oil in the diets while maintaining isonitrogenous and isolipidic levels since larvae frass had 6% crude lipid, which is higher than replacement ingredients (SBM, CM and WM). Cholesterol is the most abundant sterol in insects. This explains the significant increase in cholesterol levels in fish fed the highest dietary inclusion level of frass. Ekpo et al. [45] studied the content of cholesterol in the fat of the termite *Macrotermes bellicosus* and the caterpillar *Imbrasia belina*. They found that the average cholesterol content in the lipid fraction was 3.6%. Increased plasma cholesterol content was reported in yellow catfish, *Pelteobagrus fulvidraco*, fed a diet containing BSFL meal at high replacement levels [46].

Even though no significant differences were observed, both serum protein and immunoglobulin levels were highest in fish fed 30% frass. Increased serum protein level was also observed in sheep fed a high level of mealworm frass [37]. Similarly, lysozyme did not show any significant difference between dietary treatments, but a tendency for higher activity in fish fed diets with frass compared to fish fed diet without frass was observed. Henry et al. [47] observed increased lysozyme activity, albeit not significant, in European sea bass fed mealworm meal compared to fish fed a control diet. A significant increase in complement activity was obtained in catfish fed the diet with 10% and 20% frass. Fish fed the diets containing 20 and 30% frass also showed better survival against an *F. covae* (formerly known *F. columnare*) challenge as compared to the control or lower dietary levels of frass. Complement-dependent bacterial killing is one of the most rapid ways to eliminate an invading bacterium [48]. A significant increase in complement activity and better survival against *F. covae* and *Streptococcus iniae* challenges was also observed in tilapia fed the diet with 30% frass [20].

Insects are one of the richest sources of antimicrobial peptides/proteins (AMPs) [49], might have played an important role in increasing fish resistance to *F. covae* infection. AMPs have a broad range of antimicrobial activity against bacteria, fungi and viruses (reviewed by Lei et al. [50]). Using extracts of hemolymph, antimicrobial activity has been demonstrated in vitro against several Gram-negative bacteria [51–54], Gram-positive bacteria and yeast [55]. In general. peptides have weak membrane permeability, thereby limiting their bioavailability during oral administration. However, Wong et al. [56] demonstrated the BSFL proteins can be easily absorbed by the GI tract. Serum from shrimp fed dietary frass (20%) significantly increased the inhibition of *Vibrio parahaemolyticus* growth [24].

It is well known that dietary insect meal and frass have an abundance of chitin—a fairly potent adjuvant—which is also capable of activating the immune system [57]. The benefits of dietary chitin and/or chitosan has been reported in fish and shellfish (reviewed by Sakai [25]). Incorporating chitin into fish diets has been reported to stimulate macrophage activity in rainbow trout (*Oncorhynchus mykiss*) [25], increased lysozyme and respiratory burst activities in common carp (*Cyprinus carpio*) [58] and enhanced immune activity through the non-specific modulation of hemolytic complement activity, leucocyte respiratory burst activity and cytotoxicity in gilthead seabream (*Sparus aurata* L.) [22]. Furthermore, chitin may function as a prebiotic (non-digestible by the host) by selecting autochthonous bacteria that may have the potential to prevent the growth and colonization of pathogenic bacteria in the digestive tract. Dietary insect meal elicits species-specific differential responses of structural and functional dynamics in gut microbial communities [59]. Few studies have indicated that the inclusion of insect meal modulates the composition of fish gut microbiota diets [18,60–63] and improves the distal histomorphology in catfish [19].

5. Conclusions

In conclusion, the dietary inclusion of larval frass, particularly at higher levels, appears to be beneficial in improving hematological and immunological indexes, and in increasing the resistance of channel catfish to *F. covae* infection. However, the composition of larval frass, and thus their nutritional value, are highly dependent on the insect species [64], the substrate used to feed the insects [65], and its preparation and processing before

consumption [17]. Additionally, knowledge on the effect of larval frass or larvae meal itself on the immune system is very limited. Therefore, the effects of dietary larval frass on transcriptomics and the immunological gene expression of systemic and mucosal immunity of fish need further investigation.

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