

# EFFECT OF SUPPLEMENTED *Cordyceps militaris* EXTRACT ON DISEASE RESISTANCE AND SURVIVAL RATE OF STRIPED CATFISH (*Pangasius hypophthalmus*) FINGERLINGS

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➤ Supporting Information



**ABSTRACT:** The effects of supplemented *Cordyceps militaris* extract in diets has been studied on the survival and immune response of striped catfish (*Pangasius hypophthalmus*) fingerlings with bacterial challenge conditions. The experiment was conducted for 60 days with six treatments and three replicates, including negative and positive controls with four dietary supplementation levels of *C. militaris* extract (0.18%, 0.24%, 0.36%, and 0.47%). After the feeding trial, striped catfish were challenged with *Edwardsiella ictaluri* at a dose of 10<sup>5</sup> CFU/mL. Results showed that survival rates of striped catfish increased significantly with higher extract levels, reaching the highest value (70%) at 0.47% supplementation compared with only 16.67% in the positive control group. Hematological indices such as red blood cell (RBC) and white blood cell (WBC) including lymphocytes, monocytes, and neutrophils increased proportionally with extract concentration, which can indicate enhanced immune responses. In conclusion, the findings suggest that dietary supplementation of *C. militaris* extract at 0.47% improves the disease resistance and health status of striped catfish fingerlings against *E. ictaluri* infection. This result highlights the potential application of *C. militaris* extract as a natural immunostimulants to enhance survival and reduce antibiotic use in catfish aquaculture.

**Keywords:** *Cordyceps militaris*, *Edwardsiella ictaluri*, Immune response, *Pangasius hypophthalmus*, Survival rate.

## INTRODUCTION

*Cordyceps* species are parasitic fungi that grow on insect larvae, pupae, or adult forms. The most well-known species, *Cordyceps sinensis*, was first identified in the high-altitude regions of the Tibetan Plateau, parasitizing larvae of moths (*Thitarodes* spp.) (Lo et al., 2013; Sheng and Gerasimova, 2024; Kumar and Sharma, 2025). Another related species, *Cordyceps militaris*, also parasitizes insects of the same genus and has attracted great attention for its rich bioactive compounds, particularly cordycepin and adenosine (Lo et al., 2013; Park, 2025; Jędrejko et al., 2021).

There is substantial evidence showing that *Cordyceps* fungi possess medicinal properties and contain a variety of bioactive compounds with therapeutic potential (Das et al., 2021). These compounds have been used in traditional medicine for treating malaria, palpitations, cancer, fever, diabetes, chronic kidney disease, and other disorders (Sharma et al., 2023). Beyond human medicine, the application of *Cordyceps militaris* as a dietary supplement has been extended to terrestrial animals such as pigs (Li et al., 2024; Cheng et al., 2016; Boontiam et al., 2020) and chickens (Wang et al., 2025; Barido et al., 2024; Lan et al., 2021), where it has shown positive effects on immune response and health status.

In aquaculture, *Cordyceps* supplementation has been investigated for several aquatic species (Tian et al., 2025; Sun et al., 2021; Lan et al., 2025; Da-oh et al., 2024; Choosong et al., 2025; Van Doan et al., 2018). Van Doan et al. (2017a); Van Doan et al. (2017b) demonstrated that adding powdered *C. militaris* at 10 g/kg feed improved the growth and health of Nile tilapia (*Oreochromis niloticus*). Similarly, Vietnamese aquaculture practitioners have reported beneficial effects of *Cordyceps* supplementation on shrimp hatchery performance, including improved post-larvae immunity, reduced stress in broodstock, and enhanced larval survival (Deng et al., 2015).

The striped catfish (*Pangasius hypophthalmus*) is one of Vietnam's most economically important aquaculture species, particularly in the Mekong Delta. However, its production faces major challenges from bacterial diseases such as bacillary necrosis of the kidney and liver (BNKL), caused by *Edwardsiella ictaluri*. This disease can lead to substantial mortality in nursery and grow-out stages, threatening farm productivity (Hoa et al., 2021; Tran et al., 2025). Therefore, the search for safe and natural immunostimulants to enhance disease resistance is critical for sustainable production.

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Given the proven bioactivity of *Cordyceps militaris* in promoting immunity and growth in terrestrial animals, this study aimed to evaluate the effects of dietary supplementation with *C. militaris* extract on the survival and immune responses of striped catfish fingerlings challenged with *Edwardsiella ictaluri*. The findings are expected to provide scientific evidence for the application of *Cordyceps* extracts as natural immunostimulants in catfish aquaculture.

## MATERIALS AND METHODS

### Experimental design

The experiment was conducted following a completely randomized design with six treatments and three replicates per treatment. Two control groups were used:

- Negative control: fish fed a commercial diet without extract and not challenged.
- Positive control: fish fed a commercial diet without extract and challenged with *E. ictaluri*.

Four other treatments included diets supplemented with *C. militaris* extract at 0.18%, 0.24%, 0.36%, and 0.47%, corresponding to 1.8 g, 2.4 g, 3.6 g, and 4.7 g extract per kg feed. Fish were reared in 500-L composite tanks at a density of 200 fish per tank for 60 days.

### Feed preparation and feeding management

The *C. militaris* extract was dissolved in 100 mL distilled water and sprayed onto the feed to achieve the desired inclusion level for each treatment. The feed was then mixed thoroughly, air-dried at 60 °C for 6 hours (mixed every 2 hours), and stored at -20 °C until use. Fish were fed ad-libitum twice daily (07:30 and 17:00).

### Bacterial challenge

After 60 days of feeding, fish were challenged with *Edwardsiella ictaluri* (isolated in An Giang University) by immersion in a 500 mL bacterial suspension at a dose of LD<sub>50</sub> 10<sup>5</sup> CFU/mL (Hang et al., 2013; Hang and Phuong, 2020) for 30 minutes (except the negative control). After challenge exposure, fish were returned to their respective tanks for 14 days of observation. Mortality was recorded daily, and moribund fish were collected for bacterial re-isolation to confirm infection.

### Hematological analysis

Ten fish per tank were randomly sampled before infection (0 h), 48 h post-infection, and 14 days post-infection for hematological analysis. Red blood cells (RBC), white blood cells (WBC), lymphocytes, monocytes, and neutrophils were counted using standard hemocytometer techniques following methods by Hrubec et al. (2000); Dung (2010) and Hang et al. (2025a).

### Statistical analysis

Data were analyzed using the General Linear Model (GLM) in Minitab 16.0 (Minitab, 2010). One-way ANOVA was applied to compare mean differences among treatments, followed by Duncan's multiple range test at a significance level of  $P < 0.05$ . Results were expressed as mean  $\pm$  standard error (SE).

## RESULTS AND DISCUSSION

### Survival rate

The survival rate of fingerlings significantly increased with higher levels of *C. militaris* extract ( $P < 0.05$ ) (Table 1). The survival rate of *Pangasius* fingerlings was increased correspondingly with the increase in the rate of supplementing cordyceps extract. The highest survival (70%) was observed at 0.47% extract treatment, compared to 16.67% in the positive control treatment. Meanwhile, in the negative control treatment, the survival rate reached 100%, because the infection method and the setup conditions did not affect the experimental results. This proves that the diseased and dead fish in the remaining experiments were caused by *E. ictaluri* bacteria. Therefore, it can be concluded that 0.47% *Cordyceps* extract can be used to supplement the diet of *Pangasius* fry to help increase the survival rate when there is pathogenic *E. ictaluri* bacteria. The improvement in survival indicates that *C. militaris* extract enhances the host's resistance against *E. ictaluri*. Similar immunoprotective effects have been observed with inulin (Hang and Phuong, 2020) and *Lactobacillus plantarum* supplementation (Hang et al., 2022).

Research on *Cordyceps* extract on aquatic animals is still very new and can be said to be the first research at An Giang University and the Mekong Delta region. The results have shown that *Cordyceps* extract has great potential in the application to prevent diseases in *Pangasius* fry today. Compared with other studies on the effects of herbal extracts, probiotics, prebiotics or other immune stimulants, *Cordyceps* extract also has similar potential.

Many studies have demonstrated the effectiveness of supplementing beneficial biological substances and microorganisms to the diet to enhance resistance and reduce mortality in fish species when infected with bacteria.

Specifically, Adeshina et al. (2024) reported that supplementing alpha-lipoic acid at a dose of 1.5 to 2 g/kg of feed to the diet of catfish infected with *Edwardsiella tarda* significantly reduced mortality, to only 3.3% compared to 86.7% in the control group. In addition, the use of *Lactobacillus plantarum* at a density of  $10^{12}$  CFU/kg of feed also showed positive effects, helping fish recover health after infection and achieving a survival rate of 56.7%.

Meanwhile, tests on *Pangasius* showed that inulin is a potential additive in improving growth and immunity. According to Hang and Phuong (2020) supplementing 1% inulin every two weeks helps fish grow well, while improving resistance to *Edwardsiella ictaluri* bacteria. Another study by Kattakdad et al. (2025) reported that supplementing 0.8% inulin in an effective functional feed additive for enhancing growth, immune defense, and tissue integrity in striped catfish culture. In addition, the survival rate of *Pangasius* fingerlings in this study was lower than the results of the trial of supplementing EPS to the diet of whiteleg shrimp (97.3%) and swamp eel fingerlings (96.67%) (Thanh et al., 2025; Hang et al., 2025a). Similarly, the study added 0.2% MOS and 0.6% MOS to the diet of carp (98.08%, 83.3%) (Staykov et al., 2005; Culjak et al., 2006).

Compared with the above results, the survival rate of *Pangasius* fingerlings in the present study reached 70%. This shows the obvious effectiveness of increasing the survival rate of the method of supplementing *Cordyceps sinensis* extract into the diet of *Pangasius* fingerlings. At the same time, it opens up new potential for practical application in *Pangasius* fingerlings.

**Table 1 - The survival rate of catfish.**

Treatments	Survival rate (%)
Negative control	100.00 <sup>a</sup>
Positive control	16.67 <sup>d</sup>
0.18%	56.67 <sup>c</sup>
0.24%	60.00 <sup>c</sup>
0.36%	60.00 <sup>c</sup>
0.47%	70.00 <sup>b</sup>
SEM	1.92
p-Value	0.000

<sup>a,b,c,d</sup>Different letters following the mean values in the same column are statistically different ( $p < 0.05$ )

#### Red blood cells (RBC)

RBC counts showed no significant differences among treatments before infection with *Edwardsiella ictaluri* ( $P > 0.05$ ). However, 48 hours and 14 days post-challenge, RBC counts were significantly higher in all extract-supplemented groups compared to the positive control, suggesting that *C. militaris* mitigated infection-induced anemia. The RBC concentration in fish fed 0.47% extract ( $2.35\text{--}2.38 \times 10^6$  cells/mm<sup>3</sup>) was consistent with healthy catfish values reported by Dung (2010). The elevation in RBCs indicates better oxygen transport and physiological status, consistent with reports by Wilhelm Filho et al. (1992) and Hang and Hoa (2020).

**Table 2 - Red blood cells of *Pangasius* before and after infection with *Edwardsiella ictaluri* bacteria ( $10^6$  cell /mm<sup>3</sup>)**

Treatments	RBC0H	RBC48H	RBC14D
Negative control	2.00	1.96 <sup>b</sup>	1.95 <sup>a</sup>
Positive control	2.27	1.16 <sup>b</sup>	1.32 <sup>b</sup>
0.18%	2.29	2.24 <sup>a</sup>	2.28 <sup>a</sup>
0.24%	2.29	2.19 <sup>a</sup>	2.24 <sup>a</sup>
0.36%	2.30	2.15 <sup>a</sup>	2.27 <sup>a</sup>
0.47%	2.31	2.35 <sup>a</sup>	2.38 <sup>a</sup>
SEM	0.08	0.08	0.12
P-value	0.135	0.000	0.001

RBC0H: red blood cell before infection; RBC48H: red blood cell after infection 48 hours; RBC14D: red blood cell after infection 14 days; <sup>a,b,c,d</sup>Different letters following the mean values in the same column are statistically different ( $p < 0.05$ ).

Red blood cell (RBC) counts showed no significant differences among treatments before infection with *Edwardsiella ictaluri* ( $P > 0.05$ ). This also shows that the red blood cell density before infection did not affect the experimental results. However, 48 hours and 14 days post-challenge with bacteria causing pyogenic liver and kidney disease, the red blood cell density results fluctuated and were different between the treatments, especially the positive control treatment ( $P < 0.05$ ).

After 48 hours and 14 days of infection, the positive control treatment had the lowest red blood cell density, respectively  $1.16 \times 10^6$  cells /mm<sup>3</sup> and  $1.32 \times 10^6$  cells /mm<sup>3</sup>, which was different from the remaining treatments. In particular, the experiments supplemented with *Cordyceps sinensis* extract showed that the red blood cell density did not differ between the treatments, but at the highest supplement concentration of 0.47%, the red blood cell density reached the highest level of  $2.35 \times 10^6$  cells/mm<sup>3</sup> and  $2.38 \times 10^6$  cells/mm<sup>3</sup>. RBC counts were significantly higher in all extract-supplemented groups compared to the positive control, the red blood cell density increased correspondingly with the concentration of *Cordyceps sinensis* extract, suggesting that *C. militaris* mitigated infection-induced anemia. The RBC concentration in fish fed 0.47% extract ( $2.35\text{--}2.38 \times 10^6$  cells/mm<sup>3</sup>) was consistent with healthy catfish values reported by Dung (2010).

Red blood cells play a very important role in the metabolism of fish, transporting oxygen and nutrients to nourish cells in the body and eliminating CO<sub>2</sub> from the fish body (Tu, 2000). The elevation in RBCs indicates better oxygen transport and physiological status, consistent with reports by Wilhelm Filho et al. (1992) and Hang and Hoa (2020). According to Wilhelm Filho et al. (1992), the red blood cell density of bony fish is determined to be  $2.289 \times 10^6$  cells/mm<sup>3</sup>. Meanwhile, according to the research results of Hii et al. (2007) on freshwater eels (*Monopterus albus*), the red blood cell density is only about  $1.05\text{--}1.10 \times 10^6$  cells/mm<sup>3</sup>. For healthy catfish, the red blood cell density is determined to be  $1.79\text{--}2.75 \times 10^6$  cells/mm<sup>3</sup> (Galagarza et al., 2017). At the same time, the number of red blood cells in the blood of freshwater fish varies greatly, depending on the stage of development, physiological status and activity of the fish, ranging from 1 - 3.5 million cells/mm<sup>3</sup> (Tu, 2000).

Compared with other studies on supplementing products that help stimulate fish immunity, the results of red blood cell density in this study were also higher and still within the appropriate range. When *Pangasius* had white liver and white gill disease, the red blood cell density decreased to only  $0.1 \times 10^6$  cells/mm<sup>3</sup> (Hrubec et al., 2000). Compared with the study of adding 1% inulin to *Pangasius* feed, the red blood cell density only reached  $1.99 \times 10^6$  cells/mm<sup>3</sup> (Hang and Phuong, 2020). Supplementation vitamin C in feed on the hematology parameters of Mekong Giant Catfish (*Pangasianodon gigas*) got  $2.34\text{--}2.64 \times 10^6$  cells/mm<sup>3</sup> (Pimpimol et al., 2012). Or the study of adding 3% pomegranate extract to *Pangasius* feed, the red blood cell density reached  $2.99 \times 10^6$  cells/mm<sup>3</sup> (Hang and Hoa, 2020), which is equivalent to the results of adding cordyceps extract in this result. However, the red blood cell density when supplementing *Cordyceps sinensis* extract was lower than the study of supplementing probiotic *Lactobacillus acidophilus* on catfish fry at  $3.10 \times 10^6$  cells/mm<sup>3</sup>. At the same time, it was lower than the research results of Hang et al. (2025b) when adding 8 g/kg EPS to the feed of swamp eel fingerling at  $3.03 \times 10^6$  cells/mm<sup>3</sup>. Besides, the red blood cell results in this study were also much lower than the study of Hu et al. (2020) when adding vitamin C to the diet of eel, the red blood cell density reached  $7.6 \times 10^{12}$  cells/mm<sup>3</sup>.

### White blood cells (WBC) and immune indices

#### White blood cells (WBC)

WBC counts increased significantly with extract supplementation after infection ( $P < 0.05$ ). Fish fed 0.47% extract recorded WBC counts of  $201.43 \times 10^3$  cells/mm<sup>3</sup> at 48 hours and  $644.35 \times 10^3$  cells/mm<sup>3</sup> at 14 days, the highest among all treatments (Table 3).

**Table 3 - White blood cells of *Pangasius* before and after infection with *Edwardsiella ictaluri* bacteria ( $10^3$  cell /mm<sup>3</sup>).**

Treatments	WBC0H	WBC48H	WBC14D
Negative control	68.32 <sup>c</sup>	67.96 <sup>c</sup>	78.32 <sup>c</sup>
Positive control	86.29 <sup>bc</sup>	65.33 <sup>c</sup>	100.48 <sup>c</sup>
0.18%	91.99 <sup>ab</sup>	141.83 <sup>b</sup>	303.57 <sup>b</sup>
0.24%	90.92 <sup>ab</sup>	136.67 <sup>b</sup>	369.19 <sup>b</sup>
0.36%	94.15 <sup>ab</sup>	152.34 <sup>b</sup>	525.46 <sup>a</sup>
0.47%	106.89 <sup>a</sup>	201.43 <sup>a</sup>	644.35 <sup>a</sup>
SEM	3.89	5.72	27.26
P-value	0.0001	0.0001	0.0001

WBC0H: white blood cell before infection; WBC48H: white blood cell after infection 48 hours; WBC14D: white blood cell after infection 14 days; <sup>a,b,c,d</sup>Different letters following the mean values in the same column are statistically different ( $p < 0.05$ ).

Similar to the results of red blood cell density, white blood cell density also fluctuated when infected with bacteria causing pyogenic liver and kidney disease after 48 hours and 14 days. The results showed that the positive control experiment had the lowest white blood cell density and was different from the remaining experiments ( $P < 0.05$ ). Meanwhile, the experiments supplemented with *Cordyceps* extract tended to increase gradually according to the concentration of the extract, so at a concentration of 0.48%, it reached the highest level after 48 hours and 14 days of bacterial infection, respectively ( $201.43 \times 10^3$  cells/mm<sup>3</sup> and  $644.35 \times 10^3$  cells/mm<sup>3</sup>). This result shows that adding *Cordyceps* extract to the diet of *Pangasius* fish at a concentration of 0.48% after only 48 hours of exposure to the

pathogen has been effective in preventing the disease. The function of white blood cells in fish blood is to destroy pathogens that attack the fish body and the number of white blood cells in fish blood is 10-100 times smaller than the number of red blood cells (Tu, 2000). According to Dung (2010), the density of white blood cells in the blood of healthy catfish is determined to be  $1.0 \times 10^6$  cells/mm<sup>3</sup>. But when catfish have white liver and white gill disease, the white blood cell density is only  $0.01 \times 10^3$  cells/mm<sup>3</sup> (Hrubec et al., 2000). Compared with the results of the study on supplementing probiotic *Lactobacillus acidophilus* into the catfish diet, the white blood cell density is only  $25 \times 10^3$  cells/mm<sup>3</sup> (Al-Dohail et al., 2009), much lower than the addition of 0.48% Cordyceps extract in this study. But it is much lower than the result of adding 68.60 mg/kg of vitamin to the feed of freshwater eels, reaching  $16.68 \times 10^{10}$  cells/mm<sup>3</sup> (Hu et al., 2020). In addition, the result of leukocyte density when adding 0.48% of cordyceps extract is also much lower than the result of the study adding 8 g/kg EPS to the diet of freshwater eels, reaching  $1.4 \times 10^6$  cells/mm<sup>3</sup> (Hang et al., 2025b). At the same time, it is also lower than the study of adding 1% inulin, reaching  $232.45 \times 10^3$  cells/mm<sup>3</sup> (Zhou et al., 2020). At the same time, the results of determining the white blood cell density of this study were also higher than the results of the study adding 3% pomegranate extract to *Pangasius* feed (Hang and Hoa, 2020) or the study adding 1.2% inulin in diets of striped catfish (Kattakdad et al., 2025). The results recorded in this study show that 0.48% of cordyceps extract can be used to supplement the diet of *Pangasius* to bring high disease prevention efficiency.

### Immune indices

Lymphocytes, monocytes, and neutrophils followed similar trends of WBC, indicating enhanced cellular immunity (Table 4). The increase in lymphocytes implies a stronger adaptive immune response (Houston, 1997; Groff and Zinkl, 1999); , while elevated monocyte and neutrophil counts reflect activation of innate defenses (Yano, 1992; Magnadóttir, 2006). Comparable immune enhancements were reported in fish fed  $\beta$ -glucan (Hoang et al., 2024) and *Eclipta alba* extract (Harikrishnan et al., 2009).

**Table 4 - Immune indices (lymphocytes, monocytes, and neutrophils) of *Pangasius* before and after infection with *Edwardsiella ictaluri* bacteria ( $10^3$  cell /mm<sup>3</sup>)**

Treatments	Negative control	Positive control	0.18%	0.24%	0.36%	0.47%	SEM	P-value
<b>Immune indices</b>								
<i>Lymphocytes</i>								
Lym0H	6.62 <sup>d</sup>	4.75 <sup>d</sup>	11.00 <sup>cd</sup>	23.9 <sup>bc</sup>	33.14 <sup>b</sup>	53.50 <sup>a</sup>	2.90	0.0001
Lym48H	8.89 <sup>d</sup>	8.61 <sup>d</sup>	32.50 <sup>c</sup>	36.29 <sup>c</sup>	53.53 <sup>b</sup>	81.95 <sup>a</sup>	3.17	0.0001
Lym14D	16.64 <sup>d</sup>	16.03 <sup>d</sup>	84.07 <sup>cd</sup>	117.40 <sup>c</sup>	223.60 <sup>b</sup>	315.50 <sup>a</sup>	18.61	0.0001
<i>Monocytes</i>								
Mono0H	6.45 <sup>bc</sup>	5.90 <sup>c</sup>	10.52 <sup>bc</sup>	10.21 <sup>bc</sup>	12.78 <sup>b</sup>	21.80 <sup>a</sup>	1.37	0.0001
Mono48H	8.74 <sup>c</sup>	7.33 <sup>c</sup>	16.96 <sup>b</sup>	17.79 <sup>b</sup>	21.11 <sup>b</sup>	33.44 <sup>a</sup>	1.40	0.0001
Mono14D	18.96 <sup>d</sup>	18.70 <sup>d</sup>	98.08 <sup>cd</sup>	136.97 <sup>c</sup>	260.87 <sup>b</sup>	368.08 <sup>a</sup>	21.71	0.0001
<i>Neutrophils</i>								
Neu0H	10.92 <sup>c</sup>	14.21 <sup>c</sup>	20.22 <sup>c</sup>	20.23 <sup>c</sup>	48.89 <sup>b</sup>	68.79 <sup>a</sup>	3.97	0.0001
Neu48H	12.93 <sup>d</sup>	9.65 <sup>d</sup>	36.40 <sup>c</sup>	40.64 <sup>c</sup>	59.96 <sup>b</sup>	91.79 <sup>a</sup>	3.53	0.0001
Neu14D	23.46 <sup>d</sup>	21.38 <sup>d</sup>	112.10 <sup>cd</sup>	156.50 <sup>c</sup>	298.10 <sup>b</sup>	420.70 <sup>a</sup>	24.81	0.0001

Lym0H: Lymphocytes before infection; Lym48H: Lymphocytes after infection 48 hours; Lym14D: Lymphocytes after infection 14 days; Mono0H: Monocytes before infection; Mono48H: Monocytes after infection 48 hours; Mono14D: Monocytes after infection 14 days; Neu0H: Neutrophils before infection; Neu48H: Neutrophils after infection 48 hours; Neu14D: Neutrophils after infection 14 days; <sup>a,b,c,d</sup> Different letters following the mean values in the same row are statistically different ( $p < 0.05$ ).

Lymphocytes play an essential role in the immune defense mechanism of fish, especially in recognizing and destroying pathogens such as bacteria and parasites. They are one of the key cell types involved in specific immunity, contributing to stimulating macrophages to produce antibodies and enhancing the overall immune response (Houston, 1997). In some fish species, lymphocytes account for a high proportion of the total leukocytes, up to 85%, indicating their prominent role in the immune system of fish (Groff and Zinkl, 1999). The increase in the number of lymphocytes may be related to the ability to adapt and better respond to adverse environmental conditions. In addition, the increase in the number of lymphocytes after infection is a normal physiological phenomenon in fish species, demonstrating the core role of this cell in the specific immune mechanism (Magnadóttir, 2006).

The results from Table 4 show that the number of lymphocytes fluctuated in a significant increasing trend corresponding to the concentration of Cordyceps extract supplementation and gradually increased over time. In the positive control group, the number of lymphocytes remained low at 48 hours and 14 days after infection. In contrast, the treatment supplemented with 0.47% Cordyceps extract gave outstandingly high results, especially at the time after 12

days of infection, reaching the highest level of  $315.5 \times 10^3$  cells/mm<sup>3</sup>, proving that this concentration strongly stimulates the immune system and has a high disease prevention effect.

For healthy *Pangasius*, the number of lymphocytes is  $81 \times 10^3$  cells/mm<sup>3</sup>, but in diseased *Pangasius* with white liver and white gills, the number was only  $0.25 \times 10^3$  cells/mm<sup>3</sup> (Dung, 2010). This result shows that the number of lymphocytes in healthy *Pangasius* fish will be equivalent to the number of lymphocytes in *Pangasius* fish supplemented with 0.47% cordyceps extract after only 48 hours of infection with bacteria causing pyogenic liver and kidney disease. However, the lymphocyte results in this study were much lower than the study of supplementing 8 g/kg EPS to the diet of young eels, reaching  $2.5 \times 10^6$  cells/mm<sup>3</sup>. In addition, Oligochitosan (COS) and oligo- $\beta$ -glucan ( $\beta$ OG) show strong potential as immunostimulants and growth-enhancing additives for *Pangasianodon hypophthalmus*, as they are capable of stimulating innate immune parameters, improving disease resistance, and promoting growth performance in aquaculture systems (Nguyen et al., 2017). At the same time, the results of this study are also consistent with the results of (Rattanachaiakunsopon and Phumkhachorn, 2009), when using *Andrographis paniculata* extract on tilapia (*Oreochromis niloticus*), also showed a significant increase in the number of lymphocytes in the blood of the fish. Similarly, the study by (Harikrishnan et al., 2009) on *Sparus aurata* fish when using *Eclipta alba* plant extract showed a sharp increase in the number of lymphocytes and enhanced antibody production, helping the fish to improve their ability to prevent diseases caused by *Vibrio harveyi*.

Thus, the results of lymphocytes in this study have opened up the potential for applying *Cordyceps* extract to stimulate immunity in *Pangasius* fish with the most effective addition concentration of 0.47%. The results in Table 4 showed that the number of monocytes increased significantly according to the concentration of supplementation after 48 hours and 12 days of infection. In which, the positive control group had the lowest number of monocytes and was different from the remaining treatments, while the 0.47% supplemented treatment reached the highest number at 48 hours and 12 days, respectively, at  $33.44 \times 10^3$  cells/mm<sup>3</sup> and increased sharply at 14 days after infection to  $368.08 \times 10^3$  cells/mm<sup>3</sup>. This clearly reflects the stimulation of the fish's immune system. Because monocytes are white blood cells that play an important role in the fish's immune system when pathogens invade (Tu, 2000). When compared with other research results on aquatic species, the quantity and function of monocytes also tend to increase when there is a pathogen attack or when there is an addition of immunostimulants in the fish diet (Aly et al., 2008; Hang et al., 2025b; Misra et al., 2006). For example, according to the results of Aly et al. (2008), tilapia (*Oreochromis niloticus*) when supplemented with the immunostimulant  $\beta$ -glucan was recorded to not only increase the number of monocytes, but also increase the ability of macrophages. Similarly, Misra et al. (2006) reported that common carp (*Labeo rohita*) after infection with *Aeromonas hydrophila* had a significant increase in monocytes, reflecting the core role of this cell line in the cellular immune response. In addition, the results of (Harikrishnan et al., 2011) also showed that the use of plant extracts such as *Withania somnifera* in sea bream (*Sparus aurata*) increased the density of monocytes, thereby enhancing the ability to fight against pathogenic bacteria. This result was also consistent with the increasing trend of monocytes, especially in the treatments with high concentrations of 0.36% and 0.47%.

The results of neutrophil counts also showed a similar trend as monocytes, lowest in the positive control and highest in the 0.47% treatment (Table 4). This suggests that, when supplemented at a concentration of 0.47%, fish may have strongly mobilized the number of neutrophils. This is the first important cell type in detecting and destroying pathogens (Tu, 2000). This result is consistent with previous studies such as those of Reyes-Becerril et al. (2011), which recorded a significant increase in the number of neutrophils in sea bream (*Sparus aurata*) when injected with *Aeromonas hydrophila*, a component of Gram-negative bacteria. Similarly, Magnadóttir (2006) described the key role of neutrophils and macrophages in the acute inflammatory response in fish, especially the ability to phagocytose and secrete enzymes to destroy bacteria. Studies on grass carp (*Ctenopharyngodon idella*) supplemented with  $\beta$ -glucan also showed an increase in the number and activity of neutrophils, contributing to enhanced non-specific defense (Yano, 1992).

These findings suggest that *C. militaris* extract stimulates both specific and non-specific immune mechanisms, leading to improved disease resistance against *E. ictaluri*. The dose-dependent pattern also supports the hypothesis that bioactive compounds such as cordycepin and adenosine contribute to hematological and immunostimulatory effects.

## CONCLUSION

The results of the study showed that the addition of *Cordyceps militaris* extract to the diet of *Pangasius* fingerlings significantly improved the survival rate and enhanced the immune response when the fish were infected with *Edwardsiella ictaluri* bacteria causing pyogenic liver and kidney disease. At the addition concentration of 0.47%, the fish achieved the highest survival rate (70%) and had significantly increased red blood cell, white blood cell, lymphocyte, monocyte and neutrophil indices compared to the positive control. This proves that *Cordyceps militaris* extract has the ability to stimulate the immune system and enhance the natural resistance of *Pangasius* fish. Therefore, it is recommended to use *Cordyceps militaris* extract at a rate of about 0.47% in the diet of *Pangasius* fingerlings to increase disease prevention and reduce dependence on antibiotics in aquaculture.

## DECLARATIONS

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### Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

### Authors' contribution

Conceptualization, resources, supervision and funding acquisition: Lan T.T. and Nhi N.H.Y.;  
Methodology, validation, and data curation: Lan T.T., Nhi N.H.Y., and Hang N.T.T.;  
Formal analysis and investigation: Nhi N.H.Y., Lan T.T., Hanh N.T.B., Vy P.N.T., Mai V.P., and Hang N.T.T.;  
Writing—original draft preparation, visualization: Nhi N.H.Y. and Hang N.T.T.;  
Writing—review and editing: Nhi N.H.Y., Lan T.T. and Hang N.T.T.;  
Project administration: Lan T.T.  
All authors have read and agreed to the published version of the manuscript.

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### Competing interests

The authors declare no competing interests in this research and publication.

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