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Synbiotic application to enhance growth, immune system, and disease resistance toward bacterial infection in catfish (*Clarias gariepinus*)

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ABSTRACT

This research comprehensively evaluated the additional extract of *Solanum ferox* (SFE) and probiotic *Lactobacillus casei* (LC) on catfish (*Clarias gariepinus* var.) feed to enhance growth, feed efficiency, and resistance toward *Aeromonas hydrophila* and *Pseudomonas fluorescens* bacterial infection. Five different pellet types were made by adding various composition of SFE and LC as follows: pellet 1 (0 SFE + 0 LC), pellet 2 (0 SFE + 10 g kg^{-1} LC), pellet 3 (2 g kg^{-1} SFE + 10 g kg^{-1} LC), Pellet 4 (4 g kg^{-1} SFE +10 g kg^{-1} LC), Pellet 5 (6 g kg^{-1} SFE +10 g kg^{-1} LC). In total, 150 Catfish (initial weight ± 4 g) were randomly distributed into 15 plastic boxes and cultured for 12 weeks. Catfish were fed with different composition pellets for two weeks, followed by a regular pellet. The observation of growth was examined in weeks four and eight after feeding time. In week 8, the bacterial challenge against *A. hydrophila* and *P. fluorescens* was performed, and the observation was continued until week 12. The results showed that catfish grew to weigh an average of 45 g in week 8. Catfish fed higher concentrations of SFE and LC extractions showed a specific growth rate significantly higher (8–9%) than that of the control-treatment catfish. Additionally, SFE and LC extractions enhanced immune system function between weeks four and eight in comparison with other treatments. Resistance against bacterial infection increased with the SFE extraction to reach 99.65–100%. Fish feed containing only the LC extraction also showed higher resistance than control-treatment feed. In conclusion, higher concentrations of SFE and LC extractions in catfish feed has been shown to enhance growth, with no attendant risk. Importantly, the use of the synbiotics enhanced both the immune system and resistance to *A. hydrophila* and *P. fluorescens* bacterial infections.

1. Introduction

Prebiotics (Hardi et al., 2018a, 2018b; Kurniasih et al., 2013; Nugroho and Fotedar, 2015; Ringø and Song, 2015; Zhang et al., 2013), probiotics (Akbari Nargesi et al., 2020; Kurniasih et al., 2013; Nayak, 2010; Verschuere et al., 2000; Widanarni et al., 2014), and synbiotics (Amenyogbe et al., 2020; Praseto, 2015; Yılmaz et al., 2020) have been previously used in aquaculture to enhance growth, immune systems, and disease resistance, reduce feeding costs, restore the cultivation environment, and help sustainable aquaculture evolve.

The product of mixing probiotics and prebiotics is commonly known as synbiotics, and was first introduced by Schrezenmeir and de Vrese (2001) to support the survival rate and growth of beneficial bacteria in the digestive system. Synbiotics are a balanced combination of probiotics and prebiotics which have the potential to rapidly enhance aquaculture production through increased fish growth and improved immune systems, digestion and absorption, disease management, and water quality control (Okey et al., 2018; Torrecillas et al., 2018; Van Nguyen et al., 2019; Waagbø and Remø, 2020).

The extraction of *S. ferox* (SFE; more commonly known as "hairy eggplant") as an immunostimulant raw material for fish has been widely investigated (Hardi et al., 2016a, 2016b, 2017, 2018a, 2018b, 2018c, 2019) for its potential to supply nutritious quantities of carbohydrates, flavonoids, and alkaloids. For example, SFE has produced satisfying results through constitution as fish feed for tilapia and goldfish, significantly elevating digestibility in comparison to non-extraction fish

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feed. Prebiotics can take the form of mannan oligosaccharides, fructose oligosaccharides, galactooligosaccharides, and isomaltooligosaccharides, and improve fish health by boosting the work of the intestinal microbiota, intestinal histology, and hematological parameters, and increasing disease resistance (Ringø and Song, 2015).

Lactobacillus bacteria is a probiotic which undoubtedly enhances the digestive fish feed mechanism (Okey et al., 2018). Additionally, synbiotics in aquaculture have been broadly reported to improve production, as measured by growth rates, enhanced immune systems, improved digestion and absorption, increased disease resistance, and greater water quality control (Carnevali et al., 2006; Hussein et al., 2020; Okey et al., 2018; Thompson et al., 1999; Verschuere et al., 2000).

However, little research has yet been conducted on the application of prebiotics and probiotics specifically on catfish growth, immune function, and disease resistance. Thus, the present research evaluated the effects of prebiotic and probiotics application in catfish feed. For this study, a single *S. ferox* extract combined with the L. *casei* (LC) probiotic was administered to catfish and evaluated for its effects on growth, immune system function, and resistance to bacterial *A. hydrophila* and *P. fluorescens* infection.

2. Materials and methods

2.1. S. ferox extract (SFE) and L. casei probiotic (LC) preparation

The *S. ferox* extract (SFE) was prepared based on the previous method performed by Hardi et al. (2016a, 2016b) using ethanol solvent. The *S. ferox* was cleaned and washed up from the soil. The cleaned *S. ferox* was then finely chopped (0.3–0.5 cm) using a chopper. The chopped *S. ferox* was dried at 40–45 °C for 48 h in an oven. To obtain SFE, the dried *S. ferox* was continuously blended and soaked in ethanol (96%) for 48–72 h with a ratio of one kg of *S. ferox* powder soaked in 10 L of ethanol (1:10). The SFE was added to commercial fish pellets at 2, 4, and 6 g kg^{-1} of fish feed formula.

The L. *casei* bacteria (LC) was obtained from EM4 commercial product, which was isolated using Tryptone Soya Agar/TSA (Merck®) media and characterized by a biochemical test. The bacterial density used in the feed composition was 10^8 CFU mL^{-1}. The LC was then added to fish feed formulated at a concentration of 10 g kg^{-1}, following the previous study by Irianto and Austin (2002).

2.2. Catfish feed

The use of SFE and LC for catfish feed was applied using the methods of Van Doan et al. (2019). The SFE and LC were added to fish feed formula at various ratios as follows: Pellet 1 (0 SFE + 0 LC), Pellet 2 (0 SFE + 10 g kg^{-1} LC), Pellet 3 (2 g kg^{-1} SFE + 10 g kg^{-1} LC), Pellet 4 (4 g kg^{-1} SFE +10 g kg^{-1} LC), Pellet 5 (6 g kg^{-1} SFE + 10 g kg^{-1} LC). Catfish feed was formulated and elaborated as shown in Table 1. (See Table 2.)

In preparation for pelletizing, SFE, LC, and all other ingredients were thoroughly mixed, and water (300 mL kg^{-1}) was added to produce a stiff dough. The dough was then put into the extruder machine to produce fish pellets. Furthermore, the pellets were dried in an oven at 50 °C to a moisture content of approximately 10%, and stored in plastic bags at 4 °C until used.

2.3. Fish and experimental design

A total of 150 catfish (initial weight 4 g) were obtained from Rama Jaya Mahakam Company's hatchery at Kutai Kartanegara regency, East Kalimantan, Indonesia. All fish were acclimated to the natural environment for seven days in a laboratory. Fish were fed two times a day with commercial feed. Prior to experimentation, 10 fish were randomly caught and checked by isolating their gills, livers, and kidneys in GSP media (Merck®) to determine bacteria contaminant (*Aeromonas* and *Pseudomonas*). If bacterial growth was not found, fish were used for the experiment. In contrast, if bacterial growth was found, the fish were soaked in formalin 30% for five minutes once a day for seven days.

Table 1
The formulated composition of the Catfish feed.

Composition (g kg^{-1})	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Pellet 5
fish flour	270	270	270	270	270
Corn starch	200	200	200	200	200
soy flour	270	270	270	270	270
wheat flour	60	60	60	60	60
Bran	150	150	150	150	150
SFE	0	0	2	4	6
LC	0	10	10	10	10
Cellulose	30	20	18	16	14
soy oil	5	5	5	5	5
fish oil	10	10	10	10	10
vitamin C	5	5	5	5	5
Approximate feed composition (%)					
Water content	8,27	9	8,22	8,3	8,22
Ash	15,18	16,12	17,23	16,13	17,42
Crude protein	30,22	32,1	35,84	34,2	35,53
Crude lipid	2,26	2,2	3,2	3,4	3,1
Carbohydrate	43,07	40,58	35,51	37,97	35,73

Table 2
Survival Rate (SR), Mortality Rates (MR) and Relative Percentage of Survival (RPS) of catfish according to challenge test against *A. hydrophila* and *P. fluorescens* bacteria in week 12.

Parameters	Pellet 1	Pellets 2	Pellets 3	Pellets 4	Pellets 5
SR (%)	45	72.2	100	87.3	80.5
MR (%)	55	27.8	0	12.7	19.5
RPS (%)		49.5	100.0	76.9	64.5

The healthy fish were randomly distributed into fifteen plastic boxes (5 groups in triplicate), with each box consisting of 10 fish. Fish were fed ad libitum twice a day at 8:00 a.m. and 16:00 p.m. Fish were cultured for eight weeks to monitor growth due to feeding with different compositions. Challenge tests with pathogenic bacteria were carried out at week eight, and the fish continued to be cultured until week 12 for observation of their immune systems, survival rate (SR), and relative percentage of survival (RPS).

2.4. Growth performance

Growth performance was examined following the methods of Van Doan et al. (2019). Growth performance indicators such as weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR) were measured at weeks four and eight after feeding time.

$$\mathrm{WG\ (g)} = \mathrm{final\ weight\ (g)} - \mathrm{initial\ weight\ (g)};$$

$$\mathrm{SGR\ (\%)} = 100 \times (\ln\ \mathrm{final\ weight} - \ln\ \mathrm{initial\ weight}) / \mathrm{Duration\ of\ experim}$$

$$\mathrm{FCR} = \mathrm{feed\ offered\ (dried\ weight)} / \mathrm{weight\ gain\ (wet\ weight)};$$

$$\mathrm{SR\ (\%)} = (\mathrm{final\ fish\ number} / \mathrm{initial\ fish\ number}) \times 100.$$

2.5. Immunological parameters

Immune system parameters were detected following previous methods, described as follows: Total of leukocyte/TL (Cell mm^{-3}) was detected using the methods of Blaxhall and Daisley (1973). Lysozyme ac-

tivity/LA (μg mL^{-1}), phagocytic index/PI (%), and respiratory burst activity/RBA (μg mL^{-1}) were identified following the protocols of Van Doan et al. (2017).

2.6. Challenge test

The challenge test was administered by intramuscular injection with *A. hydrophila* (EA-01) and *P. fluorescens* (EP-02) combination bacteria with bacterial density of 10^5 CFU mL^{-1}. Injections of as much as 0.1 mL were given to each fish. Bacteria had been properly prepared following the methods of Hardi et al. (2016a, 2016b). Challenge tests were carried out at week eight after feeding with different formulations, and mortality observations were checked from 24 h after the first injection until week 12. The rate of resistance against both bacteria was measured using RPS (Amend, 1981). Eventually, the rate of protection against pathogen bacteria was also measured at week 12.

$$\text{RPS} = 1 - (\text{test mortality}/\text{control mortality}) \times 100$$

2.7. Water quality measurement

Water quality indicators such as temperature, pH, dissolved oxygen (DO), and total ammonia nitrogen NH_3-N (TAN) were measured twice a day using a multi-parameters checker, while TAN was determined using spectrophotometer.

2.8. Statistical analysis

The derived data were analyzed using a MINITAB computer program for significant data, followed by DUNCAN test. The average score was considered significantly different if $P < 0.05$.

3. Results

3.1. Growth performance

Catfish growth was observed in weeks four and eight post-feeding. The WG and SGR increased significantly ($P < 0.05$) in week 8 post-feeding for pellets containing SFE (Fig. 1).

The highest SGR and WG values were seen in fish fed with SFE of 2 and 6 g Kg^{-1} of feed and LC of 10 g. A significant difference ($P < 0.05$) was also found for this parameter with fish fed either SFE or LC only. Values of FCR were significantly ($P < 0.05$) lower in fish fed with 2 and 6 g kg^{-1} SFE + 10^8 CFU g^{-1} LC.

3.2. Immunological parameters

Observation of the immunological parameters was conducted at weeks four and eight post-feeding (Fig. 2), showing that the additional 2 g kg^{-1} SFE and 10 g kg^{-1} LC in pellet 3 significantly enhanced (the highest) all immunological parameters measured in weeks four and eight, with significant increases ($P < 0.05$) recorded in RBA, LA, PI, and TL.

The administration of Pellet 2 showed significant ($P < 0.05$) increases in RBA, IP, LA, and TL compared to fish fed with the pellet control (Fig. 2). The highest values were recorded in fish fed SFE (2 g kg^{-1} feed) and LC compared to other concentrations. However, no significant difference ($P > 0.05$) was observed between the combination of SFE + LC and LC alone (Fig. 2). Further, the increase in immunological parameters occurred in all treatments of fish fed SFE + LC and LC alone compared to controls without SFE or LC. Significantly higher ($P < 0.05$) results were found in the catfish group fed 2 g kg^{-1} of SFE, when compared to fish fed with other SFE concentrations.

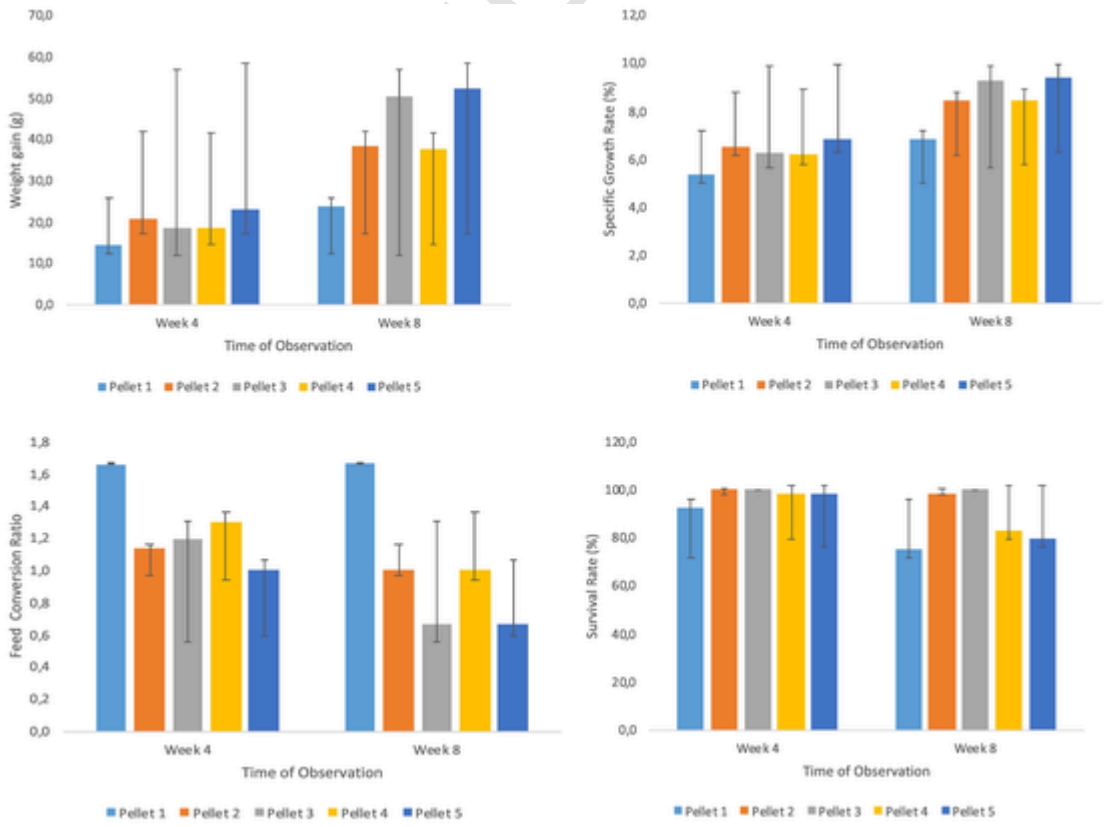

Fig. 1. Catfish growth (WG, SGR, FCR, and SR) treated with *Solanum ferox* extract (SFE) in week 4 and 8.

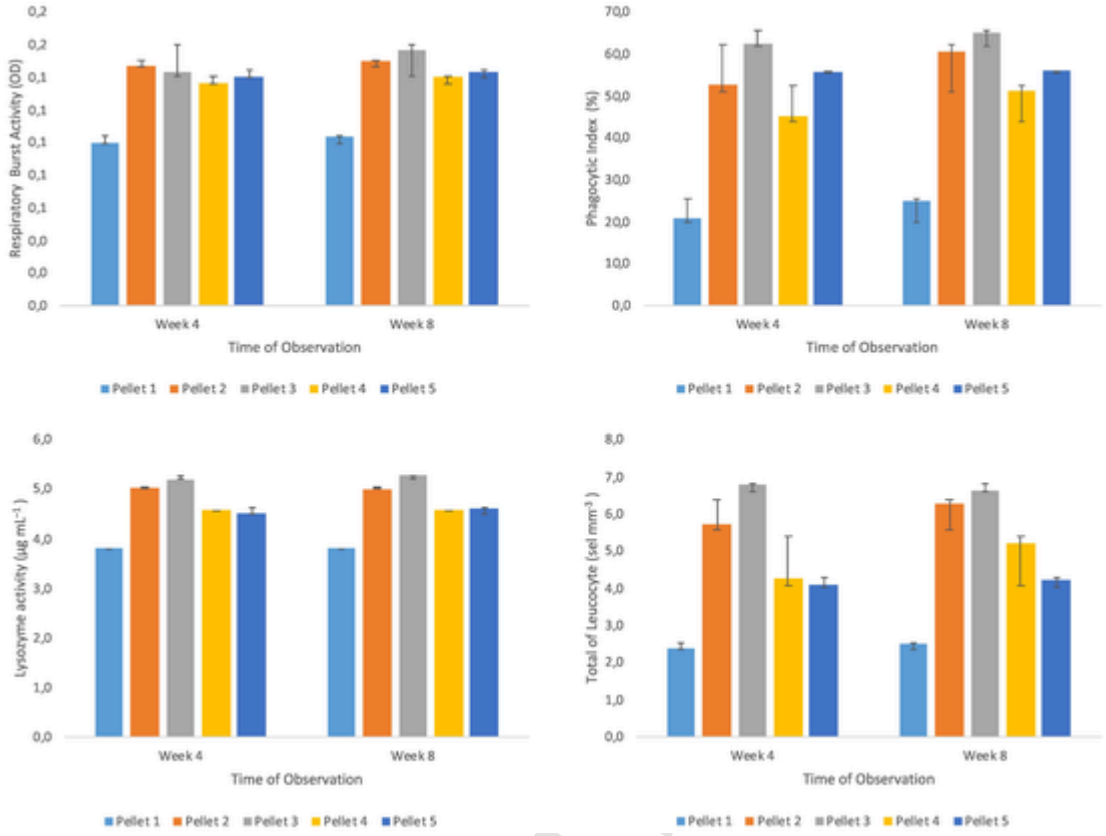

Fig. 2. Immunological parameters (RBA, IP, LA, TL) of catfish which treated by additional *Solanum ferox* extract (SFE) and *Lactobacillus casei* (LC) on the week 8.

3.3. Challenge test

Testing of catfish survival and mortality rates, as well as the RPS observed in week 12, were completed. Surprisingly, the results showed that the addition of SFE 2 g kg^{-1} and LC 10 g kg^{-1} feed functioned to protect against up to 100% of bacterial infections.

Generally, the addition of SFE and LC was able to increase fish resistance to bacterial infection by 64–100%, at a significant increase ($P < 0.05$) compared to using a single LC or feed control. However, the combination of 2 g kg^{-1} SFE and 10 g kg^{-1} of LC is the best feed formulation for disease protection in catfish.

3.4. Water quality

No significant difference was observed for water quality in the catfish culture media. Temperatures were recorded at 28 ± 0.95 °C and the DO was approximately 7.6 ± 0 mg L^{-1}, with pH ranging from 7.2 ± 0.5 and TAN at about 0.69 ± 0.22 mg L^{-1} throughout the experiment.

4. Discussion

The use of prebiotics and probiotics in fish cultivation has been developed to demonstrated effects, such as enhancing growth (Kathia et al., 2017; Rodrigues et al., 2018) maximizing the immune system (Lara-Flores et al., 2003), and reducing the number of pathogenic bacteria developed toward an antagonistic system (Gatesoupe, 1991; Gildberg and Mikkelsen, 1998; Li and Gatlin, 2005; Mohamed et al., 2007). The present study evaluated the effects of SFE and LC application in catfish feed on growth, immune system function, and protection from pathogenic bacteria.

The present study significantly demonstrated that the addition of SFE and LC in fish feed has a positive effect on fish growth and immune function. A similar result was obtained by Van Doan et al. (2019); their study involved giving orange peel-derived pectin and the probiotic *Lactobacillus plantarum* to Nile tilapia, which had a substantial impact on growth, skin mucus, and serum immune response. Further, the current findings emphasize the growth of catfish in week eight, especially for those catfish treated with SFE at concentrations of 2 and 6 g kg^{-1}. In this case, SFE did operate as a growth-stimulating additive in the cultivation mard of catfish. As with Beluga's fish feed (*Huso huso*), an additional (1–3%) dose of inulin (prebiotic) produced greater WG, SGR, energy retention, feed efficiency, and protein retention, as well as a higher protein efficiency ratio (Abd Elmonem et al., 2002; Okey et al., 2018). Mardliyah et al. (2018) found that 400 mg L^{-1} of SFE in LC culture was able to increase the number of LC bacteria (7.62 10^{14} CFU mL^{-1}) within 24 h. Furthermore, the oral administration of SFE mixed with LC (ratio 2:1) produced optimum growth in catfish, reaching 12 g for 30 days of culture. Similar findings were also obtained by Widanarni et al. (2014), who stated that carbohydrate (oligosaccharide) produced from steamed sweet potato flour could work as a prebiotic in white shrimp through the process of accelerating intestinal food absorption. Prebiotics from plant extract increase fish and shrimp weight by reducing the absorbance of glucose (Grundy et al., 2016) and postponing gastric emptiness (Schwartz et al., 1982; Ho et al., 2017; Naqash et al., 2017).

The present study also found that SFE was able to modulate the growth of intestinal microbiota such as LC bacteria. Catfish growth may result from modulation of gut microbiota by increasing the levels of beneficial bacterial populations and further improving digestive function. The presence of microbes in the intestine has a positive influence on the enzymes of food metabolism such as amylase, lipase, and protease (Eshaghzadeh et al., 2015; Hoseinifar et al., 2016), which can accelerate the process of digestion of fish feed.

Furthermore, the dietary application of mannoprotein from *Saccharomyces cerevisiae* yeast cell walls and L. *plantarum* has been shown to increase mucosal and serum immunity, disease resistance against

pathogens, and survival rates in fish and shrimp (Rodrigues et al., 2018; Van Doan et al., 2019). It can be inferred for the present research that the enhanced immune system and disease resistance observed in catfish was highly affected by the simultaneous application of SFE and LC.

Previous studies on the utilization of SFE have suggested that 400 and 600 mg L^{-1} could successfully enhance Nile tilapia's immune function and improve disease resistance against *A. hydrophila* and *P. fluorescens* bacterial infections (Hardi et al., 2017, 2018a, 2018b, 2018c). The essentials of flavonoid and levamisole significantly intensified monocyte and macrophage in the phagocitation antigen. Moreover, the increased leucocyte levels, which were boosted by SFE either singularly or in conjunction with another extract, consistently accelerated the pathogen elimination process inside the bodies of Nile tilapia (Hardi et al., 2018a, 2018b, 2018c, 2019). Furthermore, Chen et al. (2020) emphasized that additional probiotics compounding feed supplement successfully increased the resistance of grass carp fish (*Ctenoparyngodon idella*) to *A. hydrophila* bacterial infections.

Additionally, the concentrations of SFE and LC extract for inclusion in fish feed significantly increased the RPS value of catfish following the challenge test against *A. hydrophila* and *P. fluorescens* bacteria. The highest RPS value (100%) for this experiment was demonstrated by fish fed the pellet containing 2 mg L^{-1} SFE and 10 mg L^{-1} LC. This is related to immune system increases observed in the catfish pellet 2 group. TL, RBA, LA, and PI were increased in weeks two and four after feeding time, along with diseases resistance. The enhancement of the immune response caused adhesion and colonization of probiotics in fish intestines. This result is in accordance with the research of Ausubel (2005), who stated that the interaction between probiotic cells and immune systems are through microbe-associated molecular patterns (MAMPs). In this study, the SFE had a positive effect on L. *casei* probiotic growth, not only increasing immune system function but also simultaneously producing a synergistic effect on digestive health and thus overall fish health (Baik et al., 2015). Correspondingly, this demonstrated that dietary administration of SFE and LC in catfish not only enhanced growth but also improved non-specific immunity and resistance against *A. hydrophila* and *P. fluorescens* bacteria. SFE and LC extract are useful additions to fish feed for fish health management in catfish aquaculture.

5. Conclusion

The present study concluded that administration of a combination between SFE and LC probiotics in catfish feed significantly enhanced growth, feed efficiency, immunity, and disease resistance against *A. hydrophila* and *P. fluorescens* bacterial infection. Further study needs to be conducted to molecularly determine the effects of the SFE and LC probiotics combination on the immune system of the fish.

Uncited reference

Bernard et al., 2015

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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