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Feasibility of Probiotic *Lactobacillus casei* FNCC 0090 to Improve Growth and Immune Response of Catfish (*Clarias gariepinus*) toward *Aeromonas hydrophila* Infection

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ABSTRACT

Aeromonas hydrophila can cause motile Aeromonas septicemia, leading to high mortality in catfish. An effective alternative to treat this bacterial infection is administering the probiotic *Lactobacillus casei* FNCC 0090. However, proper fisheries management is crucial for long-term success. The aim of this research is to determine the effect of administering the probiotic *L. casei* FNCC 0090 on the growth parameters and immune system of catfish. 200 catfish were divided into 5 different treatments in 5 replicates. The treatments were KN (negative control: without probiotic and infected), KP (positive control: without probiotic and not infected), A (5% probiotic and infected), B (10% probiotic and infected), C (probiotic 15% and infected). The density of probiotics administered is 10^8 CFU/ml. The fish were kept for 42 days; on the 35th day, the fish were infected with 1 x 10^8 CFU/ml *A. hydrophila* intramuscularly. The parameters observed in this study are fish growth (specific growth rate, SGR; feed conversion ratio, FCR; and survival rate, SR) and the immune system (phagocytic activity and the amount of lysozyme enzyme). The data obtained were analyzed with SPSS One Way ANOVA. The results showed that the administration of 15% probiotic *L. casei* FNCC 0090 had a significant effect on the growth parameters and immune system.

1. Introduction

Catfish (*Clarias gariepinus*, Burchell 1882) is one of the most widely cultivated and consumed freshwater fish in Indonesia. The need for catfish will increase from year to year. Catfish are widely cultivated for the reason that the cultivation system is easy to adapt (Das *et al*. 2022), does not require high enough water quality (Rahman *et al*. 2022), fish can utilize the feed provided effectively, and can be cultivated on limited land and water sources (Setiadi *et al*. 2019). Constraints accompany the increasing need for catfish in catfish production, and many catfish are attacked by disease (Gobi *et al*. 2018). Diseases in cultivated fish generally arise when the cultivation conditions are unfavorable, such as decreased water quality, overfeeding, and too high fish density (Hassan *et al*. 2023).

One of the causes of disease in catfish is bacterial infection. One of the bacteria that causes pathogens in catfish and other freshwater fish is A*eromonas*

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hydrophila (Meidong *et al*. 2018). *A. hydrophila* can cause Motile Aeromonas Septicemia (MAS) disease, which infects the external body parts of fish and enters systemically into the fish's blood circulation (Paetman *et al*. 2018). This can lead to dermatitis, orbital cellulitis, and ocular rupture (Houseiny *et al*. 2023). Ultimately, the catfish will die (Silarudee *et al*. 2019). The problem of this bacterial infection in catfish can be overcome by administering synthetic antibiotics which administering antibiotics can cause the emergence of resistant pathogenic bacteria-strain pollute the environment and harm consumers (Juniora *et al*. 2019; Zhang *et al*. 2020).

Probiotics are live microbes in the host's gastrointestinal tract (Hamid *et al*. 2021). The addition of probiotics to fish feed can improve the balance of microbiota in the fish's digestive tract and inhibit the development of pathogens in the fish's intestinal tract (Tabassum *et al*. 2021; Malik *et al*. 2023). Using probiotics in aquaculture can also increase growth, improve food digestion and absorption of nutrients, enhance the immune system, modulate the balance of microbiota in the digestive tract, and improve water quality (Mondal *et al*. 2020; El Saadony *et al*. 2021). The microbial genus that often acts as a probiotic consists of *Lactobacillus*, *Bacillus*, *Enterococcus*, and *Saccharomyces* (Hamka *et al*. 2020). The effect of adding probiotics to fish feed can optimize the role of digestive enzymes or stimulate enzyme secretion so that the food digestion process becomes more optimal (Luo *et al*. 2022). Included in the bacteria that are proven to have a role as probiotics are *L. casei* (Aini and Hariani 2019; Wang *et al*. 2021).

L. casei bacteria are resistant to bile salts and low pH, so they can live in the intestine and stomach organs (Siddik *et al*. 2022). In addition, *L. casei* bacteria have antimicrobial activity and are able to enhance the fish's immune system (Song *et al*. 2023). The aim of this research is to determine the effect of administering the probiotic *L. casei* FNCC 0090 with various concentrations on the growth and immune system of catfish infected with *A. hydrophila*.

2. Materials and Methods

2.1. Ethical Statement

The study was conducted according to the Ethical Commission, Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia No.: 2.KE.163.09.

2.2. Probiotics and Diet Preparation

Reculture of *L. casei* FNCC 0090 bacteria using De Man Regosa Broth, MRSB media and *A. hydrophila* bacteria using Nutrien Broth, NB media. Bacterial isolates of *L. casei* were obtained from the Center for Food and Nutrition Studies, Gadjah Mada University, while *A. hydrophila* were obtained from the Center for Brackish Water Fisheries Culture, Jepara, Central Java. The bacterial cultures were incubated for ± 48 hours at 35^{o}C and 30^{o}C, respectively, until a cell density of at least 108 CFU/ml was obtained. The results of bacterial reculture were measured using a spectrophotometer to see the relationship between turbidity and the number of bacteria by adjusting the length of incubation time with the OD value on the standard curve that has been made, as shown in Figure 1. The re-cultured bacteria were centrifuged at 5000 rpm for 5 minutes. Then, the pellet formed was separated from the supernatant. The pellet was then diluted with physiological NaCl solution to obtain bacterial suspension with an OD value equivalent to a cell density of 108 CFU/ml. The feed used in this study was a commercial feed containing 33% protein; before being given to the fish, the pellets were placed in a basin and sprayed using probiotics according to the treatment (0.5%, 10%, and 15%). The addition of probiotics was done by spraying using a spray bottle. The feed that has been sprayed is then homogenized and allowed to stand for 30 minutes in a closed container.

2.3. Fish and Feeding Trial

The catfish used is 25-30 cm long with an average weight of 150-180 grams. Fish were placed in an aquarium for 5x24 hours for acclimation. During acclimation, the fish were fed Hi-Pro-vite 1000 commercial feed produced by PT Central Proteina Prima, Tbk (33% protein). After acclimation, each aquarium is filled with 8 fish taken randomly. Fish are fed with an amount of food equal to 3% of body weight twice a day at 6 AM and 6 PM. Water changes are carried out every 3 days by taking ±30% water. Fish kept for 42 days. On the 35th day of rearing, catfish were infected with *A. hydrophila* as much as $1x10^8$ CFU/ml by intraperitoneal injection as much 0.1 ml.

2.4. Growth Performance Parameters

Catfish biomass was measured once a week. The weight of the catfish is known from the difference in weight between the container without catfish and the container with catfish. Growth parameters such as

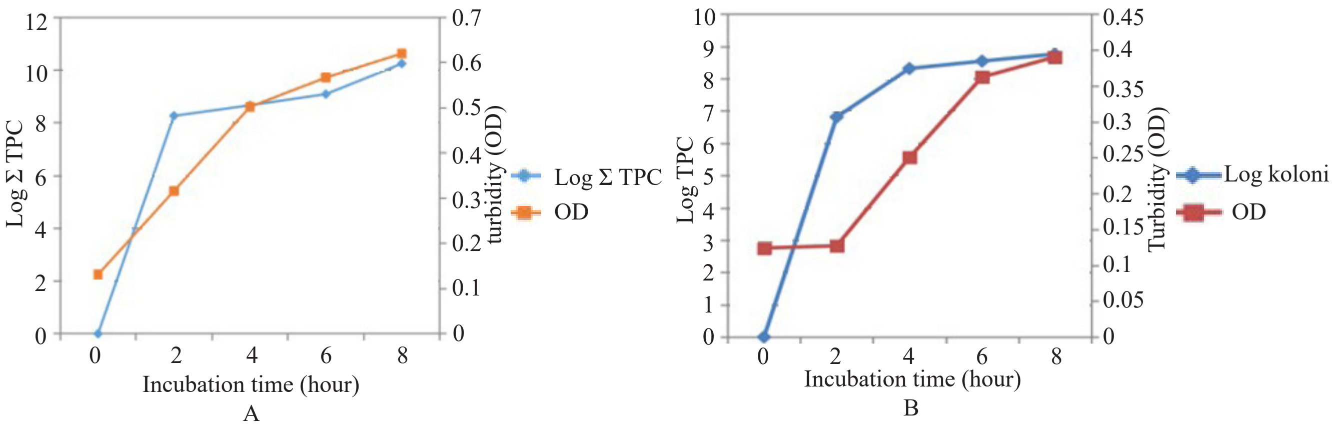

Figure 1. Graph of the relationship between *L. casei* FNCC 0090 (A) *A. hydrophila* (B) growth and turbidity (OD) at different incubation times

initial body weight (IBW), final body weight (FBW), percent weight gain (PWG), average daily gain (ADG), specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR) were calculated using the following formula:

PWG (%) = 100 × (FBW - IBW)/IBW
SGR (%/days) = 100 × (ln FBW - ln IBW)/days
ADG = (FBW-IBW)/rearing period
FCR = Dry feed consumed/weight gain
SR (%) = 100 × (final fish number/initial fish number)

2.5. Immune Response

Catfish were infected with *A. hydrophila* 0.1×10^8 CFU/ml intraperitoneally. After one hour, peritoneal fluid was obtained from the peritoneal cavity using a syringe. The preparation of phagocytes was carried out by smearing intraperitoneal fluid on glass objects and drying them. Apply fixative with methanol for five minutes. Apply Giemsa stain for 20 minutes and rinse with distilled water. Then observed under a light microscope (Aini *et al*. 2024). Phagocytic activity was determined by comparing the number of macrophages actively carrying out the phagocytosis process with 100 macrophages. Active macrophages were observed to be lighter in color, larger in size, and visible phagocytic bacterial cells in the macrophages. Lysozyme levels were calculated according to the protocol contained in the Fish Elisa Lysozyme Kit (Sigma Aldrich).

2.6. Statistical Analysis

The results obtained were then analyzed using the SPSS 16 application using ANOVA, and the Duncan test was continued to determine the most effective probiotic concentration in increasing the growth and immune system of fish. Results are presented as means ± SD ($p<0.05$).

3. Results

3.1. Growth Performance

The initial average weight (IBW) of the catfish used in the study ranged from 158.70 g to 167.86 g. At the end of the study, the weight of the fish was measured, and the final average weight was obtained between 187.27 g - and 213.08 g. During the 42 days of the study, the fish experienced weight gain represented in ADG values. During the 42 days of the study, the fish experienced weight gain represented in the ADG value. The lowest ADG value was found in the positive control treatment KP, which amounted to 0.62 g/fish/day. While the highest ADG value was treatment C, which amounted to 1.39 g/fish/day. This treatment is significantly different from the control treatment. Other growth parameters in the form of PWG showed that treatment C produced the highest PWG value of 29.53% and was significantly different from the control treatment, both positive control and negative control. The average SGR, FCR, and SR of catfish after being given the probiotic *L. casei* FNCC 0090 is shown in Table 1. Based on Table 1, it is known that the KP treatment caused the lowest SGR value, namely 0.456±0.13. For treatment C, the SGR value was the highest compared to all treatments, namely 0.790±0.16. Based on Table 1, it can be seen that there were no significantly different treatments for FCR parameters. Even though it didn't look significantly different, treatment C with 15% probiotics was a better treatment compared to the controls and other treatments. The average survival rate (SR) of catfish after being given probiotics *L. casei* FNCC 0090 and pathogenic bacteria *A. hydrophila* is presented in Table

1. Based on Table 1, KP treatment has a low SR value, namely 87.5±8.84%. For treatment C, the SR value was the highest compared to all treatments, 100.0±0.00%. The average weekly body weight of catfish given various concentrations of probiotic *L. casei* FNCC 0090 and infected with *A. hydrophila* during the rearing period is shown in Figure 4. The results of the study showed that with the provision of probiotics, the average increase in fish weight was higher compared to the treatment without probiotics

3.2. Immune Response

The average phagocytic activity of catfish that had been given probiotics and exposed to *A. hydrophila* is presented in Figure 2. Based on Figure 2, it can be seen that the group without probiotics (KN) had the lowest phagocytosis activity, namely 42±7.12%. The treatment with the highest average phagocytic activity (60.3±5.38%) was treatment C, which was 15% probiotics.

Based on Figure 3, it can be observed that treatment group C, with 15% probiotic administration, significantly increases fish lysozyme levels ($p<0.05$).

The SGR level of catfish is related to the weight gain of catfish during the rearing period, as shown in Table 1.

4. Discussion

Fish is an organism that lives in an aquatic environment that requires the fish's body to deal directly with various microbes (Shawky *et al*. 2023). Fish survival depends on the immune response to defend against pathogen attack (Hardi *et al*. 2022). The immune system will protect against infection by bacteria through a layered defense. Non-specific

Table 1. Effect of probiotics on growth performance

Treatment group	Growth performance						
	IBW (g)	FBW (g)	ADG (g/fish/day)	PWG (%)	SGR (%/day)	FCR	SR (5)
KN	161.72	202.86	1.2±0.42^{a}	25.44 ±9.91^{ab}	0.456±0.13^{a}	0.83±0.44^{a}	97.5±5.59^{a}
KP	165.48	187.27	0.62±0.41^{ab}	13.16±9.41^{a}	0.774±0.20^{b}	0.64±0.14^{a}	87.5±8.84^{a}
A	158.70	207.00	1.08±0.36^{ab}	20.44± 8.47^{ab}	0.684±0.18^{ab}	0.57±0.13^{a}	92.5±6.85^{ab}
B	167.86	206.48	1.10±0.38^{ab}	23.01±7.82^{ab}	0.38±0.43^{ab}	0.58±0.16^{a}	97.5±5.59^{b}
C	164.50	213.08	1.39±0.35^{c}	29.53± 6.90^{c}	0.790±0.16^{bc}	0.51±0.11^{a}	100.0±0.00^{b}

* Value with different shoulder letters indicates a significant difference ($p<0.05$)

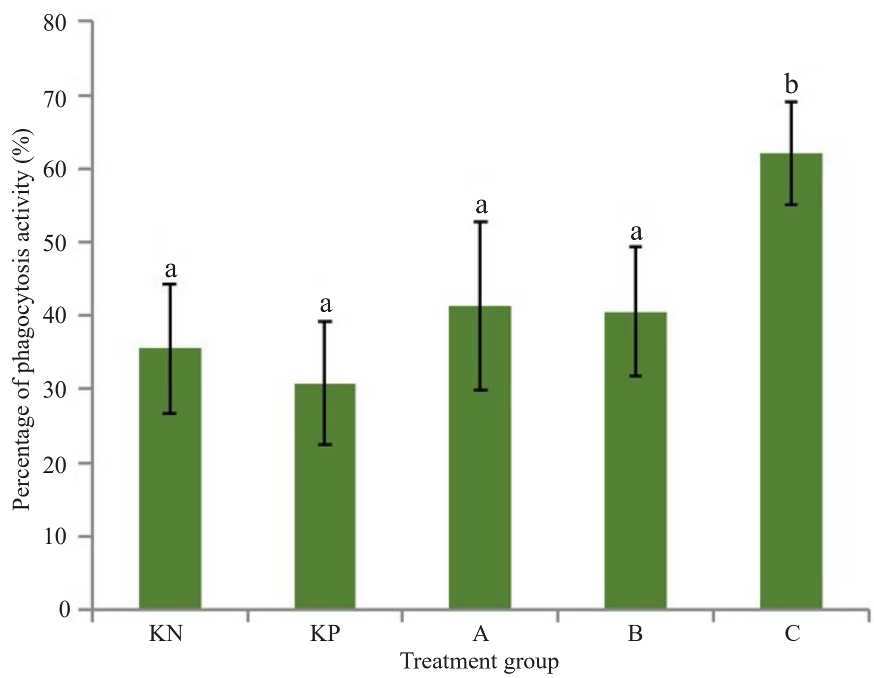

Figure 2. Diagram of the average phagocytic activity of catfish between treatment groups after administration of probiotic *L. casei* FNCC 0090 and exposure to *A. hydrophila*. Description: KN: negative control; KP: positive control; A: probiotics 5%; B: probiotics 10%; C: 15% probiotics. Different letters indicate significant differences between treatment groups

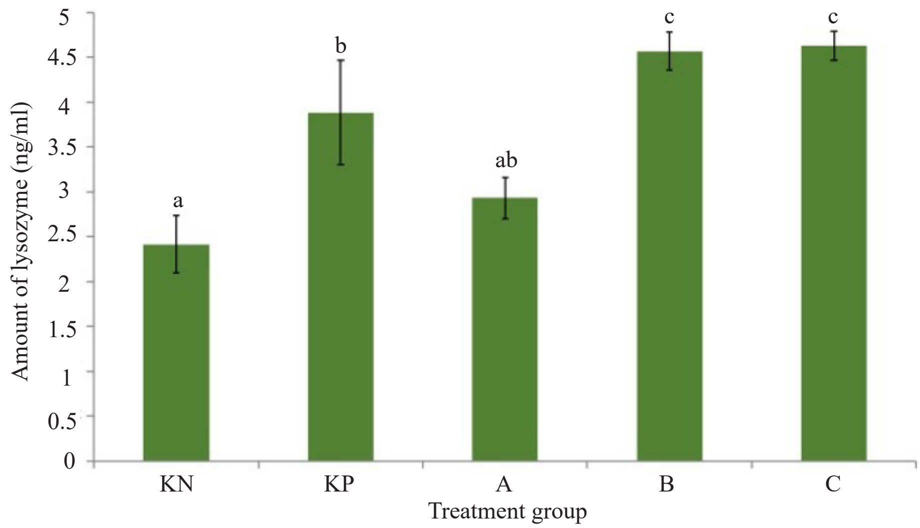

Figure 3. Diagram of average catfish lysozyme levels between treatment groups after administration of probiotic *L. casei* FNCC 0090 and exposure to *A. hydrophila*. Description: KN: negative control; KP: positive control; A: probiotics 5%; B: probiotics 10%; C: 15% probiotics. Different letters indicate significant differences between treatment groups

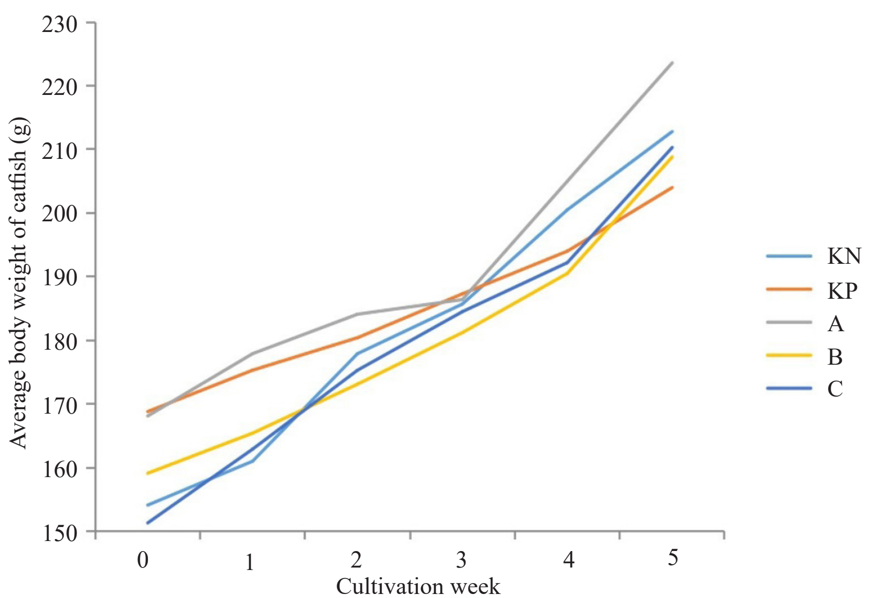

Figure 4. The average weekly body weight of catfish that have been given various concentrations of probiotic *L. casei* FNCC 0090 and infected with *A. hydrophila* during the rearing period

immunity is the first defense that responds quickly to microbes without distinguishing certain microbial groups (Aini *et al*. 2020). The fish immune system includes 3 types they are physical barriers, cellular, and humoral components (Chen *et al*. 2020). Microbes that enter the body must be able to pass through external barriers formed by the skin and mucous membranes, which are physical barriers. If the pathogenic microbe succeeds in penetrating, then the pathogen must face a second non-specific defense, namely the humoral defense. The humoral immune system includes peptides, lysozyme, complement, transferrin, pentraxin lectins, and antiproteases.

The immune system's cellular components are neutrophils and macrophages, which play a role in phagocytic pathogens, Natural Killer Cells (NKC), mast cells, and basophil cells (El Kady *et al*. 2022). Based on the results, it is known that the administration of probiotic *L. casei* FNCC 0090 showed a difference in phagocytosis activity in the control and treatment treatments. The control treatment group, both positive control (KP: without infection and without probiotics) and negative control (KN: infected with the disease and not given probiotics), were catfish without probiotics having a lower percentage of phagocytosis activity when compared to group C with 15% administration probiotic *L. casei* FNCC 0090. Treatment C was the best treatment for increasing phagocytosis activity. This is because the more probiotics there are, the more bacteria interact with enterocytes, which will activate macrophages. An enterocyte is a cell in the small and large intestines that absorbs nutrients and defends against microbial invasion. Active macrophages will be more responsive when facing pathogenic bacteria that are recognized as having antigens and secreting toxins. There was an increase in the percentage of phagocytosis activity by administering probiotics using the *Bacillus aureus* B81e bacteria in catfish infected with the pathogen *A. hydrophila* (Meidong *et al*. 2018).

Phagocytes are able to recognize pathogens, destroy them, and kill them through the process of phagocytosis. The mechanism used by phagocytic cells during the phagocytosis process is that pathogenic bacteria will be destroyed by the role of lysozyme secreted by neutrophils or macrophages. Another mechanism is that phagocytic cells produce reactive oxygen species (ROS): superoxide anions (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl free radicals (OH-) as respiratory burst activities that can damage pathogenic bacteria (Chen *et al*. 2020).

Fish food contains complete and complex nutrients. As much as 5% crude fiber and 13% ash are carbon sources that support the growth of probiotic bacteria. *L.casei* FNCC 0090 is able to convert sugar into lactic acid through a fermentation process. Other fermentation products are acetic acid and carbon dioxide in small amounts. The presence of lactic acid as a result of metabolism causes the catfish's digestive tract to have a lower pH. This condition will benefit the host because probiotic bacteria play a role in inhibiting the growth of pathogenic bacteria and putrefactive bacteria due to low pH. Low pH will also increase the secretion of proteolytic enzymes in the intestines to optimize protein metabolism (Aini *et al*. 2020).

The presence of proteolytic and cellulolytic enzymes released by probiotics has a role in facilitating the process of digesting food. Optimal enzyme secretion stimulated by the presence of probiotics causes catfish to have a low feed conversion ratio (FCR). If the feed conversion value is low, the feed efficiency value will be better, and vice versa. Feed conversion describes the level of feed utilization efficiency achieved. Feed conversion shows how much feed is converted into fish body biomass (Sumon *et al*. 2022).

In this study, the lowest FCR value was found in the treatment with the addition of 15% probiotics. Even though there was no significant difference between treatments A, B, and C, treatment C had a lower FCR value compared to the control treatment. The insignificant difference between the treatments was most likely due to the long duration of probiotic administration. Another reason is some probiotic bacteria simply pass through the fish gut without colonizing or adhering to the intestinal mucosa, thereby failing to provide optimal functionality. A low FCR value is proof that fish feed is used optimally for metabolism and growth. A low FCR value also indicates that the feed is used by fish efficiently and is categorized as good quality feed. This kind of feed will cause the fish growth rate to be faster, which is marked by the increase in fish body weight.

The survival of catfish between one feed treatment and another has a fairly fluctuating value, but in general, the survival of fish increases with the addition of probiotic concentrations. The improved survival of catfish is thought to be due to the role of the probiotic *L. casei* FNCC 0090, which was added to the feed. Feed containing probiotics that enter the fish's digestive tract is beneficial for the host. This is because the presence of probiotics can improve the balance of microorganisms in the catfish digestive tract (Wang *et al*. 2021). The balance of microorganisms in the fish's digestive tract is a good sign because it has a good impact on health by improving the fish's immune system (Sequeiros *et al*. 2022). A good immune system increases the survival value of fish.

On the other hand, the presence of probiotics in the fish digestive tract can inhibit the development and growth of pathogenic microorganisms (Safari *et al*. 2022). Probiotics will grow and develop in the fish's intestinal walls and then form colonies along the intestinal walls. Probiotic colonies attached to the fish's intestinal walls will release antibacterial substances. This substance is antagonistic to pathogens, so it is able to suppress pathogenic growth. This causes the catfish's intestines to become healthier (Zhang *et al*. 2020). Healthy digestive tract conditions will support optimal absorption of nutrients in feed while increasing the survival rate of catfish (Hien *et al*. 2021).

The novelty of this research is that there has been no research using *L. casei* FNCC 0090 to be applied to catfish, both in infected and non-infected catfish with the pathogen *A. hydrophila*. *L. casei* FNCC 0090 isolate is a pure culture obtained from cheese, however, this study shows that the bacterial isolate obtained can be applied to the field of aquaculture, especially in the process of catfish cultivation. The results also showed that the addition of *L. casei* FNCC 0090 can increase catfish growth, as seen from the parameters of SGR, FCR, SR, ADG, and PWG. In addition, the addition of *L. casei* FNCC 0090 can also improve the immune system of catfish, as seen from lysozyme enzyme levels phagocytosis activity.

The advantage and contribution of this research for science is to utilize *L. casei* FNCC 0090 bacteria as probiotic agents that can replace antibiotics in the process of catfish farming, considering the use of antibiotics has a good effect on catfish, consumers, and the environment. In addition, through this research, this author informs that probiotic bacteria successfully isolated from cheese can be applied in the field of aquaculture and have tremendous potential to be further developed in the future.

In conclusion, Probiotic *L. casei* FNCC 0090 shows promising potential for an immune response (lysozyme levels and phagocytic activity) in catfish infected with *A. hydrophila*. The addition of 15% probiotics is the best treatment to improve the fish's immune system. However, administration of probiotics had no significant effect on the growth, feed conversion, and survival of catfish infected with *A. hydrophila* but had a significant effect on ADG and PWG parameters, with the best treatment being treatment C with 15% probiotic concentration.

Suggestions for further research are the need to isolate and identify bacteria that grow dominantly in the intestines of catfish to determine their potential as natural probiotic agents so that they can be developed and re-given to catfish to be able to play a role in improving the immune system and growth of catfish. In addition, it is necessary to conduct research related to the potential of *L. casei* FNCC 0090 for other freshwater fish farming.

Author Contribution

NA, SPA,: Conception and design of the study, drafted and revised the manuscript. NA, MB: Collected data. NA, SPA, F, SA, HDKD: analysis and interpretation data. All authors have read, reviewed and approved the final manuscript.

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