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Immunomodulatory effects of *Carica papaya* leaf powder in the diet of *Labeo catla* challenged with *Aeromonas hydrophila*

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Abstract

The present study evaluated the immunomodulatory effects of dietary *Carica papaya* leaf powder on the immune response and disease resistance of *Labeo catla* fry challenged with *Aeromonas hydrophila*. A 60-day feeding experiment was conducted using five dietary treatments containing different inclusion levels of papaya leaf powder: T₀ (control), T₁ (2.5%), T₂ (5%), T₃ (7.5%), and T₄ (10%). Fish were stocked in twenty experimental tanks and fed diets containing approximately 30% crude protein. At the end of the feeding trial, immune response was assessed using the disc diffusion method by measuring the zone of inhibition against *A. hydrophila* at 24, 48, and 72 hours. The results indicated that antibacterial activity increased with higher inclusion levels of papaya leaf powder, with the highest inhibition zone recorded in T₄ (14.28 ± 0.36 mm) after 72 hours. A bacterial challenge test was subsequently performed by intramuscular injection of *A. hydrophila* (1 × 10⁵ CFU/mL), and fish survival was monitored for 10 days. The highest survival rate was observed in T₄ (95.00 ± 5.77%), followed by T₃ (80.00 ± 8.16%), T₂ (67.50 ± 9.57%), T₁ (55.00 ± 12.91%), and T₀ (52.50 ± 5.00%). Water quality parameters remained within suitable ranges throughout the experiment. The findings indicate that dietary supplementation with papaya leaf powder, particularly at 7.5-10%, enhances immune response and improves resistance against bacterial infection in *Labeo catla* fry. Therefore, papaya leaf powder can be considered a promising natural immunostimulant for sustainable aquaculture practices.

Keywords: *Carica papaya* leaf powder, *Labeo catla*, *Aeromonas hydrophila*, Immunomodulation, Disease resistance, Aquaculture nutrition

Introduction

Aquaculture plays a crucial role in global food security and economic development. According to FAO reports, the combined global production of fisheries and aquaculture reached approximately 223.2 million tonnes in 2022, with aquaculture alone contributing about 130.9 million tonnes (FAO, 2024). With the world population projected to reach nearly 10 billion by 2050, the demand for high-quality protein sources is expected to rise substantially. In this context, aquaculture has significant potential to expand aquatic food production and meet the increasing nutritional requirements of a growing and more affluent population. Consequently, aquaculture is particularly important for strengthening food security, especially in low-income and food-deficient regions (Khan *et al.*, 2023; Hassan *et al.*, 2025) [18, 16]. Over the past few decades, aquaculture production has grown rapidly, and continued expansion is considered essential to satisfy future food demands (Rajabiesterabadi *et al.*, 2020; Mabrouk *et al.*, 2025) [29, 20].

The intensification of aquaculture systems—characterized by high stocking densities, monoculture practices, and confined production environments—often creates favorable conditions for the rapid transmission of pathogens. Stress factors such as overcrowding, poor water quality, and inadequate nutrition can weaken the immune responses of fish, increasing their susceptibility to infectious diseases (Awad & Awaad, 2017; Quesada *et al.*, 2013; Reverter *et al.*, 2014) [4, 27, 30]. Despite significant progress in the farming of Indian major carps, several environmental and economic challenges continue to threaten the sustainability of the sector (Barrett *et al.*, 2019) [7].

In many Asian aquaculture systems, diseases frequently arise from primary or opportunistic pathogens that proliferate under stressful environmental conditions (Lategan & Gibson, 2003; Fang *et al.*, 2004) [9, 14]. Traditionally, antibiotics and synthetic chemicals have been used to prevent and control disease outbreaks. However, their extensive application has led to the emergence of antimicrobial resistance and the accumulation of chemical residues in fish tissues, raising concerns regarding food safety and public health (Okocha *et al.*, 2018; Imtiaz *et al.*, 2024) [23, 17].

Among the bacterial pathogens affecting aquaculture, *Aeromonas hydrophila* is a facultatively anaerobic, opportunistic, rod-shaped, non-spore-forming bacterium belonging to the family Aeromonadaceae. It is widely distributed in aquatic environments and is capable of infecting a broad range of hosts, including freshwater and marine organisms. This bacterium has been extensively investigated due to its occurrence in water, food, and estuarine environments, as well as its antibiotic resistance and pathogenic potential (Odeyemi *et al.*, 2012; Radu *et al.*, 2003) [22, 28]. Infections caused by *A. hydrophila* are responsible for severe diseases in fish, including dropsy, epizootic ulcerative syndrome (EUS), and ocular lesions (Roque *et al.*, 2009) [31].

Consequently, there is increasing interest in alternative strategies that can enhance disease resistance while reducing reliance on antibiotics and improving feed utilization efficiency (Du *et al.*, 2022) [11]. Various natural and biological approaches, including probiotics, plant-derived compounds, prebiotics, and synbiotics, have shown promising results in aquaculture due to their environmental safety, sustainability, and cost-effectiveness (Mabrouk *et al.*, 2025; Aboleila *et al.*, 2022) [20, 1]. Medicinal plants, in particular, are considered valuable due to their availability, long-term safety, and therapeutic efficacy (Yilmaz *et al.*, 2024). More than 60 plant species have been evaluated for their ability to enhance fish health and control diseases in aquaculture systems (Bulfon *et al.*, 2015) [8].

Carica papaya L. (papaya), a member of the family Caricaceae, is widely cultivated throughout Asia. Numerous investigations have explored the biological activities of different parts of the plant, including fruits, leaves, seeds, shoots, roots, and latex (Otsuki *et al.*, 2010) [25]. Papaya leaves, often regarded as agricultural by-products, contain a variety of bioactive compounds with significant pharmacological potential. These leaves exhibit immunomodulatory (Alhassan *et al.*, 2024) [3], antioxidant (Sharma *et al.*, 2022) [3], and anti-inflammatory (Cao *et al.*, 2024) properties due to the presence of phenolic compounds (Espadas *et al.*, 2019) [12], flavonoids (Nugroho *et al.*, 2017) [21], and enzymes such as papain (Babalola *et al.*, 2023) [5]. Papain is a cysteine protease capable of breaking down complex proteins into smaller peptides and amino acids,

thereby facilitating digestion. Additionally, papaya leaves contain several bioactive constituents, including alkaloids, flavonoids, phenolic acids, and papain, which collectively contribute to their broad therapeutic potential (Palanisamy & Basalingappa, 2020) [26]. These compounds have also been reported to enhance antioxidant activity and reduce lipid peroxidation in biological systems (Otsuki *et al.*, 2010) [25].

Therefore, the present study aimed to evaluate the effect of dietary supplementation with *Carica papaya* leaf powder on the immune response of *Labeo catla* when challenged with *Aeromonas hydrophila*.

Materials and Methods

Experimental Design and Fish

The feeding trial was conducted for a period of 60 days using twenty experimental tanks, each supplied with adequate aeration to maintain appropriate dissolved oxygen levels. Prior to the experiment, all tanks were thoroughly washed and disinfected with potassium permanganate (KMnO₄) to ensure hygienic conditions. After disinfection, each tank was filled with approximately 40 L of filtered and disinfected freshwater.

Fry of *Labeo catla* were obtained from Aqua-Fish Farm, Nal Sarovar (Nani Kathechi, Gujarat). The fry were transported to the laboratory in oxygenated polyethylene bags by road. Upon arrival, the fish were transferred to 500-L FRP tanks and acclimatized under continuous aeration. During acclimation, the fry were fed three times daily to facilitate adjustment to the new environment. Healthy individuals with an average body weight of 0.381 ± 0.001 g were selected for the feeding experiment.

Five dietary treatments were prepared for the study: T₀ (control diet without *Carica papaya* leaf powder), T₁ (2.5%), T₂ (5%), T₃ (7.5%), and T₄ (10%) inclusion levels of dried papaya leaf powder in the formulated diets.

Collection of *Carica papaya* Leaves and Preparation of Leaf Powder

Leaves of *Carica papaya* were collected from a nearby farm and washed thoroughly with tap water to remove dust and impurities. The cleaned leaves were sun-dried for 3-4 days until completely dehydrated. The dried leaves were then ground into a fine powder using a grinder. The powdered material was sieved to obtain a uniform texture and subsequently stored in airtight polyethylene bags to preserve its quality until use.

Ingredient Proximate Analysis

Proximate composition of the feed ingredients was determined using standard analytical procedures described by AOAC (2000). The proximate composition of the ingredients is presented in Table 1.

Table 1: Proximate composition of ingredients

Ingredients (%)	Crude protein (%)	Crude fat (%)	Moisture (%)	Ash (%)
Papaya Leaf Powder	11.67	2.51	7.35	5.98
Fishmeal	58.34	10.39	7.63	21.68
GNOC	40.45	7.50	11.72	4.51
Wheat Flour	10.67	0.74	5.95	1.15
Tapioca Flour	0.89	10.23	7.63	0.25

Diet Preparation

Experimental diets were formulated using the Pearson square method. Five diets (T₀-T₄) were prepared, where treatments T₁, T₂, T₃, and T₄ contained varying levels of papaya leaf powder, while T₀ served as the control diet without papaya leaf powder. The required quantities of feed ingredients were weighed accurately according to the formulation described in Tables 2 and 3.

All ingredients were thoroughly mixed with an appropriate amount of water in a plastic tray to obtain a homogeneous mixture. The feed mixture was then subjected to steam

cooking at 121 °C under a pressure of 15 lbs for 15 minutes. After steaming, vitamin and mineral premixes were incorporated into the mixture and mixed thoroughly. Subsequently, papaya leaf powder was added to the diets according to the treatment levels and mixed uniformly.

The prepared feed mixture was pelletized using a mechanical pelletizer to produce pellets of uniform size and shape. The pellets were spread evenly on steel trays and sun-dried for 2-3 days until the moisture content was reduced to below 12%. The dried pellets were then stored in properly labelled airtight plastic containers until use.

Table 2: Treatment Details

Experimental ingredient: Papaya (<i>Carica papaya</i>) leaf powder					
Treatment group	T ₀	T ₁	T ₂	T ₃	T ₄
	Control	2.5%	5%	7.5%	10%
	Grams of herbal products per 100 grams of feed				
	0.0	2.5	5.0	7.5	10.0

Table 3: Composition of Experimental Diet

Ingredients	Diet (30%)				
	T ₀	T ₁	T ₂	T ₃	T ₄
Papaya leaf powder	0.0	2.5	5.0	7.5	10.0
Sterilized fishmeal	27.2	26.6	26.0	25.0	24.0
GNOC	27.0	26.0	25.0	25.0	24.4
Wheat flour	27.0	26.0	25.0	25.0	24.4
Tapioca flour	13.8	12.9	12.0	13.0	11.6
Plant oil	2.0	2.0	2.0	2.0	2.0
Fish oil	2.0	2.0	2.0	2.0	2.0
Vitamin & minerals	2.0	2.0	2.0	2.0	2.0
Total	100.0	100.0	100.0	100.0	100.0

Experimental Tank Management

Stocking

Labeo catla fry were stocked in the twenty experimental tanks at a density of 20 individuals per tank.

Feeding

Fish were fed twice daily at 08:00 h and 17:00 h throughout the experimental period. Feed was provided at a rate of 10% of the fish body weight. The formulated diets contained approximately 30% crude protein. The feed quantity was adjusted every two weeks based on the growth performance of the fish to maintain an appropriate feeding regime.

Water Quality Management

Continuous aeration was maintained throughout the experiment to ensure adequate dissolved oxygen levels. The tanks were cleaned daily before feeding by siphoning to remove uneaten feed and accumulated waste materials. Approximately 10% of the tank water was replaced regularly with filtered and disinfected freshwater during the 60-day experimental period.

Minimum Inhibitory Concentration Test

At the end of the feeding trial, the immune response of *Labeo catla* fry fed papaya leaf powder was evaluated using *Aeromonas hydrophila* as the test pathogen. Bacterial isolates were first cultured in liquid medium and subsequently transferred onto nutrient agar plates. Blood samples were collected from the caudal peduncle of fish using a 1-mL syringe containing EDTA as an anticoagulant. The samples were transferred into Eppendorf tubes and centrifuged at 5000 rpm for 5 minutes.

Sterile paper discs were immersed in the supernatant for 15 minutes to ensure proper absorption. These discs were then placed on nutrient agar plates inoculated with *Aeromonas hydrophila* using sterile forceps. The plates were incubated at 35 °C for 72 hours. Zones of inhibition around the discs were measured and recorded after 24, 48, and 72 hours.

Effect of Papaya Leaf Powder on Disease Resistance of *Labeo catla* Fry

At the end of the feeding trial, a bacterial challenge test was conducted. Fish from each treatment group were injected intramuscularly with 0.1 mL of *Aeromonas hydrophila* suspension at a concentration of 1×10^5 CFU/mL. The control group was injected with phosphate buffer solution (PBS). Ten fish from each replicate were selected for the challenge test.

Following infection, fish survival was monitored for 10 days. During this period, fish were observed for clinical signs of disease and abnormal behaviour. Due to the small size of the fry, the commonly used intraperitoneal injection method was not feasible; therefore, the bacterial challenge was administered via intramuscular injection.

Statistical Analysis

The experimental data were analyzed using standard statistical procedures described by Snedecor and Cochran (1994)^[35].

Results and Discussion

1. Minimum Inhibitory Concentration (MIC) Test

The immune response of *Labeo catla* fry under different dietary treatments was assessed using the disc diffusion

method, and the results are presented in Table 4. Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition at 24, 48, and 72 hours. The observations revealed significant variations among the treatments at all sampling intervals. Furthermore, Figure 1 illustrates the differences in inhibition zones among the treatments after 48 hours of incubation.

After 24 hours, the highest zone of inhibition was recorded in treatment T₄ (12.60 ± 0.24 mm), followed by T₃ (12.15 ± 0.21 mm), T₂ (11.58 ± 0.26 mm), and T₁ (11.30 ± 0.16 mm), while the lowest value was observed in the control group T₀ (10.95 ± 0.13 mm). At 48 hours, a similar trend was

recorded, with T₄ exhibiting the largest inhibition zone (13.65 ± 0.52 mm), followed by T₃ (13.25 ± 0.47 mm), T₂ (12.33 ± 0.29 mm), T₁ (11.95 ± 0.37 mm), and T₀ (11.23 ± 0.10 mm).

After 72 hours, the inhibition zone further increased across treatments, with T₄ showing the highest value (14.28 ± 0.36 mm), followed by T₃ (13.48 ± 0.36 mm), T₂ (12.65 ± 0.50 mm), T₁ (12.08 ± 0.79 mm), and T₀ (11.35 ± 0.13 mm). Overall, the results clearly indicated that treatment T₄ produced the strongest antibacterial response, followed by T₃, T₂, T₁, and T₀.

Table 4: Immune response of *Labeo catla* fry recorded in different treatments after 60 days

24 Hours					
	T ₀ (mm)	T ₁ (mm)	T ₂ (mm)	T ₃ (mm)	T ₄ (mm)
Mean	10.95 ± 0.13 ^d	11.3 ± 0.16 ^c	11.58 ± 0.26 ^c	12.15 ± 0.21 ^b	12.60 ± 0.24 ^a
48 Hours					
	T ₀ (mm)	T ₁ (mm)	T ₂ (mm)	T ₃ (mm)	T ₄ (mm)
Mean	11.23 ± 0.10 ^c	11.95 ± 0.37 ^b	12.33 ± 0.29 ^b	13.25 ± 0.47 ^a	13.65 ± 0.52 ^a
72 Hours					
	T ₀ (mm)	T ₁ (mm)	T ₂ (mm)	T ₃ (mm)	T ₄ (mm)
Mean	11.35 ± 0.13 ^d	12.08 ± 0.79 ^c	12.65 ± 0.50 ^c	13.48 ± 0.36 ^b	14.28 ± 0.36 ^a

The progressive increase in inhibition zones over time indicates an enhancement in immune response due to the dietary inclusion of papaya leaf powder. In the present study, the incorporation of papaya leaf powder at 7.5% and 10% levels significantly improved the antibacterial activity of fish serum against *Aeromonas hydrophila*.

These findings are consistent with the results reported by Ballad *et al.* (2011)^[6], who observed that papaya leaf extract at a concentration of 10 mg/ml produced an inhibition zone of 14.33 mm, indicating strong antibacterial activity. Similarly, Fakoya *et al.* (2019)^[13] demonstrated that methanolic and ethanolic extracts of papaya leaves produced

significant inhibition zones against *A. hydrophila* using the disc diffusion technique.

The enhanced antibacterial activity observed in the present study may be attributed to the presence of bioactive phytochemicals in papaya leaves, including saponins, alkaloids, tannins, glycosides, and phenolic compounds, which are known for their antimicrobial, antibacterial, and antipathogenic properties (Doughari *et al.*, 2007; Sales *et al.*, 2013; Verma *et al.*, 2013)^[10, 32, 36]. Therefore, dietary supplementation with papaya leaf powder appears to strengthen the immune defense of fish and may serve as a natural immunostimulant in aquaculture systems.

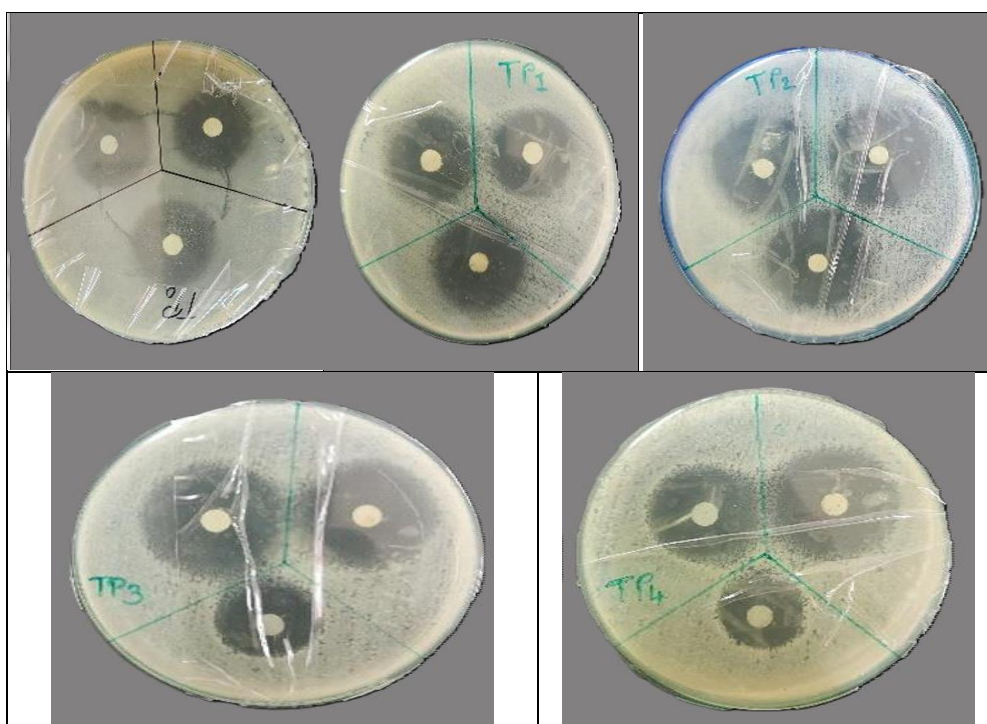


Fig 1: Zone of inhibition after hours

2. Effect of Papaya Leaf Powder on Disease Resistance of *Labeo catla* Fry

Following the completion of the 60-day feeding trial, fish were subjected to a bacterial challenge test using *Aeromonas hydrophila*. Each fish received an intramuscular injection of 0.1 ml bacterial suspension containing 1×10^5 cells/ml, and survival was monitored for 10 days. The survival data obtained after the challenge test are presented in Table 5.

Table 5: Effect of *C. papaya* incorporated diet on survival of *L. catla* fry after the challenge test

Details	Treatments				
	T ₀	T ₁	T ₂	T ₃	T ₄
10 days	21/40	22/40	27/40	32/40	38/40
Survival (%)	52.50 ± 5.00 ^d	55.00 ± 12.91 ^{cd}	67.50 ± 9.57 ^{bc}	80.00 ± 8.16 ^b	95.00 ± 5.77 ^a

The higher survival rate observed in the higher inclusion levels of papaya leaf powder indicates improved disease resistance in *L. catla* fry. Similar findings were reported by Silaen *et al.* (2023) ^[34], who observed a 95% survival rate in Koi fish (*Cyprinus rubrofuscus*) when treated with papaya leaf extract against *A. hydrophila*. Likewise, Olusola and Nwokike (2018) ^[24] reported a survival rate of 83% in *Clarias gariepinus* when fish diets were supplemented with 3% papaya leaf and bitter leaf extracts, which improved resistance to *A. hydrophila* infection.

The increased survival rates may be attributed to the antimicrobial and immunostimulatory properties of phytochemicals present in papaya leaves, such as saponins, alkaloids, tannins, glycosides, and phenolic compounds, which have been widely reported for their antibacterial and antipathogenic activities (Doughari *et al.*, 2007; Sales *et al.*, 2013; Verma *et al.*, 2013) ^[32, 36].

The present findings therefore suggest that dietary supplementation with papaya leaf powder, particularly at 7.5% (T₃) and 10% (T₄) levels, can significantly enhance disease resistance and immune response in *Labeo catla* fry. Such plant-based additives can serve as effective and eco-friendly alternatives to antibiotics in aquaculture,

The highest survival rate was recorded in T₄ (95.00 ± 5.77%), followed by T₃ (80.00 ± 8.16%), T₂ (67.50 ± 9.57%), T₁ (55.00 ± 12.91%), and T₀ (52.50 ± 5.00%). Statistical analysis indicated significant differences among the treatments ($P < 0.05$), suggesting that dietary inclusion of papaya leaf powder significantly enhanced resistance against bacterial infection.

contributing to sustainable fish health management.

3. Physico-Chemical Water Parameters

The physico-chemical characteristics of the experimental water during the study period are presented in Table 6. Throughout the experimental duration, all water quality parameters remained within acceptable ranges suitable for the culture of *Labeo catla* fry.

Water temperature varied between 24.2 °C and 29.7 °C across treatments. The pH ranged from 6.8 to 7.9, indicating slightly acidic to neutral conditions suitable for fish growth. Dissolved oxygen levels ranged from 5.4 to 6.6 ppm, which were adequate to support normal metabolic activity and fish health.

Total hardness values ranged from 140 to 230 ppm, while ammonia concentrations were maintained between 0.05 and 0.11 ppm, and nitrite levels ranged from 0.17 to 0.42 ppm. These values remained within permissible limits and did not exhibit significant variations among treatments.

The stable water quality conditions observed during the experiment ensured that fish growth, immune response, and survival were primarily influenced by the dietary treatments rather than environmental stress factors.

Table 6: Physio-chemical parameter during the experiment.

Parameters	T ₀	T ₁	T ₂	T ₃	T ₄
Water temperature (°C)	25.3-28.0	24.2-29.7	25.7-28.9	24.5-28.7	24.9-28.5
pH	6.8-7.7	7.2-7.8	7.0-7.9	7.2-7.8	7.0-7.6
DO (ppm)	6.0-6.3	5.4-6.5	5.7-6.3	5.6-6.6	5.8-6.5
Total Hardness (ppm)	150-210	170-230	140-200	160-230	180-220
Ammonia (ppm)	0.05-0.10	0.06-0.09	0.05-0.10	0.07-0.10	0.05-0.11
Nitrite (ppm)	0.17-0.31	0.18-0.30	0.19-0.42	0.18-0.34	0.20-0.31

Conclusion

The present study demonstrated that dietary supplementation with *Carica papaya* leaf powder significantly enhances the immune response and disease resistance of *Labeo catla* fry. Fish fed diets containing papaya leaf powder showed higher antibacterial activity against *Aeromonas hydrophila*, as evidenced by increased zones of inhibition in the disc diffusion assay. Among the treatments, the highest inclusion level (10%, T₄) produced the strongest immune response and the greatest inhibition zone at all observation intervals. Similarly, the challenge test revealed improved survival rates in fish receiving papaya-supplemented diets, with the highest survival recorded in T₄ (95%), followed by T₃ (80%). These findings indicate that papaya leaf powder acts as a natural immunostimulant, likely due to the presence of bioactive

phytochemicals such as flavonoids, phenolic compounds, alkaloids, and tannins. Furthermore, water quality parameters remained within acceptable ranges throughout the experimental period, ensuring suitable rearing conditions. Overall, the results suggest that dietary inclusion of papaya leaf powder, particularly at 7.5-10%, can be an effective, eco-friendly alternative to antibiotics for improving fish health and disease resistance in aquaculture systems.

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