International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

Effects of Dietary Immunostimulants on the Serum Chemistry of Catla Catla Challenged with Aeromonas Hydrophila

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Abstract

This study assessed the immunomodulatory activities of dietary immunostimulants in Catla catla infected with Aeromonas hydrophila. Diets supplemented with vitamin C, vitamin E, chitin, chitosan, and levamisole were given orally. Serum biochemical and immunological parameters, including total protein, albumin, globulin, A/G ratio, IgM, lysozyme activity (LA), alkaline phosphatase (AKP), and aspartate aminotransferase (AST), were analyzed under normal and pathogen-infected conditions to determine innate and adaptive immunity. Supplementation with immunostimulants significantly ($p < 0.05$) elevated serum fractions of proteins, IgM levels, and lysozyme activity compared to the controls. Vitamin E and levamisole significantly increased albumin, globulin, and A/G ratio, indicative of improved immune stability. Increased activities of AKP and AST levels revealed activated macrophage functions and metabolic defense mechanisms. Generally, levamisole and vitamin E promoted both innate and adaptive immunity effectively, confirming their usefulness as aquaculture dietary supplements to enhance disease resistance, prevent antibiotic overuse, and ensure sustainable fish health management.

Keywords: Catla Catla, Immunostimulant, Serum biochemistry, Aeromonas hydrophilla

1. Introduction

Mass mortalities from infectious diseases, which result in significant losses for fish farmers, are critical constraints to the advancement and sustainability of aquaculture methods. The prevalence of diseases on fish farm is attributed to a variety of factors related to raising practices, environmental conditions, and fluctuations [8]. Bacterial infections continue to pose a significant challenge in the aquaculture sector [1]. A. hydrophila exists in aquatic environments, causing illness in stressed fish [3]. The immune system of fish eliminates bacteria using both innate and acquired components, as well as humoral and cellular processes that collaborate to try and stop the spread of illnesses. The nonspecific pathways encompass the synthesis of various antibacterial chemicals, acute-phase inflammatory proteins; complement activation via the alternative pathway, cytokines, phagocytosis, and inflammation [10]. The humoral innate elements, triggered by alternative and lectin pathways, include inhibitory substances such as transferrin, antiproteases, lysozyme, C-reactive protein, antibacterial peptides, and proteins in the complement network, which impede bacterial development. Defence cells, like phagocytes, neutrophils, and macrophages, are very important because they have a lot of lysosomal enzymes and make reactive oxygen species when they phagocytose [11]. Antibodies, on the other hand, are glycoproteins, often referred to as immunoglobulins, that are released by plasma cells, which are B

lymphocytes that have been stimulated by an antigen, and expressed on the membrane of the B cells as receptors (BCR) or freely circulated in body fluids [6].

A highly promising approach to disease management in aquaculture involves enhancing the immune defences of fish via the prophylactic use of immunostimulants [16]. Recent advancements in immuno-nutrition research have demonstrated that some nutrients are associated with immunological condition of fish [9]. This has attracted the interest of fish nutritionists in the immunoprotection of fish in addition to growth. Sustainable aquaculture relies on an optimal equilibrium between fish growth and health status. Moreover, serological markers indicate the health condition of fish, as they respond more rapidly than other frequently assessed indicators and can identify symptoms of stress resulting from environmental circumstances, infections, or diet, among other factors [7]. These metrics serve as a crucial instrument for disease diagnostics, indicating the health condition of fish [12]. Nutritional parameters, including feeding frequency, food quantity, and quality influence fish health [17]. Hence, the present study evaluates the effects of dietary administration of different immunostimulants such as vitamin C, vitamin E, chitin, chitosan, and levamisole on the potential serological markers in C. catla under healthy and Aeromonas infected conditions.

2. Materials and Methods

A. Experimental Design

This study was performed to assess the possible immunostimulatory effects of five distinct compounds: vitamin C, vitamin E, chitin, chitosan, and levamisole, on the serum biochemistry of A. hydrophilaexposed C. catla.The control group was fedwith GrowFin feed without immunostimulants. The second group was divided into five experimental groups whichfed withdifferent immunostimulants.Fish in the third group were fed a control diet and infected with A. hydrophila(10^6 CFU/mL).The fourthgroup was divided into five experimental groups which infected with A. hydrophila and fed with five different immunostimulants individually.Following 30 days of exposure, fish were sacrificed with the objective ofmeasuring total serum protein, albumin, globulin, IgM, lysozyme activity, alkaline phosphatase, and aspartate aminotransferase for all the treatment groups. Blood drawn from every group was put into EDTA vials, centrifuged at 7000 rpm for 10 minutes, and stored at –20°C to be analysed later.

B. Evaluation of Serum Biochemistry

The total serum protein concentration was evaluated using the Biuret method. The total serum albumin levels in the fish samples were evaluated using bromocresol binding method [4] using serum albumin detection kit (Atlas medical kit).The total serum globulin concentration was determined by subtracting serum albumin from total serum protein [2].Serum IgM levels in the fish samples were estimated qualitatively using IgM ELISA kit (Cusabio, USA).Serum lysozyme assay evaluated using turbidimetric assay describe by modified Ellis, (1999) method.Serum alkaline phosphatase activity (AKP) was estimated as per the method given by Thengodkar and Sivakami, (2010).Sizer and Jenkins, (1962) method was used to determine on the aspartate aminotransferase activity.

3. Statistical Analysis

Each experiment was carried out three times, and the results were shown as the mean ± standard deviation. The results were examined using descriptive statistics and regression correlation. The turkey's HSD test was conducted to assess the comparative pairwise analysis between control feed and each

immunostimulant mixed feed under normal and Aeromonas infected conditions. A 'P' value of less than 0.05 is considered statistically significant. All statistical analyses were conducted using SPSS Windows version 10.0.

4. Results

A. Total Serum Protein

Figure 1 compiles serum protein levels of immunostimulant-supplemented feedings of C. catla under healthy and A. hydrophilia-infected conditions. Levels of serum protein tended to be higher for immunostimulants-fed fish than for controls, and slightly higher for infected groups compared to healthy groups. Of the tested feedings, vitamin C, vitamin E, and levamisole provided consistently higher serum protein levels, whereas relatively low effects were found for chitin and chitosan. Overall, findings suggest that dietary immunostimulants markedly enhanced serum protein content for healthy and infected fish, and that was also validated by means of Tukey's HSD test ($p<0.05$) (Table 1).

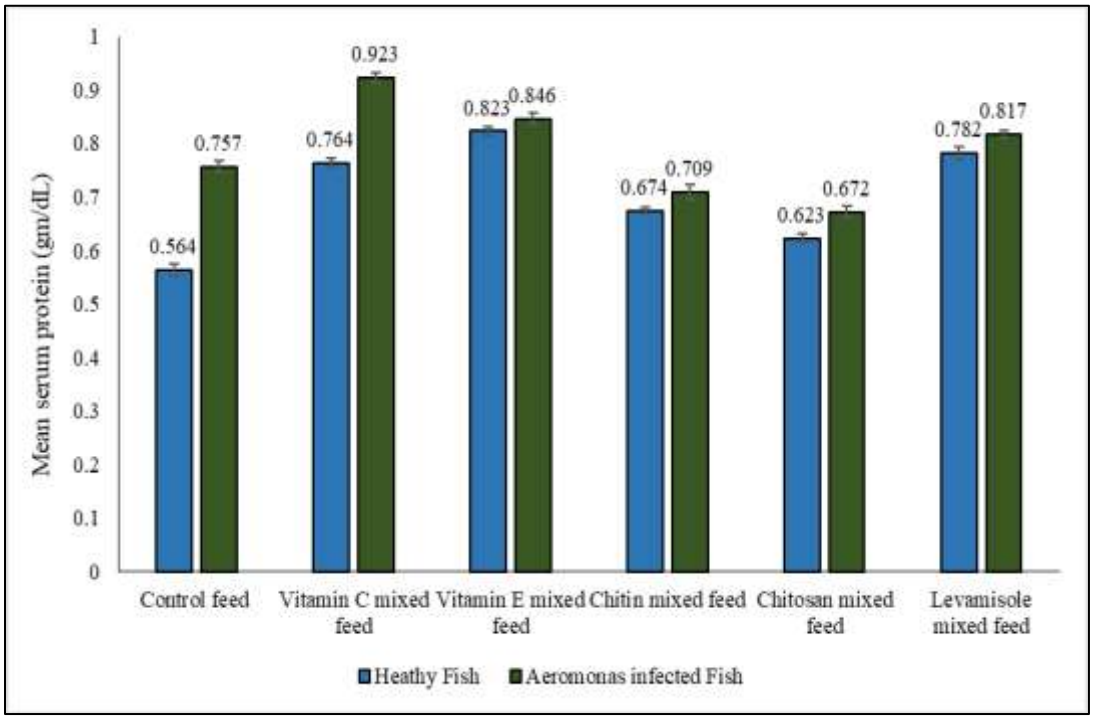

Figure 1: Serum Protein Sugar Content In The Fishes Fed With Different Immunostimulant Mixed Feeds Under Normal And Aeromonas Infected Conditions

B. Serum Albumin

Figure 2 shows the serum albumin levels of C. catla on various immunostimulant-supplemented diets under healthy and A. hydrophila-infected states. Levels of albumin were significantly different among treatments, varying from about 0.33 to 0.47 g/dL in healthy and 0.32 to 0.48 g/dL in infected fish. The highest level was recorded in infected fish that was fed vitamin E, and the lowest in infected fish that was fed chitosan.Generally, serum albumin levels were significantly greater ($p<0.05$) in infected fish than in healthy ones. Immunostimulant diets also boosted albumin in healthy groups compared with controls, where vitamin C, vitamin E, and levamisole produced the most significant effects. Among infected fish, these three immunostimulants boosted albumin, while chitin and chitosan decreased it.

Tukey's HSD test revealed that the majority of immunostimulant-fed groups differed significantly ($p<0.05$) from control-fed fish (Table 2).

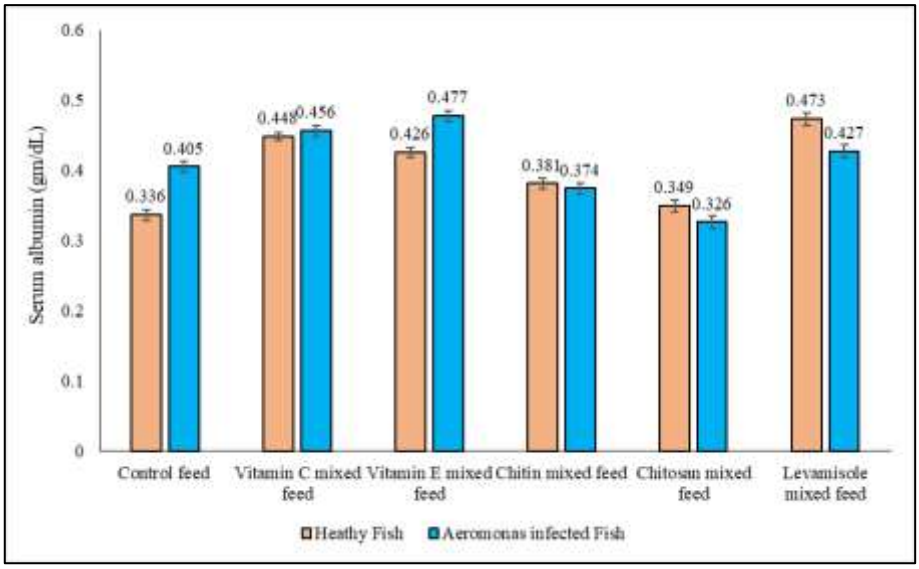

Figure 2: Serum Albumin Content in the Fishes Fed With Different Immunostimulant Mixed Feeds Under Normal and Aeromonas Infected Conditions

C. Serum Globulin

Figure 3 illustrates serum globulin of control and immunostimulant (vitamin C, vitamin E, chitin, chitosan, levamisole)-supplemented C. catla under normal and A. hydrophila-infected conditions. Healthy fish recorded 0.247±0.027 to 0.361±0.002 g/dL, while infected fish varied from 0.335±0.008 to 0.461±0.013 g/dL, with the maximum in vitamin C-fed infected fish and the minimum in control-fed healthy fish. Infection greatly raised globulin ($p<0.05$), and immunostimulant diets increased globulin in healthy fish relative to controls. Of the immunostimulants, vitamin C, vitamin E, and levamisole most strongly raised globulin upon infection, with chitin and chitosan having intermediate effects. These findings suggest that dietary immunostimulants increase baseline immune activity in healthy fish and yield protective effects under pathogenic challenge, with vitamin C, vitamin E, and levamisole being the most active immunostimulants (Table 3).

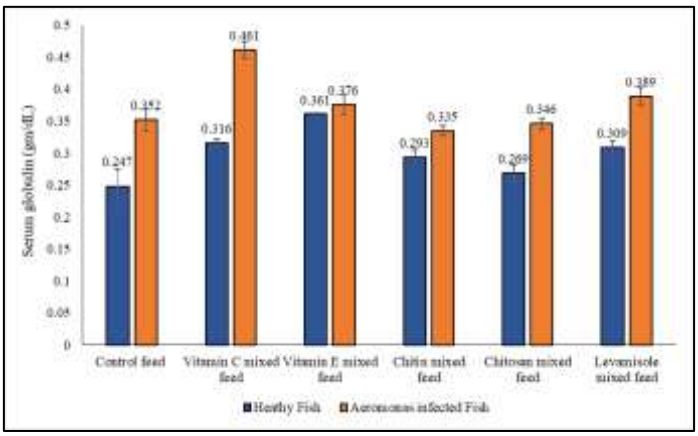

Figure 3: Serum Globulin Content in the Fishes Fed With Different Immunostimulant Mixed Feeds Under Normal and Aeromonas Infected Conditions

D. Albumin Globulin Ration (A/G ratio)

Serum A/G ratio of fish challenged with five immunostimulant-supplemented diets and control feeds under normal and A. hydrophila infection is given in Figure 4. It was found that the A/G ratio significantly ($p<0.05$) dropped in infected fish relative to healthy fish. Vitamin C and levamisole diets elevated the A/G ratio above control in healthy fish, whereas in infected fish, vitamin E supplementation had a higher ratio than control.

In the healthy groups, values of A/G ratio were 1.370±0.149 (control), 1.416±0.027 (vitamin C), 1.284±0.023 (vitamin E), 1.305±0.083 (chitin), 1.302±0.09 (chitosan), and 1.531±0.064 (levamisole). In infected ones, they were 1.15±0.073 (control), 0.989±0.016 (vitamin C), 1.267±0.037 (vitamin E), 1.118±0.018 (chitin), 0.944±0.039 (chitosan), and 1.097±0.061 (levamisole).

In total, feeding with immunostimulants greatly reduced A/G ratio in comparison to the control except in case of healthy fish fed vitamin C and infected fish fed vitamin E. Tukey's HSD test also indicated that the majority of experimental groups fed immunostimulants possessed considerably lower A/G ratios ($p<0.05$) than the controls (Table 4).

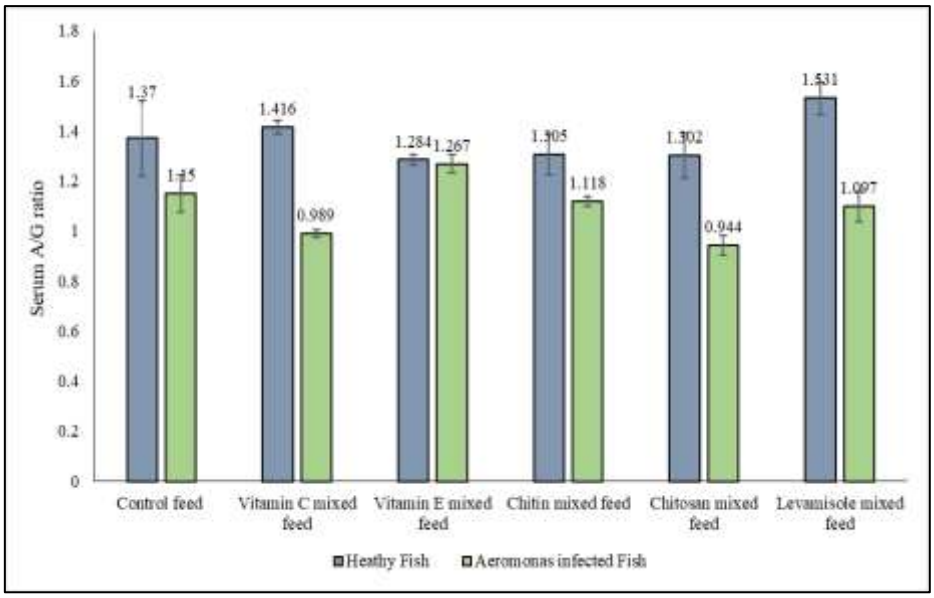

Figure 4: Serum A/G Ratioin the Fishes Fed With Different Immunostimulant Mixed Feeds Under Normal and Aeromonas Infected Conditions

E. Serum IgM

The serum IgM concentrations in fishes that received immunostimulant-mixed and control diets under normal and Aeromonas infection have been presented in figure 5. The serum IgM was found to be much higher ($p<0.05$) in infected fishes than in healthy ones, and the immunostimulant supplementation also increased IgM levels in both cases compared to control. In healthy fish, serum IgM varied between 0.55±0.04 to 0.86±0.02 mg/mL, whereas in infected fish it ranged between 0.72±0.04 to 0.97±0.01 mg/mL. The maximum IgM (0.97±0.01 mg/mL) was found in levamisole-administered infected fish, while minimum (0.55±0.04 mg/mL) was found in the normal control group. Immunostimulants, especially vitamin C, vitamin E, and levamisole, boosted IgM response in both control and infected fishes. Tukey's HSD test verified significant differences ($p<0.05$) between most of the experimental groups and control (Table 5).

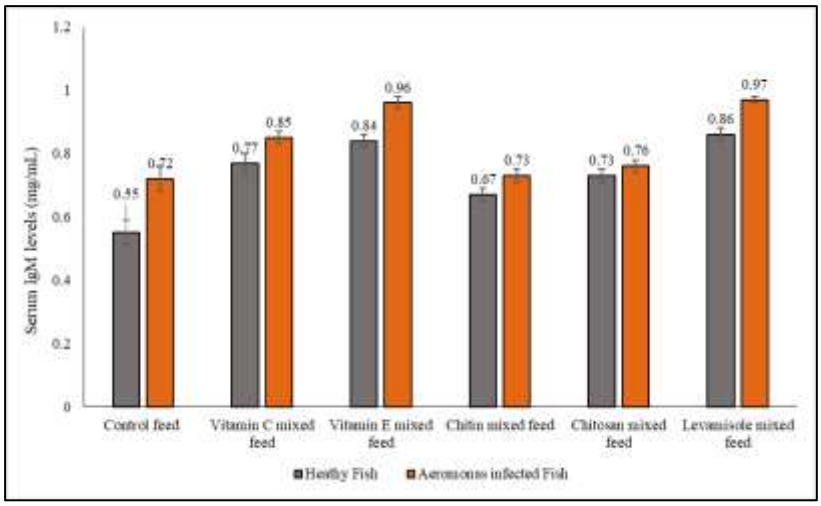

Figure 56: Serum Igm Levels in the Fishes Fed With Different Immunostimulant Mixed Feeds Under Normal and Aeromonas Infected Conditions

F. Serum lysozyme activity (LA)

Figure 6 indicates that serum LA was considerably ($p<0.05$) greater in Aeromonas-infected fishes than in the healthy ones, and immunostimulant diets augmented LA in both groups further compared to control.In the healthy fishes, LA varied from 224.83±9.13 to 416.13±9.04 U/mL, whereas in infected fishes it varied from 314.9±10.15 to 565.23±9.47 U/mL. The maximum value (565.23±9.47 U/mL) was found in levamisole-fed infected fishes and the minimum (224.83±9.13 U/mL) in healthy controls.Tukey's HSD test validated that immunostimulant-fed fishes (vitamin C, vitamin E, chitin, chitosan, levamisole) under both conditions also significantly registered higher serum LA than controls ($p<0.05$) (Table 6).

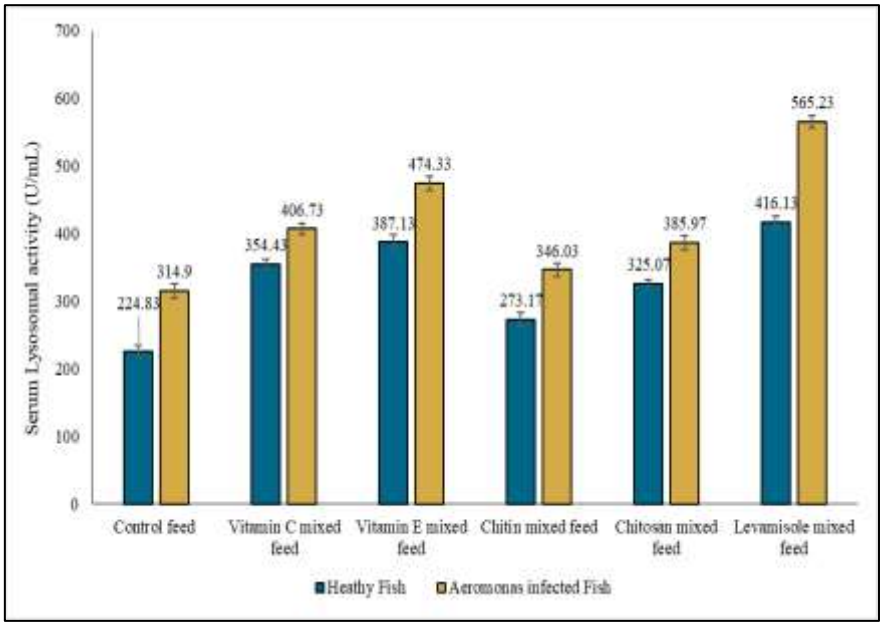

Figure 6: Serum Lysozyme Activity In The Fishes Fed With Different Immunostimulant Mixed Feeds Under Normal And Aeromonas Infected Conditions

G. Serum alkaline phosphatase (AKP)

Figure 7 presents serum AKP activity in fishes that were fed with five immunostimulant-supplemented diets and control feed with normal and Aeromonas-infected conditions. Serum AKP activity was higher significantly ($p<0.05$) with infection as well as with immunostimulant supplementation when compared to control.In healthy fishes, AKP varied between 22.3±2.41 to 63.57±1.96 U/mL, while in infected fishes it varied between 37.03±1.64 to 84.03±3.23 U/mL. The maximum activity (84.03±3.23 U/mL) was in infected fishes administered levamisole diet, and the minimum (22.3±1.64 U/mL) in healthy controls.Mean AKP levels in healthy fishes administered control, vitamin C, vitamin E, chitin, chitosan, and levamisole diets were 22.3±2.41, 44.17±3.32, 55.23±3.08, 25.43±3.52, 29.5±2.36, and 63.57±1.96 U/mL, respectively. In infected fishes, corresponding values were 37.03±1.64, 57.4±2.52, 75.33±2.73, 41.8±1.57, 52.17±2.40, and 84.03±3.23 U/mL. Tukey's HSD test also established that fishes receiving immunostimulant diets had significantly increased AST levels ($p<0.05$) than control under both normal and infected states (Table 7).

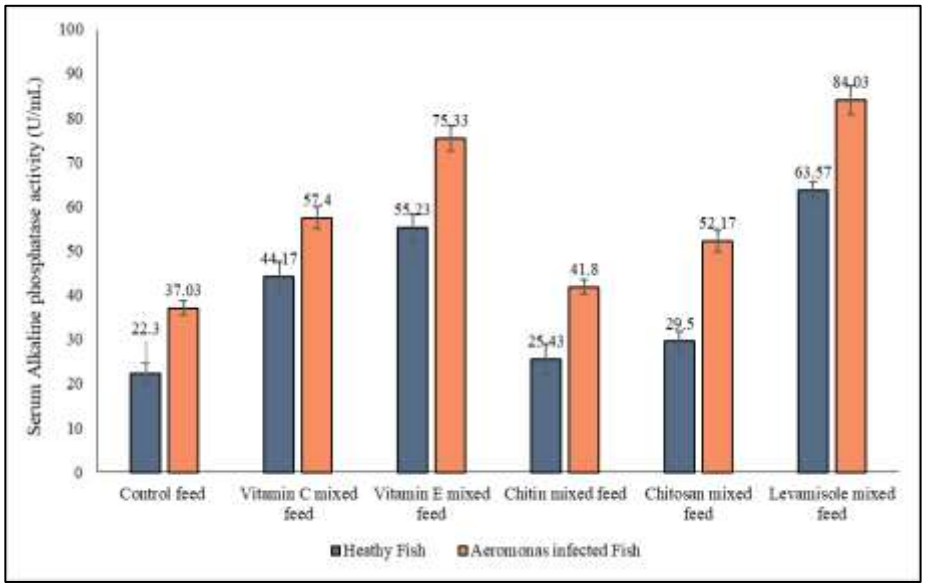

Figure 7: Serum Alkaline Phosphatase Activity in The Fishes Fed With Different Immunostimulant Mixed Feeds Under Normal And Aeromonas Infected Conditions

H. Serum Aspartate Aminotransferase (AST)

Figure 8 presents serum AST activity in fishes that were fed with five immunostimulant-supplemented diets and control feed with normal and Aeromonas-infected conditions. Serum AST activity was higher significantly ($p<0.05$) with infection as well as with immunostimulant supplementation when compared to control. In healthy fishes, AST varied between 72.3±1.61 to 117.67±2.82 U/mL, while in infected fishes it varied between 83.93±2.01 to 140.2±4.01 U/mL. The maximum activity (140.2±4.01 U/mL) was in infected fishes administered levamisole diet, and the minimum (72.3±1.61 U/mL) in healthy controls. Mean AST levels in healthy fishes administered control, vitamin C, vitamin E, chitin, chitosan, and levamisole diets were 72.3±1.61, 90.77±3.08, 107.13±2.71, 85.27±2.68, 89.93±3.13, and 117.67±2.82 U/mL, respectively. In infected fishes, corresponding values were 83.93±2.01, 110.77±2.09, 131.27±2.54, 95.43±2.01, 99.9±1.58, and 140.2±4.01 U/mL. Tukey's HSD test also

established that fishes receiving immunostimulant diets had significantly increased AST levels ($p<0.05$) than control under both normal and infected states (Table 8).

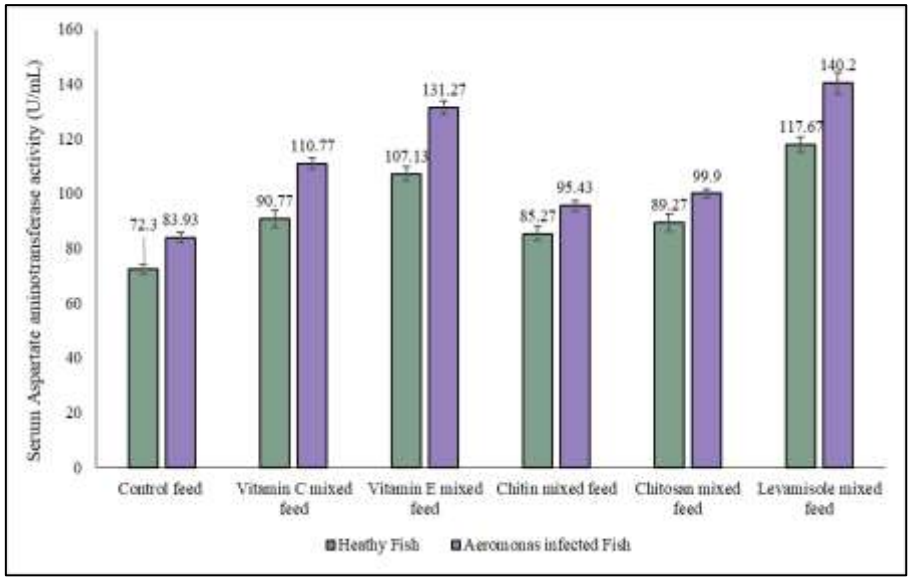

Figure 8: Serum Aspartate Aminotransferase Activity in the Fishes Fed With Different Immunostimulant Mixed Feeds Under Normal and Aeromonas Infected Conditions

Table 1: Tukey's HSD Analysis of the Serum Proteininc Catla Fed with Different Immunostimulant Diets (Normal vs. Infected)

Pairwise comparisons	Vitamin C			Vitamin E			Chitin			Chitosan			Levamisole		
	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value
T1 : T2	15.02	13.39	0.000	0.19	44.73	0.000	0.19	14.73	0.0001	0.19	44.73	0.000	0.19	44.73	0.000
T1 : T3	22.06	19.66	0.000	0.26	60.92	0.000	0.11	21.97	0.0001	0.06	13.88	0.000	0.21	52.02	0.000
T1 : T4	35.06	31.25	0.000	0.28	65.92	0.000	0.14	29.11	0.0001	0.11	24.84	0.000	0.25	61.03	0.000
T2 : T3	7.04	6.27	0.002	0.07	16.18	0.000	0.08	16.25	0.0000	0.13	30.14	0.000	0.03	6.10	0.028
T2 : T4	20.04	17.86	0.000	0.09	21.19	0.000	0.05	9.11	0.042	0.08	19.18	0.000	0.06	15.11	0.000
T3 : T4	13.00	11.59	0.000	0.02	5.0	0.013	0.04	7.14	0.062	0.05	10.96	0.000	0.04	9.01	0.005

T1- fishes fed with control feed under normal condition.

T2- fishes fed with control feed under Aeromonas infected condition.

T3- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under normal condition.

T4- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under Aeromonas infected condition.

Table 2: Tukey's HSD Analysis of the Serum Albumininc Catla Fed with Different Immunostimulant Diets (Normal vs. Infected)

Pairwise comparisons	Vitamin C			Vitamin E			Chitin			Chitosan			Levamisole		
	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value
T1 : T2	0.07	25.92	0.000	0.07	22.56	0.000	0.07	22.43	0.000	0.07	22.43	0.000	0.07	22.43	0.000
T1 : T3	0.11	41.33	0.000	0.13	40.09	0.000	0.05	15.20	0.000	0.01	4.28	0.037	0.14	41.37	0.000
T1 : T4	0.12	43.78	0.000	0.14	44.11	0.000	0.04	13.71	0.000	0.01	2.68	0.479	0.09	27.24	0.000
T2 : T3	0.04	15.41	0.000	0.06	17.73	0.000	0.02	7.23	0.001	0.06	18.36	0.000	0.07	19.82	0.000
T2 : T4	0.05	17.86	0.000	0.07	21.55	0.000	0.03	8.72	0.001	0.08	24.71	0.000	0.02	5.69	0.049
T3 : T4	0.01	2.45	0.341	0.01	3.82	0.066	0.00	1.50	0.719	0.02	6.35	0.002	0.05	14.13	0.000

T1- fishes fed with control feed under normal condition.
T2- fishes fed with control feed under Aeromonas infected condition.
T3- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under normal condition.
T4- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under Aeromonas infected condition

Table 3: Tukey's HSD Analysis of the Serum Globulin Inc Catla Fed with Different Immunostimulant Diets (Normal vs. Infected)

Pairwise comparisons	Vitamin C			Vitamin E			Chitin			Chitosan			Levamisole		
	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value
T1 : T2	0.11	16.30	0.000	0.11	15.98	0.000	0.11	15.85	0.000	0.11	15.85	0.000	0.11	15.85	0.0001
T1 : T3	0.07	11.50	0.000	0.12	17.79	0.000	0.05	7.30	0.001	0.03	4.30	0.0354	0.09	8.64	0.001
T1 : T4	0.22	34.04	0.000	0.13	19.65	0.000	0.09	13.76	0.000	0.10	15.12	0.000	0.15	14.37	0.000
T2 : T3	0.03	4.80	0.018	0.01	1.81	0.589	0.06	8.55	0.001	0.08	11.41	0.000	0.02	1.56	0.694

T2 : T4	0.12	17.74	0.000	0.02	3.68	0.082	0.01	2.09	0.471	0.00	0.59	0.974	0.04	4.18	0.042
T3 : T4	0.15	22.54	0.000	0.01	1.87	0.563	0.04	6.46	0.002	0.07	10.82	0.001	0.06	5.73	0.005

T1- fishes fed with control feed under normal condition.
T2- fishes fed with control feed under Aeromonas infected condition.
T3- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under normal condition.
T4- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under Aeromonas infected condition

Table 4: Tukey's HSD Analysis of the Serum A/G RatioinC Catla Fed with Different Immunostimulant Diets (Normal vs. Infected)

Pairwise comparisons	**Vitamin C**			**Vitamin E**			**Chitin**			**Chitosan**			**Levamisole**		
	Tukey's HSD	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**
T1 : T2	0.22	6.56	0.002	0.22	6.56	0.014	0.22	6.56	0.014	0.22	6.56	0.001	0.22	6.56	0.001
T1 : T3	0.02	0.71	0.957	0.10	3.02	0.184	0.07	1.90	0.551	0.10	2.69	0.266	0.15	3.98	0.055
T1 : T4	0.40	11.79	0.000	0.11	3.32	0.128	0.27	7.18	0.006	0.43	11.19	0.000	0.30	7.97	0.002
T2 : T3	0.25	7.27	0.005	0.12	3.48	0.105	0.15	4.11	0.046	0.12	3.10	0.167	0.38	9.89	0.000
T2 : T4	0.18	5.23	0.01	0.11	3.18	0.152	0.04	1.17	0.842	0.21	5.40	0.007	0.08	2.05	0.487
T3 : T4	0.43	12.50	0.000	0.01	0.30	0.997	0.20	5.28	0.088	0.33	8.51	0.001	0.45	11.95	0.000

T1- fishes fed with control feed under normal condition.
T2- fishes fed with control feed under Aeromonas infected condition.
T3- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under normal condition.
T4- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under Aeromonas infected condition.

Table 5: Tukey's HSD Analysis of the Serum IgMinC Catla Fed with Different Immunostimulant Diets (Normal vs. Infected)

Pairwise comparisons	**Vitamin C**			**Vitamin E**			**Chitin**			**Chitosan**			**Levamisole**		
	Tukey's HSD	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**

T1 : T2	0.18	15.13	0.000	0.18	15.13	0.000	0.18	15.13	0.000	0.18	15.13	0.000	0.18	15.13	0.000
T1 : T3	0.23	19.00	0.000	0.29	25.03	0.000	0.12	9.72	0.002	0.18	15.70	0.000	0.31	27.54	0.000
T1 : T4	0.30	25.22	0.000	0.41	34.56	0.000	0.18	14.58	0.000	0.21	18.66	0.000	0.42	36.66	0.000
T2 : T3	0.05	3.87	0.064	0.11	9.70	0.002	0.06	4.86	0.016	0.00	0.00	0.000	0.13	11.75	0.000
T2 : T4	0.12	10.09	0.000	0.23	19.24	0.000	0.00	0.00	0.000	0.03	2.96	0.196	0.24	20.87	0.000
T3 : T4	0.07	6.22	0.002	0.11	9.53	0.003	0.06	4.86	0.016	0.03	2.96	0.196	0.10	9.12	0.004

T1- fishes fed with control feed under normal condition.
T2- fishes fed with control feed under Aeromonas infected condition.
T3- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under normal condition.
T4- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under Aeromonas infected condition.

Table 6: Tukey's HSD Analysis of the Serum Lysozyme ActivityinC Catla Fed with Different Immunostimulant Diets (Normal vs. Infected)

Pairwise comparisons	**Vitamin C**			**Vitamin E**			**Chitin**			**Chitosan**			**Levamisole**		
	Tukey's HSD	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**	**Tukey's HSD**	**Q value**	**P value**
T1 : T2	87.24	25.46	0.000	87.24	25.46	0.000	87.24	25.46	0.000	87.24	25.46	0.000	87.24	25.46	0.000
T1 : T3	129.08	37.67	0.000	160.18	35.65	0.000	45.94	11.36	0.000	99.44	25.86	0.000	189.42	50.05	0.000
T1 : T4	181.54	52.98	0.000	247.62	55.11	0.000	119.66	29.58	0.000	158.76	41.29	0.000	340.48	89.97	0.000
T2 : T3	41.84	12.21	0.000	72.94	16.23	0.000	41.30	10.21	0.001	12.20	3.17	0.153	102.18	27.00	0.000
T2 : T4	94.30	27.52	0.000	160.38	35.70	0.000	32.42	8.01	0.002	71.52	18.60	0.000	253.24	66.92	0.000
T3 : T4	52.46	15.31	0.000	87.44	19.46	0.000	73.72	18.22	0.000	59.32	15.43	0.000	151.06	39.92	0.000

T1- fishes fed with control feed under normal condition.
T2- fishes fed with control feed under Aeromonas infected condition.
T3- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under normal condition.

T4- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under Aeromonas infected condition.

Table 7: Tukey's HSD Analysis of the Serum AKP Activityinc Catla Fed with Different Immunostimulant Diets (Normal vs. Infected)

Pairwise comparisons	Vitamin C			Vitamin E			Chitin			Chitosan			Levamisole		
	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value
T1 : T2	15.02	13.39	0.000	15.02	13.39	0.000	15.02	13.39	0.000	15.02	13.39	0.000	15.02	13.39	0.000
T1 : T3	22.06	19.66	0.000	32.68	31.37	0.000	2.96	2.83	0.229	7.36	7.85	0.002	41.34	41.30	0.000
T1 : T4	35.06	31.25	0.000	53.66	51.51	0.000	19.66	18.77	0.000	30.38	32.42	0.000	62.22	62.25	0.000
T2 : T3	7.04	6.27	0.002	17.66	16.95	0.000	12.06	11.51	0.000	7.66	8.17	0.002	26.32	26.29	0.000
T2 : T4	20.04	17.86	0.000	38.64	37.09	0.000	4.64	4.43	0.029	15.36	16.39	0.000	47.30	47.25	0.000
T3 : T4	13.00	11.59	0.000	20.98	20.14	0.000	16.70	15.94	0.000	23.02	24.57	0.000	20.98	20.96	0.000

T1- fishes fed with control feed under normal condition.
T2- fishes fed with control feed under Aeromonas infected condition.
T3- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under normal condition.
T4- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under Aeromonas infected condition.

Table 8: Tukey's HSD Analysis of the Serum AST activity inC Catla Fed with Different Immunostimulant Diets (Normal vs. Infected)

Pairwise comparisons	Vitamin C			Vitamin E			Chitin			Chitosan			Levamisole		
	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value	Tukey's HSD	Q value	P value
T1 : T2	11.78	12.98	0.000	11.78	12.98	0.000	11.78	12.98	0.000	11.78	12.98	0.000	11.78	12.98	0.000
T1 : T3	18.24	20.09	0.000	35.04	37.44	0.000	13.14	14.70	0.000	18.06	19.43	0.000	45.44	41.72	0.000
T1 : T4	39.20	43.18	0.000	59.78	63.87	0.000	23.00	25.73	0.000	27.54	29.62	0.000	69.06	63.40	0.000
T2 : T3	6.46	7.12	0.006	23.26	24.85	0.000	1.36	1.52	0.709	6.28	6.75	0.007	33.66	30.90	0.000

T2 : T4	27.42	30.21	0.000	48.00	51.29	0.000	11.22	12.55	0.000	15.76	16.95	0.000	57.28	52.59	0.000
T3 : T4	20.96	23.09	0.000	24.74	26.43	0.000	9.86	11.03	0.000	9.48	10.20	0.001	23.62	21.69	0.000

T1- fishes fed with control feed under normal condition.
T2- fishes fed with control feed under Aeromonas infected condition.
T3- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under normal condition.
T4- fishes fed with Vitamin C, Vitamin E, Chitin, Chitosan or levamisole mixed feed under Aeromonas infected condition.

5. Discussion

The current research assessed the immunomodulatory function of dietary vitamin C, vitamin E, chitin, chitosan, and levamisole on C. catla infected with A. hydrophila. Serum biochemistry and immune markers served as indicators of the health status and defense mechanism of fish. Generally, immunostimulant-supplemented diets strongly developed the innate and adaptive immunity compared to controls under normal and infected states.

Serum proteins, especially albumin and globulin, are also well established as indicators of immune ability in teleosts. In this research, their levels were significantly greater in immunostimulant-fed fish, indicating enhanced basal protein synthesis and nonspecific immune preparedness. These findings are consistent with existing results where immunostimulants and probiotics increased serum protein fractions and drove antibody production [15]. The albumin/globulin (A/G) ratio also gave information on immune modulation, with an overall reduction in infected fish but diet-improvement in infected groups given vitamin C, vitamin E, and levamisole, reflecting improved humoral immunity in spite of infection stress.These results aligned with the findings of Panwar et al., (2021), who indicated that the albumin-globulin ratio of C.batrachus ranged from 0.71 to 1.92 following vaccination of sheep red blood cells (SRBC).

Immunoglobulin M (IgM), the major antibody in fish, was found to be greatly elevated in all immunostimulant-fed groups, with levamisole and chitin causing the most effective stimulation. This verifies the function of dietary agents to trigger adaptive immunity and increase pathogen-specific antibody production. In accordance with the present study, Shiry et al., (2020) found a considerable rise in IgM expression in the intestines of rainbow trout following infection with streptococcus iniae.Likewise, lysozyme activity, a central innate defense enzyme, was invariably higher within immunostimulant-fed groups. Its increase indicates macrophage activation and heightened antibacterial defense and confirms previous observation of diet- or plant extract-induced elevation of lysozyme activity throughout teleosts [14].

AKP, an enzyme of the lysosome associated with macrophage activity and metabolic adaptation, also rose profoundly in treated groups, particularly under infection, suggesting its function in the detection of pathogens and protection by immunity [20]. AST, yet another biochemical marker implicated in immune stimulation, exhibited significant rises in all immunostimulant-fed fish, the level being the greatest with levamisole. Although AST is also connected with hepatic function, here its rise reflects an enhanced metabolic and immune response [22].Together, these results prove that dietary immunostimulants promote both humoral and cellular defence systems in C. catla and promote resistance to disease against

A. hydrophila. Levamisole and vitamin E produced the most significant responses most consistently, whereas vitamin C, chitin, and chitosan also exerted notable effects. The findings confirm the efficacy of immunostimulant-supplemented diets as green solutions over antibiotics in aquaculture and provide a green approach to enhancing fish health, survival, and productivity.

6. Conclusion

This research proves that vitamin C, vitamin E, chitin, chitosan, and levamisole supplementation in the diet greatly promoted innate and adaptive immunity in C.catla under normal and A. hydrophilainfected conditions. Immunostimulant-fed fish had increased serum proteins (albumin, globulin), immunoglobulin M, lysozyme activity, alkaline phosphatase, and AST, indicating better metabolic preparedness and immune capability. Of the compounds tested, levamisole and vitamin E elicited consistently the highest responses, followed by vitamin C, chitin, and chitosan, with significant effects. These observations justify the inclusion of immunostimulant-supplemented diets as environmentally friendly, eco-efficient, and sustainable substitutes for antibiotics and vaccines in aquaculture, hence augmenting disease resistance, survival, and productivity in carp culture systems.

7. Acknowledgements

Authors acknowledge the department of zoology, Acharya Nagarjuna University, Guntur for providing facilities to carry out the research work.

8. References

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