



Effect of ImmuPlus on Growth and Inflammatory Response to Freund's Complete Adjuvant in Common Carp, *Cyprinus carpio* (L.)

M. Priyadarshini¹, J.K. Manissery¹, C. V. Mohan², P. Keshavanath^{1*}

¹ Karnataka Veterinary, Animal and Fisheries Sciences University, Department of Aquaculture, College of Fisheries, Mangalore 575 002, India.

² Network of Aquaculture Centers in Asia-Pacific (NACA), P.O. Box 1040, Kasetsart Post Office, Bangkok 10903, Thailand.

* Corresponding Author: Tel.: +91.824 2249256; Fax: +91.824 2248366;
E-mail: perarkeshavanath@yahoo.co.in

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Abstract

The effect of 2 dosages (1.5 and 3.0%) of ImmuPlus, a polyherbal immunomodulator, was tested on growth and inflammatory response to Freund's complete adjuvant (FCA) in common carp, *Cyprinus carpio*. The growth study was carried out for 120 days in nine 25 m² outdoor cement tanks by feeding triplicate groups of 40 common carp fry (av. wt. 0.4±0.03 g) with a control (T₀ without ImmuPlus) and two test diets (T₁ and T₂ with 1.5 and 3.0% ImmuPlus respectively). At the end of the growth experiment, 20 fish from each of these were used for evaluating the inflammatory response to FCA. The final weight gain of fish fed the test diets was significantly (P<0.05) higher, there being no difference between the two treatments. Specific growth rate of treated fish showed a significant increase (P<0.05) over that of the control fish. Supplementation of diet with ImmuPlus improved (P<0.05) survival of fish. Protein content of fish carcass was significantly (P<0.05) higher in the fish receiving the two test diets, while fat content was higher (P<0.05) only in fish from T₂ treatment. Enhanced (P<0.05) protease and amylase activity was found in the treated fish. Even though there were no marked differences in the overall inflammatory response between the control and treated fish following FCA administration, recovery was faster in the latter, with stronger encapsulation of the adjuvant droplets. The results reflect the ability of ImmuPlus to promote growth and improve inflammatory response when administered through diet.

Keywords: *Cyprinus carpio*, immuplus, carcass composition, digestive enzymes, Freund's adjuvant.

Introduction

Over the years, aquaculture system has evolved from extensive to semi-intensive and intensive types. In such systems of culture, fish are subjected to various stresses and simultaneously get exposed to potential pathogens. Therefore, use of preventive approaches to maintain good health of cultured fish by enhancing their defence mechanism is becoming increasingly important in aquaculture. The use of natural immunostimulants in fish culture for the prevention of diseases is a promising new development (Sakai, 1999). Immunostimulants are known to activate non-specific defence mechanisms, cell-mediated immunity and specific immune responses. (Siwicki *et al.*, 1998). Bricknell and Dalmo (2005) defined immunostimulant as a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens. The biological effects of immunostimulants are highly dependent on the receptors of the target cells recognising them as

potential high risk molecules and triggering defence pathways. At least 20 different compounds are used as immunostimulants, adjuvants or vaccine carriers in fish (Anderson, 1992). Gannam and Schrock (1999) reviewed various immunostimulants and methods of their application in fish culture. While the effectiveness of different immunostimulants in protecting cultured species against diseases has been extensively researched, such studies with eco-friendly herbal immunomodulators are limited (Venkatalakshmi and Michael, 2001; Dügenci *et al.*, 2003; Jian and Wu, 2003; Rao *et al.*, 2006; Christyapita *et al.*, 2007; Divyagnaneswari *et al.*, 2007; Ji *et al.*, 2007; Sahu *et al.*, 2007; Harikrishnan *et al.*, 2009; Pratheepa *et al.*, 2010).

This study was carried out to evaluate the effect of ImmuPlus, a polyherbal immunomodulator, on the growth and immune response of common carp. ImmuPlus contains extracts of medicinal plants viz. *Ocimum sanctum* (Tulsi), *Withania somnifera* (Ashwagandha), *Tinospora cordifolia* (Guduchi) and *Embllica officianalis* (Amlaki) as major constituents. It is known to potentiate both cellular and humoral

components of the immune system as well as non-specific immunity with consequent increase in the host defence against pathogenic stimuli. Two dosages (1.5 and 3.0%) of ImmuPlus were tested through dietary administration.

Materials and Methods

Feed Ingredients and Feed Formulation

The feed ingredients viz. fish meal, groundnut cake, rice bran, tapioca flour and vitamin-mineral mixture were procured from the local market. ImmuPlus was obtained from the manufacturer, Indian Herbs Research and Supply Company, Saharanpur, Uttar Pradesh, India. Diets T₀, T₁, and T₂ were prepared separately following the method described by Jayaram and Shetty (1981) using the different ingredients (Table 1). The ingredients were mixed well with sufficient water to get the required consistency in the dough. The dough was then transferred to an aluminum container and steam cooked in a pressure cooker at 15 psi for 15 minutes. After cooling the dough, the required quantity of ImmuPlus and vitamin-mineral mixture were added to it, mixing thoroughly. Pellets (2 mm diameter size) were prepared by a hand pelletizer and were air dried in an oven at 40°C. After drying, they were packed in airtight labeled polythene bags and stored. Small quantities of these diets were transferred to airtight container jars for daily feeding as per requirement.

Experimental Set Up

The growth experiment was carried out in nine uniform sized (25 m²) cement tanks (5 x 5 x 1 m) without any soil base. The tanks were cleaned, dried and then filled with freshwater drawn from a nearby perennial well. Water level was maintained at 80±5 cm throughout the experimental period. Uniform sized (2.4±0.17 cm, 0.4±0.03 g) common carp fry

were stocked at 40 per tank. They were fed once daily in the morning at 5% body weight. Sampling of fish was carried out at 15-day intervals and the feed quantity was re-adjusted based on the body weight recorded at every sampling.

The measured physical and chemical properties of water at the start of the experiment were as follows. Water temperature 29.5°C, pH 7.2, dissolved oxygen 6.87 ppm, free carbon dioxide 1.4 ppm, total alkalinity (CaCO₃) 50 ppm and ammonia 6.07µg·L⁻¹.

Water Analyses

Analysis of water quality for temperature, pH, dissolved oxygen, free carbon dioxide, total alkalinity and ammonia was done at weekly intervals, collecting samples from the experimental tanks between 09.00 and 10.00 hr. Water temperature was recorded using a digital thermometer, while pH was measured with a digital pH meter (LI-120, ELICO, India). The rest of the parameters were determined following standard procedures (APHA 1998).

Dry weight of plankton was also determined every 15 days by filtering 100 liters of water through a plankton net of 60 µm size and drying the filtrate in a hot-air oven at 80°C, till a constant weight was obtained.

Proximate Composition

Proximate composition of feed ingredients, feed and fish carcass was analysed. Carcass was obtained upon harvest, by collecting five fish each from the triplicate tanks of different treatments and drying at 80°C to a constant weight. The dried carcass of each group was pooled and ground. Moisture and ash contents were estimated following AOAC (1995) procedures. Crude protein, fat and fibre contents were analysed using Kjeltex (Tecator, 1002 distilling unit), Soxtech (Tecator, 1043 extraction unit) and Fibretex (Tecator, 1017 hot extractor) systems.

Table 1. Ingredient proportion and proximate composition (± S.E.) of the experimental diets

Diets	Control	T ₁	T ₂
Ingredient proportion (%)			
Fish meal	30.0	30.0	30.0
Groundnut oil cake	30.0	30.0	30.0
Rice bran	29.0	29.0	29.0
Tapioca flour	10.0	10.0	10.0
Vitamin mineral mixture ¹	1.0	1.0	1.0
Immuplus	0	1.5	3.0
Proximate composition (%)			
Moisture	5.70±0.07	4.55±0.16	4.70±0.07
Crude protein	30.52±0.52	31.14±0.50	30.84±0.48
Fat	6.0±0.02	6.4±0.21	6.1±0.28
Ash	17.82±0.03	17.40±0.10	18.15±0.15
Crude fibre	17.00±0.13	16.80±0.01	17.10±0.17
Nitrogen-free extract	23.16	23.51	22.11
Gross energy (kJ·g ⁻¹)	12.47	12.90	12.44

¹Supplivite-M (Sarabhai Company Ltd., India)

Carbohydrate content was calculated as nitrogen free extract (NFE) by the difference method of Hastings (1976). The energy value of each ingredient as well as feed was obtained by multiplying protein, lipid and carbohydrate contents by factors 22.6, 38.9 and 17.2 respectively (Mayes, 1990) and expressed in kJ.

Digestive Enzyme Activity

The digestive enzymes viz. protease, amylase and lipase were analysed using three fish each from the triplicate tanks of different treatments on termination of the growth experiment, following the methods of Rick and Stegbauer (1974), Kunitz (1947) and Bier (1962) respectively. Enzyme activity is expressed as μ moles of product liberated per g tissue per min. at 30°C.

Inflammatory Response to Freund's Complete Adjuvant (FCA)

At the end of the growth experiment, 20 fish from each group (T_0 av. wt. 32.33 g, T_1 av. wt. 40.96 g, T_2 av. wt. 38.69 g) were used for evaluating the inflammatory response to Freund's complete adjuvant (FCA). Benzocaine anaesthetized fish were injected intramuscularly with 0.1 ml of FCA (Sigma, USA) and released back to the tanks. The fish were continued to be fed once daily in the morning with the respective diets. Two fish from each group were sampled at intervals of 3, 6, 9, 12 days after injection. The samples were fixed immediately in 10% formalin, after splitting open the belly region. Skin and skeletal muscle (1-1.5 cm²) from the site of injection were dissected out from formalin fixed fish for histological studies carried out by the technique of Bullock (1989).

Growth, Survival and Feed Utilization Indices

Specific growth rate (SGR), survival rate (SR), feed conversion ratio (FCR) and protein efficiency ratio (PER) were calculated on termination of the growth experiment, using the following formulae:

$$\text{SGR} = [(\text{Ln final weight} - \text{Ln initial weight}) / \text{experimental duration in days}] \times 100.$$

$$\text{SR} = \% \text{ of live fish number at harvest.}$$

$$\text{FCR} = \text{Dry weight of feed given (g)} / \text{wet weight gain (g)}.$$

$$\text{PER} = \text{Gain in wet weight of fish (g)} / \text{dry weight of protein fed (g)}.$$

Statistical Analysis

Comparison among different dietary treatments was done by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test at $P < 0.05$ (Zar, 2009).

Results

Proximate Composition of Feed Ingredients and Feed

Protein, fat and ash contents were the highest in fish meal, while fibre and NFE were maximum in rice bran and tapioca flour, respectively. ImmuPlus recorded values of 6.63% for protein and 1.5% for fat (Table 2). Protein content of the diets varied from 30.52% (T_0) to 31.14% (T_1), while fat content fluctuated between 6.0% (T_0) and 6.4% (T_1) (Table 1).

Water Quality Parameters

The range of water quality parameters monitored over the experimental period were: water temperature 29-31°C, pH 7.2-8.4, dissolved oxygen 4.87-11.08 ppm, free carbon dioxide 0-14.4 ppm, total alkalinity (CaCO₃) 50-130 ppm and ammonia 6.07-13.8 $\mu\text{g}\cdot\text{L}^{-1}$. The average plankton dry weight varied from 4.5 to 32.6 $\text{mg}\cdot 100 \text{ L}^{-1}$, increasing with the progress of the experiment.

Fish Growth

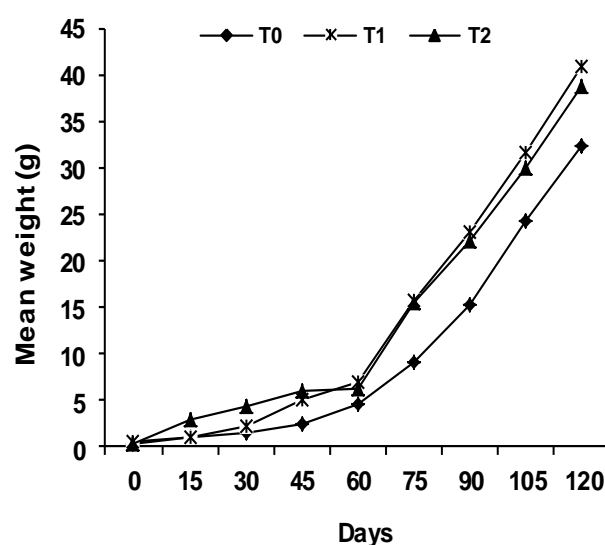
ImmuPlus resulted in significant ($P < 0.05$) growth increment of fish. The highest final average weight of common carp was recorded under treatment T_1 , followed by T_2 and T_0 (Table 3). Growth of fish picked up in the second half of the experimental period (Figure 1). SGR followed the trend of final weight of fish at harvest. There was no difference ($P > 0.05$) in FCR and PER between the control and treatments. Survival of fish was significantly higher ($P < 0.05$) in both the treatments (Table 3).

Table 2. Proximate composition (%) of ingredients used in feed formulation

Parameter	Fish meal	Groundnut oilcake	Rice bran	Tapioca flour	ImmuPlus
Moisture	7.25±0.1	8.47±0.07	7.45±0.8	9.27±0.18	4.85± 0.2
Crude protein	51.24±0.5	39.80±0.8	10.94±0.2	3.98±0.1	6.63±0.33
Fat	7.30±0.2	6.36±0.16	1.69±0.17	0.20±0.8	1.50±0.8
Ash	20.20±0.18	5.80±0.1	15.45±0.05	1.82±0.13	7.42±0.02
Crude fibre	0.80±0.18	16.8±0.19	31.8±0.03	3.4±0.15	17.0±0.02
Nitrogen-free extract	13.21	22.77	32.67	81.33	62.60
Gross energy (kJ·g ⁻¹)	15.67	14.53	8.39	14.51	12.42

Table 3. Growth parameters (\pm S.E.) of common carp fed the experimental diets

Parameter	Diets		
	T ₀	T ₁	T ₂
Final weight (g)	32.33 ^b ±2.06	40.96 ^a ±3.82	38.69 ^a ±2.43
Specific growth rate (%)	3.12 ^b ±0.17	3.40 ^a ±0.07	3.30 ^a ±0.12
Food conversion ratio	1.48 ^a ±0.29	1.35 ^a ±0.14	1.32 ^a ±0.30
Protein efficiency ratio	2.63 ^a ±0.46	2.95 ^a ±0.21	2.87 ^a ±0.44
Survival (%)	65.33 ^b ±0.82	69.16 ^a ±1.05	68.33 ^a ±0.66
Carcass proximate composition (%)			
Moisture	78.28 ^a ±0.08	78.22 ^a ±0.87	75.52 ^b ±0.65
Protein	13.61 ^b ±0.08	14.05 ^a ±0.21	14.83 ^a ±0.33
Fat	3.95 ^b ±0.3	4.16 ^b ±0.06	4.82 ^a ±0.4
Ash	1.49 ^a ±0.1	1.64 ^a ±0.21	1.48 ^a ±0.35

**Figure 1.** Mean weight (g) of common carp fed the experimental diets.

Proximate Composition of Fish Muscle

ImmuPlus treatment affected carcass proximate composition. While protein was significantly ($P < 0.05$) higher in fish from both the treatments, fat was higher only under T₂ treatment, where moisture level was significantly ($P < 0.05$) lower. Ash content did not vary ($P > 0.05$) among the control and treatments (Table 3).

Enzyme Activity

Dietary inclusion of ImmuPlus enhanced the activity of enzymes protease and amylase significantly ($P < 0.05$) both in the hepatopancreas and intestine (Table 4).

Inflammatory Response to Freund's Complete Adjuvant (FCA)

The overall histopathological changes associated with sequential progress of events between the control and treated fish were similar. However, recovery time was less in fish from the two treatments, being nine

and twelve days respectively in T₂ and T₁. Stronger encapsulation with more number of phagocytes was seen in fishes under treatment T₂, while it was comparatively weaker in T₁, followed by T₀.

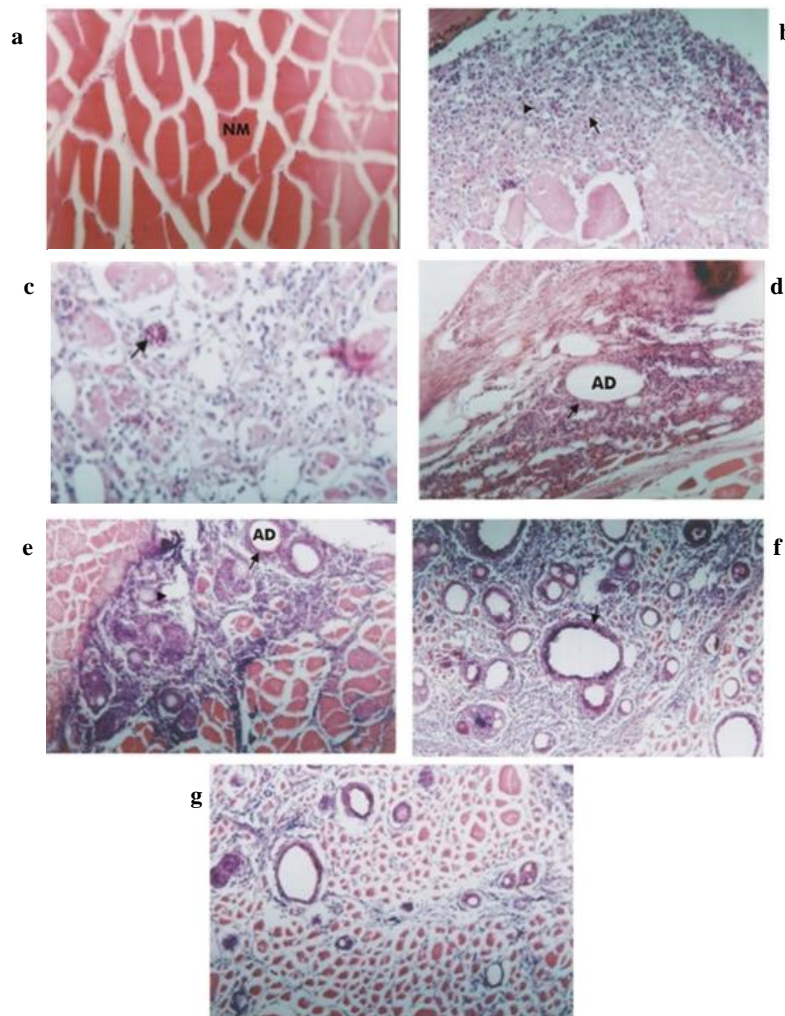
Control (T₀)

Figure 2 shows the histology of normal skeletal musculature below the dorsal fin, the site of FCA administration (Figure 2a) and the changes that occurred following adjuvant injection in the control fish. Degeneration of the muscle tissue was seen with the infiltration of large number of inflammatory cells towards the lesion area on the third day post injection (dpi) at the injection site (Figure 2b). On 6 dpi, haemorrhages were seen and the degenerated area was dominated by macrophages (Figure 2c). The centre of the lesion area showed complete degeneration of the muscle tissue along the path of movement of adjuvant droplets. Migration of inflammatory cells towards the lesion area was seen with initiation of encapsulatory response (Figure 2d). On 9 dpi, clear encapsulatory response was noticed around the adjuvant droplets, formed by numerous neutrophils (Figure 2e). On 12

Table 4. Digestive enzyme activity (total activity \pm S.E.)¹ in the gut of common carp fed the experimental diets

Diets	Protease		Amylase		Lipase	
	Intestine	Hepato	Intestine	Hepato	Intestine	Hepato
T ₀	8.50 \pm 0.10 ^b	0.63 \pm 0.01 ^c	148.38 \pm 2.91 ^b	169.04 \pm 3.19 ^b	1.79 \pm 0.13 ^a	1.66 \pm 0.23 ^a
T ₁	12.21 \pm 0.09 ^a	1.56 \pm 0.04 ^a	158.98 \pm 3.36 ^a	177.01 \pm 3.34 ^a	1.66 \pm 0.23 ^a	1.93 \pm 0.13 ^a
T ₂	11.95 \pm 0.17 ^a	1.13 \pm 0.01 ^b	156.13 \pm 3.36 ^a	182.79 \pm 3.32 ^a	1.10 \pm 0.13 ^b	1.24 \pm 0.01 ^b

¹Enzyme activity is expressed as μ moles of product liberated per g tissue per min. at 30 °C. Hepato. = Hepatopancreas. Figures in the same column with the same superscript are not significantly different (P>0.05).

**Figure 2.** Histological sections of the injection site (H&E x100).

a. Normal skeletal musculature below the dorsal fin. b. Degeneration of the muscle tissue is seen, with infiltration of a large number of inflammatory cells towards the lesion area on the third day post injection (dpi). c. Degenerated area is dominated by macrophages on 6 dpi. d. Initiation of encapsulatory response is noticed on 6 dpi. e. Advancement in encapsulatory response is observed around the adjuvant droplets on 9 dpi. f. A typical granuloma is evident, with a large number of inflammatory cells in the centre of the lesion area on 12 dpi. g. Signs of initial recovery of the muscle fibres is noticed on 12 dpi.

dpi a typical granuloma was still evident with a large number of inflammatory cells in the centre of the lesion area (Figure 2f). There were also signs of initial recovery of the muscle fibres (Figure 2g).

Treatment T₁

The histological changes recorded post FCA injection in 1.5% ImmuPlus fed fish is shown in

Figure 3. On 3 dpi, there was evidence of sarcoplasmic lysis at the injection site with the migration of neutrophils (Figure 3a). On 6 dpi, complete muscle degeneration was noticed, with large scale infiltration of inflammatory cells into the injected area. At the same time, initiation of encapsulatory response could be seen around the adjuvant droplets by phagocytes, macrophages being dominant (Figure 3b). On 9 dpi, muscular necrosis

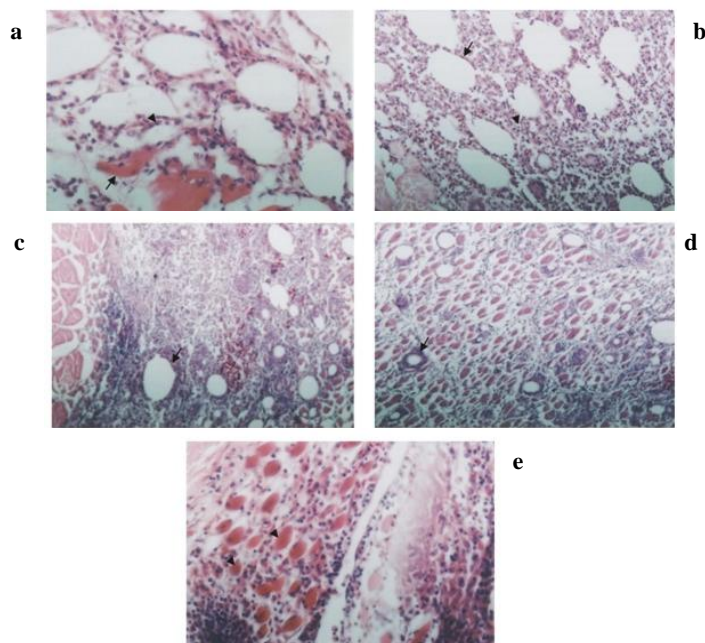


Figure 3. Histological sections of the injection site (H&E x100).

a. Sarcoplasmic lysis is seen, with the migration of neutrophils on 3 dpi. b. Initiation of encapsulatory response is seen around the adjuvant droplets on 6 dpi. c. A typical granuloma around the adjuvant droplets is formed by macrophages on 9 dpi. d. Several layers thick walls of granuloma are observed, with large number of macrophages and epitheloid cells on 9 dpi. e. Fibroblastic activity is noticed outside the lesion area and the regenerating muscle cells appear pinkish on 12 dpi

was still marked; however, a typical granuloma around the adjuvant droplets was formed by the macrophages (Figure 3c). The walls of the granuloma were several layers thick, with large number of macrophages and epitheloid cells (Figure 3d). On 12 dpi, reduced degeneration was observed. Outside the lesion area, there was fibroblastic activity and the regenerating muscle cells appeared pinkish (Figure 3e).

Treatment T₂

Figure 4 depicts the histological changes observed in 3.0% ImmuPlus fed fish post FCA injection. On 3 dpi, muscle necrosis at the centre of the lesion area was clearly seen with the migration of the inflammatory cells towards it (Figure 4a). On 6 dpi, adjuvant droplets with the initiation of encapsulatory response were seen, but lysis of the muscle tissue was still evident (Figure 4b). By 9 dpi, larger adjuvant droplets were encapsulated by macrophages (Figure 4c). The centre of the lesion area showed more inflammatory cells around the adjuvant droplets. The periphery of the injected site showed recovery of muscle fibres (Figure 4d). On 12 dpi, both larger and smaller adjuvant droplets were covered by macrophages and epitheloid cells, forming a granuloma. Section through the centre of granuloma appeared as empty spaces, while the center of the lesion area showed fibroblastic activity (Figure 4e). Outside the lesion area, strong encapsulation with increased fibroblastic activity was observed (Figure 4f), with regenerating muscle tissue appearing almost

normal.

Discussion

Dietary ImmuPlus enhanced fish growth and survival significantly ($P < 0.05$) at both the levels (1.5 and 3.0%) of incorporation. The increment in the final weight of common carp in T₁ and T₂ treatments over the control was 26.69% and 19.67% respectively. This higher growth in the treated fish can be attributed to ImmuPlus, since all other ingredients were the same in the control and test diets. Protease and amylase activity was greater in the treated fish in both the intestine and hepatopancreas. Better utilization of feed through improved digestive enzyme activity and higher deposition of protein and fat in carcass appear to be responsible for the higher growth in the treated fish. Jaya Kumari *et al.* (2007) reported higher growth of rohu (*Labeo rohita*) with lower dose of ImmuPlus and reduced growth with higher dose and related the latter to 'regulatory' mechanisms. One of the components of ImmuPlus, Amlaki, is rich in vitamin C. Dietary administration of small quantities of vitamin C is known to improve fish growth (Shobana, 1997; Misra *et al.*, 2007; Tiwary and Patra, 2008). Ji *et al.* (2007) observed improved growth in *Pagrus major* fed medicinal herbs.

The type and intensity of inflammatory response depends on a number of factors, the important one being the kind of initiating agent. FCA, being oil based adjuvant, acts as a depot for holding the antigens in tissues after injection for a slow release. It contains paraffin oil and killed, dried tubercle bacilli,

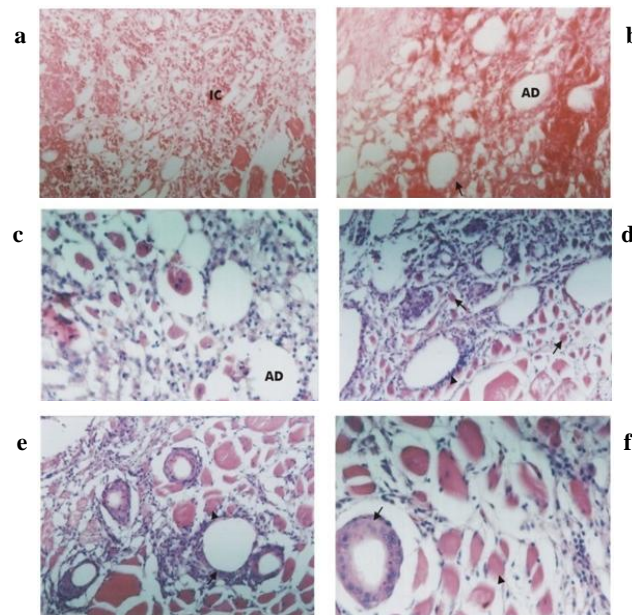


Figure 4. Histological sections of the injection site (H&E x100).

a. Muscle necrosis is seen at the centre of the lesion area, with the migration of inflammatory cells towards it on 3 dpi. b. Initiation of encapsulatory response is noticed around adjuvant droplets on 6 dpi. c. Larger adjuvant droplets are encapsulated by macrophages on 9 dpi. d. The periphery of the injected site shows recovery of muscle fibres on 9 dpi. e. The center of the lesion area shows fibroblastic activity on 12 dpi. f. Outside the lesion area, strong encapsulation with increased fibroblastic activity is observed on 12 dpi.

Mycobacterium butyricum. After the administration of FCA, the injected site showed sarcoplasmic lysis and localised haemorrhage, followed by infiltration of phagocytes dominated by macrophages. The chronic lesion developed as a central zone of necrotic cell material containing the adjuvant with a surrounding layer of macrophages and other inflammatory cells. In fish fed diet T₁, recovery of muscle fibres along with fibroblastic activity was observed on day 12, whereas in fish fed diet T₂ recovery of muscle fibres on the periphery of the injected site was observed by day 9 itself. But, in the control, epitheloid cells were evident with large number of inflammatory cells in the centre of the lesion area, with only initial signs of recovery on day 12. Here, the continuous form of inflammation might have inhibited muscle recovery by producing more muscle damage (Anderson and Roberts, 1975).

The differences between the control and treated fish in the overall histopathological changes associated with sequential progress of events in inflammatory response were not marked, except with respect to time. Further, stronger encapsulation was observed in fish from treatment T₂. These could be attributed to ImmuPlus administration. Four of the major constituents of ImmuPlus viz. Amlaki, Guduchi, Aswagandha and Tulsi are known to play immunomodulatory roles (Devasagayam and Sainis, 2002). Amlaki is rich in vitamin C. The immunostimulant and antioxidant properties of vitamin C are well documented in fish (Anderson, 1992; Sakai, 1999; Sahoo and Mukherjee, 2003). Guduchi is known to augment phagocytic cell functions and enhance protection against infection. The active ingredients of Tulsi leaves are responsible

for the antibody response and promote a non-specific defence mechanism in *Oreochromis mossambicus* against *Aeromonas hydrophila* (Venkatalakshmi and Michael, 2001). Thus, the different constituents of ImmuPlus would have collectively enhanced immunity in the treated common carp, leading to faster healing compared to that of the control, the effect being dose dependent. Jaya Kumari *et al.* (2007) have reported enhanced immunity in rohu fed ImmuPlus at various life stages. Investigations with a few plant derived immunostimulants show their immune modulating potential in different fish species when administered through diet (Rao *et al.*, 2006; Christyapita *et al.*, 2007; Divyagnaneswari *et al.*, 2007; Ji *et al.*, 2007; Sahu *et al.*, 2007; Pratheepa *et al.*, 2010).

Finn and Nielson (1971) opined that the inflammatory response is greatly influenced by temperature. The temperature that prevailed during the present study was in the range of 29-31°C. In studies carried out at temperatures close to this range, Shobana *et al.* (2002) reported regeneration of muscle fibres along with fibroblastic activity on the 9th day, in vitamin C fed *Cirrhinus mrigala*, in response to FCA, whereas Pradhan *et al.* (2008) observed that following FCA administration, the lesion area appeared repaired with regenerated muscle fibres after 10 days in *Catla catla*. Another factor that would have contributed to inflammatory response in the treated fish is the enhanced complement activity. Among the different dietary factors, ascorbic acid can significantly influence the functioning of the phagocytic cells in fish (Johnson and Ainsworth 1992). Increased serum complement activity following feeding high levels of

vitamin C has been reported in fish (Hardie *et al.*, 1991; Ortuno *et al.*, 1999).

The results of the present study reflect the ability of ImmuPlus to promote growth and improve inflammatory response when administered through diet. The percentage increase in growth was better in fish receiving 1.5% ImmuPlus than those fed 3%. However, in terms of inflammatory response, 3% proved better since in fish fed diet T₂ recovery of muscle fibres on the periphery of the FCA injected site was observed by day 9, as against day 12 post injection in fish receiving diet T₁. The increased growth and immunity would nullify the additional cost incurred in incorporating small quantity of ImmuPlus in the diet.

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References

- Anderson, D.P. 1992. Immunostimulants, adjuvants and vaccine carriers in fish: Applications to aquaculture. *Annual Review of Fish Diseases*, 2: 281-307. doi: 10.1016/0959-8030(92)90067-8
- Anderson, C.D. and Roberts, R.J. 1975. A comparison of the effects of temperature on wound healing in a tropical and temperate teleost. *Journal of Fish Biology*, 7: 173-182. doi: 10.1111/j.1095-8649.1975.tb04588.x
- AOAC, 1995. Official Methods of Analysis of AOAC International, P.A. Cunniff (Ed.), AOAC International, 16th Ed., Arlington, USA.
- APHA/AWWA/WEF, 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. Clesceri, L.S., Greenberg, A.E., Eaton, A.D. (Eds.), American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.
- Bier, M. 1962. Lipases. In: S.P. Colowick and N.O. Kaplan, (Eds.), *Methods in Enzymology*, Academic Press, New York: 627-642.
- Bricknell, I. and Dalmo, R.A. 2005. The use of immunostimulants in fish larval aquaculture. *Fish and Shellfish Immunology*, 19: 457-472. doi: 10.1016/j.fsi.2005.03.008
- Bullock, A.M. 1989. Laboratory methods-Histopathology. In: R.J. Roberts (Ed.), *Fish Pathology*, Bailliere Tindall, London: 374-384.
- Christybapita, D., Divyagnaneswari, M. and Michael, R.D. 2007. Oral administration of *Eclipta alba* leaf aqueous extract enhances the non-specific immune responses and disease resistance of *Oreochromis mossambicus*. *Fish and Shellfish Immunology*, 23: 840-852. doi: 10.1016/j.fsi.2007.03.010
- Devasagayam, T.P.A. and Sainis, K.B. 2002. Immune system and antioxidants, especially those derived from Indian medicinal plants. *Indian Journal of Experimental Biology*, 40: 639-655.
- Divyagnaneswari, M., Christybapita, D. and Michael, R.D. 2007. Enhancement of non-specific immunity and disease resistance in *Oreochromis mossambicus* by *Solanum trilobatum* leaf fractions. *Fish and Shellfish Immunology*, 23: 249-259. doi: 10.1016/j.fsi.2006.09.015
- Düğenci, S.K., Arda, N. and Candan, A. 2003. Some medicinal plants as immunostimulant for fish. *Journal of Ethnopharmacology*, 88: 99-106. doi: 10.1016/S0378-8741(03)00182-X
- Finn, J.P. and Nielson, N.O. 1971. The effect of temperature variation on the inflammatory response of rainbow trout. *The Journal of Pathology*, 105: 257-268. doi: 10.1002/path.1711050404
- Gannam, A.L. and Schrock, R.M. 1999. Immunostimulants in fish diets. *Journal of Applied Aquaculture*, 9: 53-89. doi: 10.1300/J028v09n04_06
- Hardie, L.J., Fletcher, T.C. and Secombes, C.J. 1991. The effect of dietary vitamin C on the immune response of the Atlantic salmon (*Salmo salar* L.). *Aquaculture*, 95: 201-214. doi: 10.1016/0044-8486(91)90087-N
- Harikrishnan, R., Balasundaram, C., Dharaneedharan, S., Moon, Y.G., Kim, M.C., Kim, J.S. and Heo, M.S. 2009. Effect of plant active compounds on immune response and disease resistance in *Cirrhina mrigala* infected with fungal fish pathogen, *Aphanomyces invadans*. *Aquaculture Research*, 40: 1170-1181. doi: 10.1111/j.1365-2109.2009.02213.x
- Hastings, W.H. 1976. Fish nutrition and feed manufacture. Paper presented at FAO Technical Conference on Aquaculture, Kyoto, Japan. FIR: Aq/conf/76/R.23:1-13.
- Jaya Kumari, Sahoo, P.K. and Giri, S.S. 2007. Effects of polyherbal formulation ImmuPlus on immunity and disease resistance of Indian major carp, *Labeo rohita* at different stages of growth. *Indian Journal of Experimental Biology*, 45: 291-298.
- Jayaram, M.G. and Shetty, H.P.C. 1981. Formulation, processing and water stability of two pelleted fish feeds. *Aquaculture*, 23: 355-359. doi: 10.1016/0044-8486(81)90028-4
- Ji, S.C., Takaoka, O., Jeong, G.S., Lee, S.W., Ishimaru, K., Seoka, M. and Takii, K. 2007. Dietary medicinal herbs improve growth and some non-specific immunity of red sea bream *Pagrus major*. *Fisheries Science*, 73: 63-69. doi: 10.1111/j.1444-2906.2007.01302.x
- Jian, J. and Wu, Z. 2003. Effects of traditional Chinese medicine on nonspecific immunity and disease resistance of large yellow croaker, *Pseudosciaena crocea* (Richardson). *Aquaculture*, 218: 1-9. doi: 10.1016/S0044-8486(02)00192-8
- Johnson, M.R. and Ainsworth, A.J. 1992. The effect of a megadose level of ascorbic acid on anterior kidney neutrophil response of *Edwardsiella ictaluri* in channel catfish (*Ictalurus punctatus*). *J. of Aquatic Animal Health*, 3: 266-273. doi: 10.1577/1548-8667(1991)003%3c0266:aedloa%3e2.3.co2
- Kunitz, M. 1947. Crystalline soybean trypsin inhibitor II. General properties. *Journal of General Physiology*, 30: 291-310. doi: 10.1085/jgp.30.4.291
- Mayes, P.A. 1990. Nutrition. R.K. Murray, D.K. Graner, P.A. Mayes and V.W. Rodwell (Eds.), *Harper's Biochemistry* 22nd Ed., Prentice Hall International Inc., USA: 571-579.
- Misra, C.K., Das, B.K., Mukherjee, S.C. and Pradhan, J. 2007. Effects of dietary vitamin C on immunity, growth and survival of Indian major carp *Labeo*

- rohita*, fingerlings. Aquaculture Nutrition, 13: 35–44. doi: 10.1111/j.1365-2095.2007.00451.x
- Ortuno, J., Esteban, M.A. and Megeguer, J. 1999. Effect of high dietary intake of vitamin C on non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish and Shellfish Immunology, 9: 429-443. doi: 10.1006/fsim.1998.0201
- Pradhan, P.K., Mohan, C.V. Shankar, K.M. and Kumar, B.M. 2008. Inflammatory response in Indian major carp, *Catla catla* and barb, *Puntius cauvieriensis*, to *Aphanomyces invadans* and Freund's Complete Adjuvant. Asian Fisheries Science, 21: 385-394.
- Pratheepa, V., Ramesh S. and Sukumaran, N. 2010. Immunomodulatory effect of *Aegle marmelos* leaf extract on freshwater fish *Cyprinus carpio* infected by bacterial pathogen *Aeromonas hydrophila*. Pharmaceutical Biology, 48: 1224-1239. doi: 10.3109/13880201003713598
- Rao, Y.V., Das, B.K., Jyotirmayee P. and Chakrabarti, R. 2006. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. Fish and Shellfish Immunology, 20: 263-273. doi: 10.1016/j.fsi.2005.04.006
- Rick, W. and Stegbauer, H.P. 1974. Alpha amylase measurement of reducing groups. In: H.V. Bergmeyer (Ed.), Methods of Enzymatic Analysis, 2nd Edition, Academic Press, New York: 885-889.
- Sahoo, P.K. and Mukherjee, S.C. 2003. Immunomodulation by vitamin C in healthy and aflatoxin B₁ induced immunocompromised rohu (*Labeo rohita*). Comparative Immunology, Microbiology and Infectious Diseases, 26: 65-76. doi: 10.1016/S0147-9571(01)00038-8
- Sahu, S., Das, B.K., Pradhan, J., Mohapatra, B.C., Mishra B.K. and Sarangi, A. 2007. Effect of *Magnifera indica* kernel as a feed additive on immunity and resistance to *Aeromonas hydrophila* in *Labeo rohita* fingerlings. Fish and Shellfish Immunology, 23: 109-118.
- Sakai, M. 1999. Current research status of fish immunostimulants. Aquaculture, 172: 63-92. doi: 10.1016/S0044-8486(98)00436-0
- Shobana, K.S. 1997. Modulatory effects of vitamin C on the disease resistance mechanism of Indian major carps. PhD thesis, University of Agricultural Sciences, Bangalore, India.
- Shobana, K.S., Mohan, C.V. and Shankar, K.M. 2002. Effect of dietary vitamin C on the inflammatory response of mrigal, *Cirrhinus mrigala* (Hamilton) to Freund's complete adjuvant. Journal of Fish Diseases, 25: 179-184.
- Siwicki, A.K., Morand, M., Terech-Majewska, E., Niemczuk, W., Kazuń, K. and Glabski, E. 1998. Influence of immunostimulants on the effectiveness of vaccines in fish: in vitro and in vivo study. Journal of Applied Ichthyology, 14: 225-227. doi: 10.1111/j.1439-0426.1998.tb00646.x
- Tewary, A. and Patra, B.C. 2008. Use of vitamin C as an immunostimulant. Effect on growth, nutritional quality and immune response of *Labeo rohita* (Ham). Fish Physiology and Biochemistry, 34: 251-259. doi: 10.1007/s10695-007-9184-z
- Venkatalakshmi, S. and Michael, R.D. 2001. Immunostimulation by leaf extract of *Ocimum sanctum* Linn. in *Oreochromis mossambicus* (Peters). Journal of Aquaculture in the Tropics, 16: 1-10.
- Zar, J.H. 2009. Biostatistical Analysis 5th Ed. Prentice Hall International Inc., USA. 960 pp.