Improved Growth Performance And Mucosal Immunity In Koi Carp Fed On A Diet Enriched With *Borassus Flabellifer* Sap

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Abstract
Koi carps are fascinating ornamental fish known for their vibrant colouration and graceful appearance. Due to their high popularity in the ornamental fish sector, their growth enhancement and mucosal immunity are of great concern. *B.flabellifer* sap or palm nectar was coated along with the commercial pellet diet at various concentrations, i.e. 2.0%, 5.0%, 10.0% and 15.0% respectively. In parallel, a control diet lacking palm sap was maintained for analysis to test the growth and skin mucosal immune parameters in koi carp reared in indoor culture system. Koi carp fed on the P3 diet showed a maximum weight gain of 1.95 ± 0.02g compared to a low weight gain of 0.654 ± 0.02g noticed in the control diet fed fish. The skin mucosal immune parameters, such as total immunoglobulin, lysozyme activity, bactericidal activity and alkaline phosphatase activity, were remarkably high in the skin mucus of P3 and P4 treated groups compared to control diet-fed fish.

Keywords: *Borassus flabellifer* sap, skin mucus, Mucosal immunity.

Introduction
Palmyra palm trees are used for multiple purposes such as food, drink, fibre and medicine. The sap is the most valuable product of the palm that holds a combination of sugar, protein, amino acids, and minerals that contribute to the nutritional status of this beverage (Ezeagu and Fafunso 2003). The fresh sap is a good source of vitamin B complex (Morton 1998). People considered palm sap as a part of their daily diet due to its good nutrient composition and health benefits. Antibiotic usage in aquaculture systems promotes resistant strains of bacteria and residual effects. In this juncture, people are turning to natural products to overcome the negative impacts of antibiotics (Fauci 1993). In addition, plant products have been shown to boost the immune system apart from its growth promoting properties (Sivaram *et al.,* 2004; Citarasu *et al.,* 2006). Fish mucus plays a main role in the innate immune defence as mucus holds appreciable amount of anti-microbial bioactive compounds that eliminate the pathogens (Dash *et al.,* 2018; Patel *et al.,* 2020). Apart from trapping the pathogens, mucus also contains various innate immune components such as lysozymes, complement proteins, and antimicrobial peptides (Ellis 2001, Kuppulakshimi *et al.,* 2008, Su 2011). In this view, the present study was performed to evaluate the growth-promoting potential of palm sap and its role in skin mucosal immunity in the koi carp reared in an indoor culture system.

Materials and methods
Koi carp was obtained from Jai Jai aquarium, Nagercoil, Tamil Nadu. Healthy fish were selected based on their active movement from the aquarium and further transported in oxygenated polythene bags to the culture site with the least disturbance to the fish.

Palm sap
Fresh palm sap was obtained from a palm sap tapper belonging to the Parvathi Puram area, Tamil Nadu, India. The Palm sap was brought to the experimental site in proper air-tight containers for experimental purpose.

Diet preparation
Commercial ornamental fish diet was obtained from Jay Jay Aquarium, Nagercoil that consisted of 35.0% crude protein. The diet was segregated into 5 groups separately each consisting of 100g of feed. Further, palm sap was coated individually along with the segregated feed at 2.0%, 5.0%, 10.0%, and 15.0% concentration and designated as P1, P2, P3 and P4 diets, respectively. A control diet lacking palm sap was also maintained for analysis. The palm sap-coated diet was dried in a hot air oven at 45°C and further stored in individual plastic airtight containers for feeding the fish.

Experimentation
Healthy koi carp weighing about 4.74 ± 0.12g to 6.60 ± 0.12g were segregated into respective control and experimental tanks for the experimentation (20litre capacity). During the culture period, koi carp were fed on the respective diets to

satiation. The unfed remains were regularly siphoned out from the culture tanks, and fifty per cent of the water exchange was provided daily to maintain the optimum water quality parameters. Sufficient aeration was provided to the culture system to maintain optimum dissolved oxygen levels using venus aqua air pumps (AP – 208).

Water quality analysis

The temperature in the culture system was analysed using a digital Thermometer. The pH of the water samples was determined using a digital pH meter (Systronics) and calibrated using standard buffers. The dissolved oxygen content of the culture water was analysed using the titration method (APHA 1995). Ammonia content in the culture water was determined using the (API) Ammonia kit and the colouration of ammonia was compared with the standard chart provided in the kit.

Growth Performance

Growth performance indices such as production, food consumption, FCR, FCE, SGR and G (%) were calculated based on the formulae given below.

Production

Production (g) = Final weight – Initial weight

Food consumption

Food consumption (g) = Food provided – Unfed remains

Food conversion ratio (FCR)

$$FCR = \frac{\text{Total amount of feed given (g)}}{\text{Total production of fish (g)}}$$

Food conversion efficiency (FCE)

$$FCE\ (\%) = \frac{\text{Wet weight of the fish produced (g)}}{\text{Dry weight of the feed given (g)}} \times 100$$

Specific growth rate (SGR)

$$SGR\ (\%) = \frac{\text{In final weight (g)- In Initial Weight (g)}}{\text{Experimental period}} \times 100$$

Growth Percentage (G %)

$$G\ (\%) = \frac{\text{Growth (g)}}{\text{Experimental duration}} \times 100$$

Survival (%)

$$\text{Survival}\ (\%) = \frac{\text{Number of fish harvested}}{\text{Number of fish stocked}} \times 100$$

Evaluation of Mucosal Immune Parameters

Mucus Collection

Mucus was obtained from koi carp by gently scraping the skin of the fish using a cell scraper. The collected mucus was pooled and placed in an Eppendorf vial and placed in a thermocol box provided with ice cubes for the evaluation of mucosal immune parameters

Total Immunoglobulin

Total immunoglobulin in the skin mucus of koi carp was determined according to the method of Siwicki and Anderson (1993) with minor modifications. Briefly, about 100µl of pooled skin mucus was treated with an equal volume of (PEG) Polyethene glycol and centrifuged at 1000g for 10 minutes. The protein content of the skin mucus before the addition of PEG and the final supernatant was evaluated. The values were expressed in mg/ml Concentration.

$$\text{Total immunoglobulin (mg/ml)} = \frac{\text{Total protein in mucus}}{\text{Total protein in supernatant}}$$

Bactericidal Activity
Bactericidal activity in the skin mucus of koi carp was analysed by MTT reduction principle based on the method of *Welker et al.,* (2007) with slight modifications. The colouration of formazan was read at a spectrophotometer at 560nm that corresponds to the bactericidal activity.

Lysozyme Activity (LYZ)
Lysozyme activity was quantified in the skin mucus of koi carp according to the protocol of Parry *et al.,*1965 with minor modifications. A required amount of *Micrococcus lysodeikticus* (Sigma- M 3770, ATCC No 4698) was dissolved in sodium phosphate buffer. Then 1.8ml of buffer dissolved *M. lysodeikticus* was taken in a cuvette, and to this, 100µl of pooled skin mucus was added. The reduction in absorbance was read at 450nm using Systronics spectrophotometer (Model-104) at regular time intervals and finally the LYZ activity was expressed in U/ml.

Alkaline Phosphatase Activity (ALP)
ALP activity was analysed in the skin mucus of koi carp using ERBA ALP kit. The ALP activity corresponds to the increase in optical density (OD), which was measured using a Systronics spectrophotometer (Model-104) at 405nm. The intensity of yellow colour formed corresponds to the ALP activity.

Table 1. Water quality parameters recorded in the culture tanks

Parameters	Control	P1	P2	P3	P4
Temperature (°c)	31.00 ± 0.02	31.00 ± 0.04	31.00 ± 0.04	31.00 ± 0.04	31.00 ± 0.04
pH	7.49 ± 0.14	7.32 ± 0.12	7.40 ± 0.17	7.46 ± 0.10	7.60 ± 0.12
Dissolved oxygen(mg/l)	5.62 ± 0.12	5.49 ± 0.13	5.68 ± 0.14	5.70 ± 0.12	5.62 ± 0.10
Ammonia (mg/l)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

Table 2. Growth performance of koi carp fed on different diets during experimentation

Parameters	C	P1	P2	P3	P4
Initial weight (g)	6.60 ± 0.12	5.38 ± 0.10	4.74 ± 0.12	4.88 ± 0.14	4.82 ± 0.12
Final weight (g)	7.25 ± 0.10	7.20 ± 0.12	6.60 ± 0.14	6.83 ± 0.10	5.63 ± 0.15
Production (g)	0.65 ± 0.02	1.82 ± 0.04	1.86 ± 0.04	1.95 ± 0.02	0.81 ± 0.03
Food Consumed(g)	2.08 ± 0.01	2.32 ± 0.02	2.34 ± 0.01	2.40 ± 0.04	2.10 ± 0.07
FCR	3.18 ± 0.02	1.27 ± 0.03	1.25 ± 0.02	1.23 ± 0.04	2.59 ± 0.02
FCE (%)	31.44 ± 1.06	78.44 ± 1.30	80.00 ± 1.04	81.25 ± 1.02	38.57 ± 1.04
SGR (%)	4.19 ± 0.02	4.26 ± 0.04	4.04 ± 0.06	4.14 ± 0.04	3.50 ± 0.03
G (%)	1.63 ± 0.02	4.55 ± 0.02	4.65 ± 0.04	4.87 ± 0.02	2.02 ± 0.04

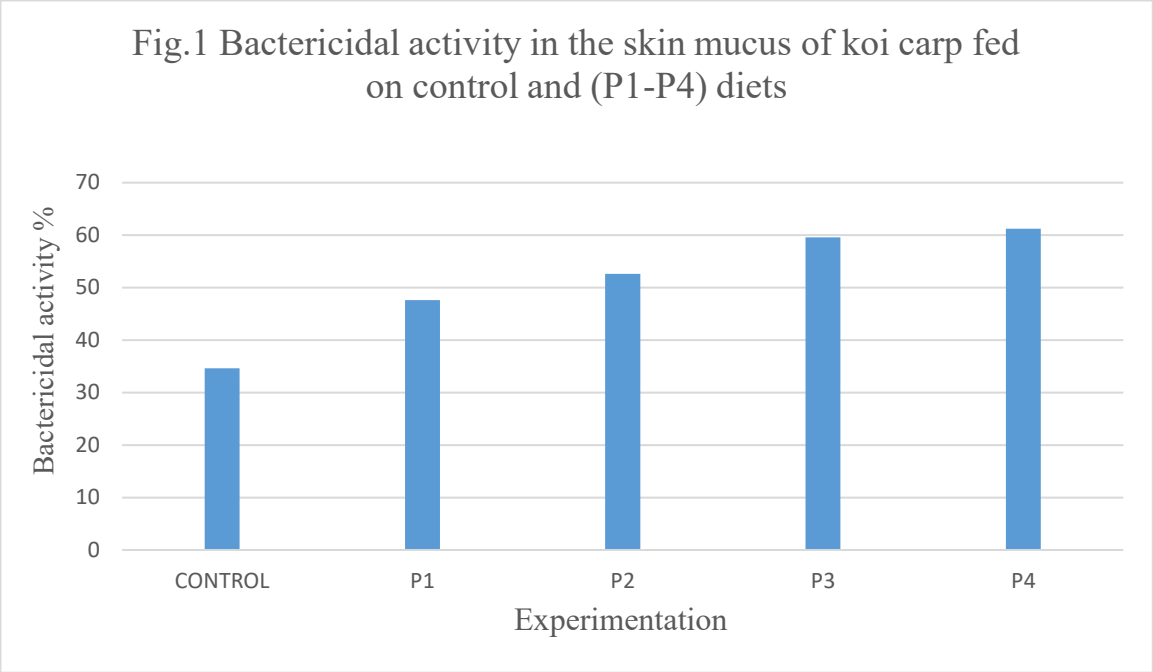
Results
Water Quality Parameters
Water quality parameters recorded in the culture system showed slight variations among the control and treated groups Table 1. For instance, the temperature ranged from 31.00 ± 0.02ºC to 31.00 ± 0.04ºC in the culture tanks. The pH showed minor variations among the control and experimental groups, i.e., it varied between 7.32 ± 0.12 and 7.60 ± 0.12. The dissolved oxygen content ranged from 5.49 ± 0.13 mg/l to 5.70 ± 0.12 mg/l whereas, the ammonia content in the culture tanks was negligible, i.e. < 0.1mg/l in the control and experimental tanks.

Growth Performances
Growth Performances of koi carp reared on control and experimental diet fed fish displayed significant variations ($P < 0.05$) among treatments Table 2. For instance, the production peaked high 1.95 ± 0.02g in the P3 diet-fed fish, in contrast to a low production of 0.654 ± 0.02g noticed in the control diet-fed group. Growth Percentage showed significant ($P < 0.05$) variations among control and treated groups, i.e., it was high 4.87 ± 0.02% in P3 diet-fed fish koi carp compared to a low G% of 1.63 ± 0.02% noticed in control diet-fed fish. The FCE was remarkably high, 81.25 ± 1.02% in the P3 treated group, compared to a low FCE of 31.44 ± 1.06% noticed in the control diet-fed fish. The FCR was satisfactory, i.e. 1.23 ± 0.04, 1.25 ± 0.02 and 1.27 ± 0.03 in P3, P2 and P1 treated groups compared to a high FCR of 3.18 ± 0.02 and 2.59 ± 0.02 noticed in control and P4 treated groups.\

Survival
Survival of koi carp was higher than 95. 00 ± 0.42 % in control and P1 - P4 treated groups.

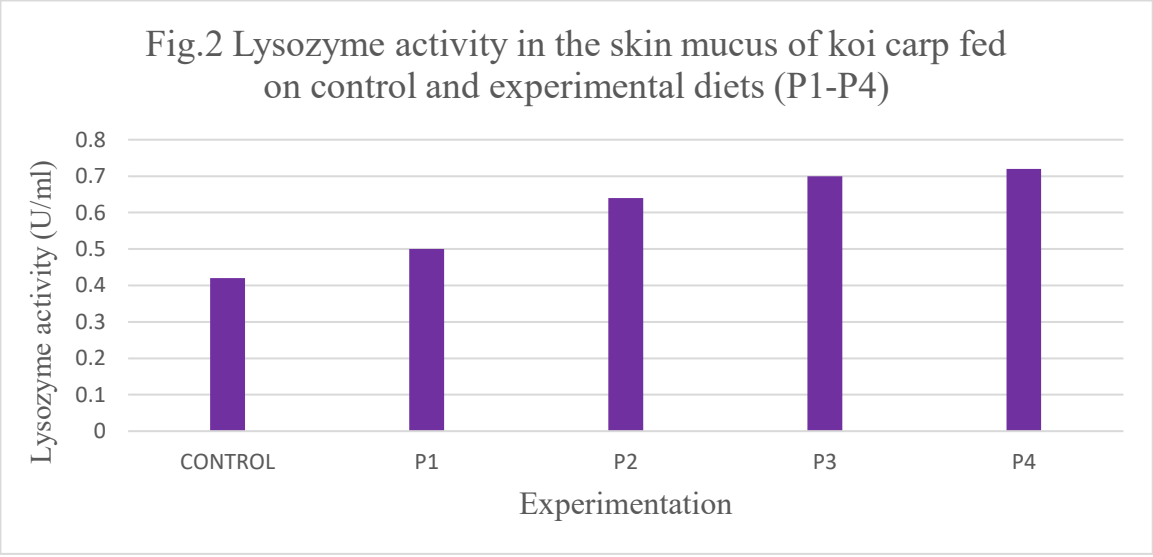
Bactericidal Activity (BCA)
Bactericidal activity in the skin mucus of koi carp showed remarkable variations ($P < 0.05$) among control and treated groups (Fig 1). For instance, the BCA activity peaked high 61.24 ± 1.20% and 60.05 ± 1.04% in the skin mucus of P4

and P3 treated group compared to a low BCA of 34.62 ± 1.02% noticed in the skin mucus of control diet fed fish however, P1 and P2 treated groups showed a better BCA activity in the skin mucus, i.e., 47.64 ± 1.14% and 52.63 ± 1.17% compared to control group. (Fig.1)



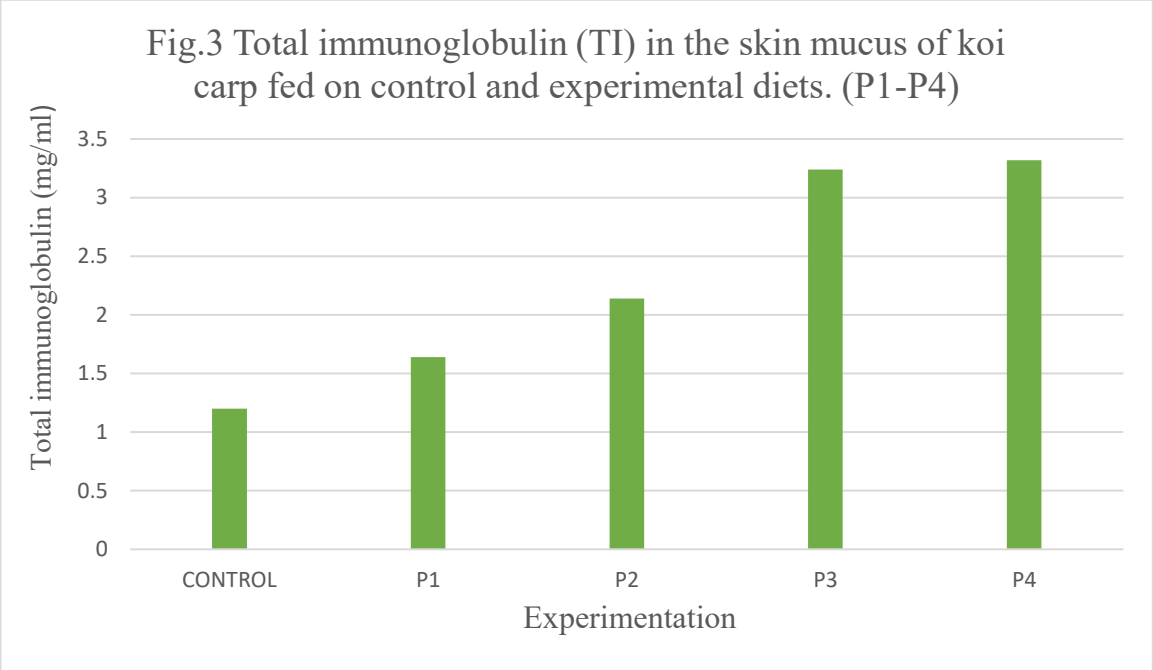
Lysozyme Activity (LYZ)

Lysozyme activity in the skin mucus of fish fed on a control diet and experimental diets (P1-P4) registered diet-dependent significant variations ($P < 0.05$) among treatments. Accordingly, a high LYZ activity of 0.72 ± 0.02 U/ml was noticed in the skin mucus of P4 diet-fed fish compared to a low LYZ activity of 0.42 ± 0.02 U/ml noticed in the skin mucus of control fish. But the LYZ activity was better in the skin mucus of P1, P2 and P3 treated groups, i.e., 0.50 ± 0.03 U/ml, 0.64 ± 0.02 U/ml and 0.70 ± 0.04 U/ml compared to the control diet-fed fish (Fig.2)



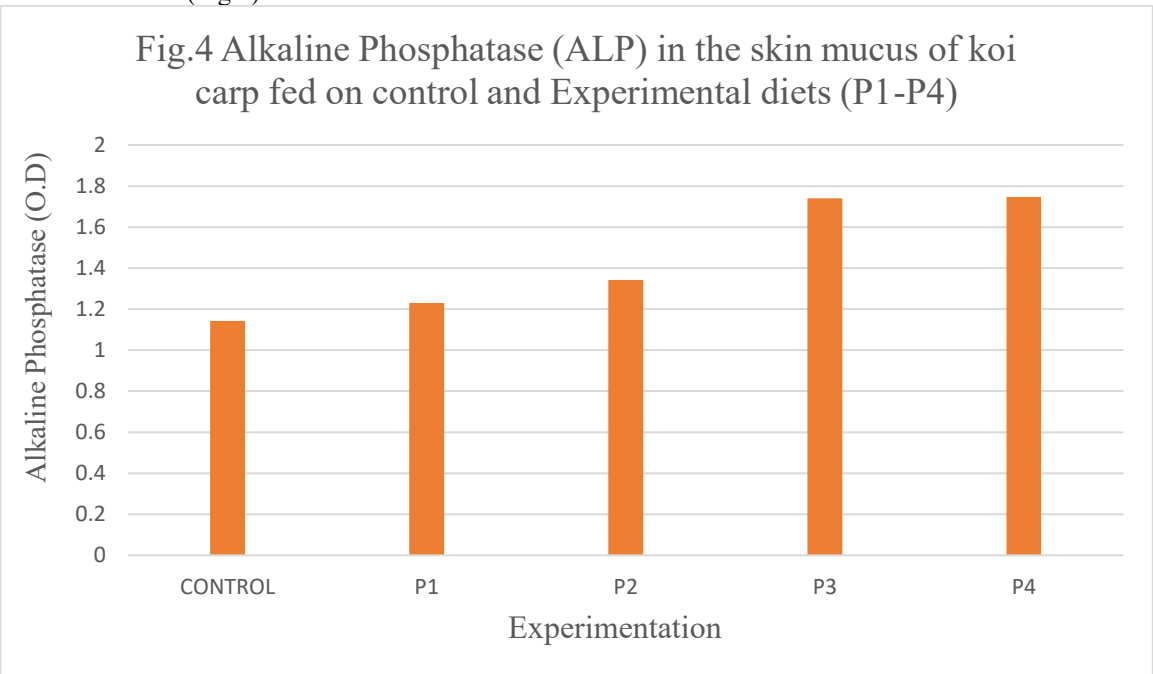
Total immunoglobulin (TI)

Total immunoglobulin in the skin mucus of koi carp showed significant ($P < 0.05$) variations among the control and treated groups, Fig 3. The TI peaked high of 3.36 ± 0.02 mg/ml in the skin mucus pf P4 diet-fed fish compared to a low TI activity of 1.20 ± 0.05 mg/ml noticed in the skin mucus of control diet fed fish. In P1, P2 and P3 treated groups, the TI in the skin mucus ranged between 1.64 ± 0.04 mg/ml to 3.24 ± 0.04 mg/ml respectively that was higher than control diet fed fish. (Fig.3)



Alkaline Phosphatase (ALP) activity

ALP activity in the skin mucus of koi carp showed a significant difference ($P < 0.05$) between the control and treated groups. Notably, the P3 & P4 diet-fed fish showed a high ALP activity of 1.746 ± 0.23 OD and 1.740 ± 0.20 OD in their skin mucus compared to a low ALP activity of 1.142 ± 0.04 OD noticed in the skin mucus of control diet-fed fish. However, the ALP activity was high in the skin mucus of P1 & P2 treated groups, i.e. 1.230 ± 0.02 OD, 1.342 ± 0.02 OD compared to control diet-fed fish. (Fig.4)



STATISTICAL ANALYSIS

The data obtained in the present analysis were expressed as Mean ± SD using One – Way ANOVA test at 5% significant level.

Discussion

The palm tree is considered as a celestial tree known for its versatile benefits; palm sap, commonly known as pathaneer in its fresh form, is white in colour and sweet in nature. The main form of sugar is sucrose, and apart from this, it holds an appreciable amount of Iron, Calcium, phosphorus, and B-complex vitamins. (Chrystopher 1985, Mogea *et al.,* 1991, Vengaiah *et al.,* 2015, Vengiah *et al.,* 2017, Siju and Babu 2020). Due to these properties, palm sap was selected in the present study to evaluate its health-promoting aspects in koi carp reared in an indoor culture system. Palm toddy is a good

probiotic with multiple nutritional benefits. Further, reports show their antibacterial activity against microbes, such as *E.coli*, *Klebsiella pneumonia* and *Staphylococcus aureus* (Theivendikirarajah and Christoper 1985, Sandhya and Kalaiselvam 2020). Similarly, in the present investigation, koi carp fed on a palm sap-coated diet exhibited better mucosal immune parameters in their skin mucus compared to control diet-fed fish. For instance, the BCA was high 61.24 ± 1.20 %, in P4 treated group whereas, it declined 34.62 ± 1.02% in control diet fed group . However, P1, P2 and P3 treated group showed a better BCA activity of 47.64 ± 1.14% 52.63 ± 1.17 % and 60.05 ± 1.04 %, compared to control diet-fed group. Fish mucus is synthesised and secreted by the epidermal goblet cells that encompass a wide range of functions (Shephard 1994). The pathogens get entrapped in the skin mucus and fnally the destruction of the pathogen takes place due to several components in the skin mucus, such as lysozyme, Immunoglobulin, antibacterial protein and peptides. (Cole *et al.,* 1997; Subramaniam *et al.,* 2007). Accordingly, in the present investigation, the TI content in the skin mucus ranged high in palm sap-treated groups, i.e., it was 3.36 ± 0.02 mg/ml in P4 diet-fed group compared to a low TI value of 1.20 ± 0.05 mg/ml noticed in the skin mucus of control diet-fed fish. However, P3, P2 and P1 treated groups registered a better TI content in their skin mucus compared to control, which could be evidenced from the data, i.e., 3.24 ± 0.04 mg/ml, 2.14 ± 0.03 mg/ml and 1.64 ± 0.04 mg/ml.The mucus of fish provides valuable information concerning the health status of fish due to immune molecules such as lysozyme, immunoglobulin and phosphatases. Lysozyme is an important antimicrobial peptide that plays a major role in providing defence in fish and also resists the pathogen entry (Pourmozaffar *et al.,* 2017; Adeshina *et al.,* 2021). In the present analysis, the skin mucosal lysozyme activity is taken into consideration for evaluating the health status of fish. Lysozyme plays a major role in restricting the entry of pathogens like bacteria, fungi and viruses. The lysozyme levels are influenced by nutritional additives such as probiotics, and in the case of any stress, they show a declining profile. In the present investigation, the lysozyme activity in the skin mucus was elevated, i.e., 0.72 ± 0.02 U/ml and 0.70 ± 0.04 U/ml in the P4 and P3 treated group, compared to a low lysozyme activity of 0.42 ± 0.02 U/ml noticed in the skin mucus of control diet-fed fish. Fish serum and skin mucus are known to encompass a wide range of antimicrobial substances such as lysozyme, alkaline phosphatase, and lectins. Alkaline phosphatase (ALP) plays a key role in the innate immune response, and it exerts its effect as a vital detoxifying agent and helps in the wound healing process under stress and infection. ALP responds to varied dietary modifications. Likewise, in the present observation, the ALP activity was high in koi carp fed on P3 and P4 diets, i.e., an increase in optical density of 1.746 ± 0.03 O. D and 1.740 ± 0.20 O. D was noticed in their skin mucus compared to a low ALP activity of 1.142 ± 0.04 O. D noticed in the skin mucus of control diet fed group.

Conclusion

The present results documented that koi carp supplemented with 10.0%, i.e. P3 diet, resulted in an improved growth performance and skin mucosal immune status. Therefore incorporation of palm sap at this particular concentration can be effectively used as a feed additive in koi carp diet that could be much favoured in the ornamental fish sector.

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